

Probing the Mechanisms Underlying the Modulation  
of Dopamine Release by Axonal Cholinergic Inputs  
in Mice and *Drosophila*



Lucille Sophie Alice Duquenoy

Department of Physiology, Anatomy and Genetics  
Medical Sciences Division, DPhil in Neuroscience  
St John's College  
University of Oxford

A thesis submitted for the degree of  
**Doctor of Philosophy**  
Hilary Term 2025

# Table of contents

|                                                                                                                                          |           |
|------------------------------------------------------------------------------------------------------------------------------------------|-----------|
| Table of contents .....                                                                                                                  | ii        |
| Statement of Authorship .....                                                                                                            | vi        |
| Conference Abstracts and Manuscripts .....                                                                                               | vii       |
| Acknowledgements.....                                                                                                                    | viii      |
| Abstract.....                                                                                                                            | ix        |
| Abbreviations.....                                                                                                                       | x         |
| <b>1. General introduction .....</b>                                                                                                     | <b>1</b>  |
| 1.1. Thesis overview .....                                                                                                               | 2         |
| 1.2. Organisation and function of the striatum .....                                                                                     | 3         |
| 1.2.1. The striatum is a major input nucleus from the basal ganglia.....                                                                 | 3         |
| 1.2.2. The integrative function of the striatum is supported by its topography .....                                                     | 6         |
| 1.2.3. The striatum consists of diverse neuronal populations .....                                                                       | 8         |
| 1.3. The meso-striatal dopaminergic system .....                                                                                         | 10        |
| 1.3.1. Midbrain dopaminergic neurons: akin yet distinct.....                                                                             | 10        |
| 1.3.1.1. Similar cell morphology but morphologically disparate projections.....                                                          | 10        |
| 1.3.1.2. Physiological properties .....                                                                                                  | 12        |
| 1.3.2. Striatal DA transmission .....                                                                                                    | 12        |
| 1.3.3. Local modulation of DA release .....                                                                                              | 14        |
| 1.3.4. Behavioural role of striatal DA release.....                                                                                      | 14        |
| 1.4. The striatal cholinergic system.....                                                                                                | 16        |
| 1.4.1. Striatal cholinergic interneurons and connectivity.....                                                                           | 16        |
| 1.4.2. Cholinergic receptors present in the striatum.....                                                                                | 19        |
| 1.4.2.1. mAChRs .....                                                                                                                    | 19        |
| 1.4.2.2. nAChRs.....                                                                                                                     | 20        |
| 1.5. ACh-DA interaction .....                                                                                                            | 23        |
| <b>2. General methods.....</b>                                                                                                           | <b>28</b> |
| 2.1. Use of mouse as an animal model to investigate dopamine release mechanisms.....                                                     | 29        |
| 2.1.1. Ethical statement for the use of animals .....                                                                                    | 29        |
| 2.1.2. Mouse strains used in this body of work.....                                                                                      | 30        |
| 2.2. Stereotaxic intracranial injections for viral transfection of genetically encoded fluorescent sensors for imaging experiments ..... | 31        |
| 2.3. Live mouse brain slices preparation and solutions .....                                                                             | 33        |
| 2.4. Electrical stimulation on mouse brain slices.....                                                                                   | 34        |
| 2.5. Electrochemical detection of dopamine <i>ex vivo</i> in live mouse brain slices using fast-scan cyclic voltammetry (FCV) .....      | 35        |
| 2.5.1. FCV as a method to detect dopamine release in acute mouse brain slices.....                                                       | 35        |
| 2.5.2. Carbon fibre microelectrode (CFM) .....                                                                                           | 39        |
| 2.5.3. FCV recordings.....                                                                                                               | 40        |
| 2.6. Using the genetic encoded GRAB <sub>ACh</sub> sensor to record evoked acetylcholine transients in live mouse brain slices .....     | 41        |
| 2.6.1. About GRAB sensors and GRAB <sub>ACh</sub> .....                                                                                  | 41        |
| 2.6.2. Animals used for GRAB <sub>ACh</sub> imaging.....                                                                                 | 42        |
| 2.6.3. Imaging parameters to detect ACh release in the striatum using GRAB <sub>ACh</sub> .....                                          | 43        |
| 2.6.4. Data extraction and analysis for GRAB <sub>ACh</sub> imaging .....                                                                | 44        |

|                                                                                                                                                                        |            |
|------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------|
| 2.7. Statistical analyses .....                                                                                                                                        | 44         |
| 2.8. Drugs .....                                                                                                                                                       | 45         |
| <b>3. The Role of Axonal Voltage-gated Potassium Channels in the Modulation of Dopamine Release by Nicotinic Acetylcholine Receptors.....</b>                          | <b>47</b>  |
| 3.1. Introduction and initial hypothesis .....                                                                                                                         | 48         |
| 3.2. Methods .....                                                                                                                                                     | 52         |
| 3.2.1. Slice preparation .....                                                                                                                                         | 52         |
| 3.2.2. FCV recordings .....                                                                                                                                            | 52         |
| 3.2.3. GRAB <sub>ACh</sub> imaging.....                                                                                                                                | 52         |
| 3.2.4. Data acquisition and analysis.....                                                                                                                              | 53         |
| 3.2.5. Drugs.....                                                                                                                                                      | 53         |
| 3.3. Results .....                                                                                                                                                     | 54         |
| 3.3.1. VGKC inhibition only partially substitutes for nAChR activation when nAChRs are inhibited by DH $\beta$ E .....                                                 | 54         |
| 3.3.2. VGKC inhibition partially prevents the nAChR-mediated STD of DA release .....                                                                                   | 59         |
| 3.3.3. Effects of VGKCs blockade on DA release are not due to the change in ACh levels.....                                                                            | 64         |
| 3.3.4. Higher external potassium seems to lower 4p/1p .....                                                                                                            | 68         |
| 3.4. Conclusion and discussion.....                                                                                                                                    | 72         |
| <b>4. The Role of Axonal Voltage-gated Sodium Channels in the Modulation of Dopamine Release by Nicotinic Acetylcholine Receptors .....</b>                            | <b>74</b>  |
| 4.1. Introduction and initial hypothesis .....                                                                                                                         | 75         |
| 4.2. Methods .....                                                                                                                                                     | 80         |
| 4.2.1. Slice preparation .....                                                                                                                                         | 80         |
| 4.2.2. FCV recordings.....                                                                                                                                             | 80         |
| 4.2.3. GRAB <sub>ACh</sub> imaging.....                                                                                                                                | 81         |
| 4.2.4. Monitoring membrane voltage changes population of striatal dopaminergic axons using fluorescent voltage sensor.....                                             | 81         |
| 4.2.4.1. About voltage sensors and ASAP5f.....                                                                                                                         | 81         |
| 4.2.4.2. Animals used for voltage sensor experiments .....                                                                                                             | 83         |
| 4.2.4.3. Imaging parameters allowing the detection of membrane voltage changes in a population of dopaminergic axons in the striatum .....                             | 83         |
| 4.2.4.4. Data extraction and analysis for voltage sensor experiments.....                                                                                              | 84         |
| 4.2.4.5. Voltage sensor characterisation for recording in a population of axons.....                                                                                   | 87         |
| <i>i. ASAP5f signal reflects changes in membrane voltage.....</i>                                                                                                      | <i>87</i>  |
| <i>ii. Axonal transients are widened by VGKCs block .....</i>                                                                                                          | <i>88</i>  |
| <i>iii. The shape of the signal is stable over time despite overall <math>\Delta F/F_0</math> attenuation.....</i>                                                     | <i>90</i>  |
| 4.2.5. Reporting Ca <sup>2+</sup> dynamics from a population of striatal dopaminergic axons using ultra-fast Ca <sup>2+</sup> imaging .....                            | 94         |
| 4.2.5.1. About jRCaMP8m sensor .....                                                                                                                                   | 94         |
| 4.2.5.2. Animals used to detect ultra-fast Ca <sup>2+</sup> transient in a population of dopaminergic axons in the striatum .....                                      | 95         |
| 4.2.5.3. Imaging parameters for ultra-fast jRCaMP8m experiments .....                                                                                                  | 95         |
| 4.2.5.4. Data extraction and analysis for the ultra-fast jRCaMP8m imaging.....                                                                                         | 96         |
| 4.2.6. Drugs.....                                                                                                                                                      | 97         |
| 4.3. Results .....                                                                                                                                                     | 99         |
| 4.3.1. nAChR activation support prolonged depolarisation of DA axons and limit spike height .....                                                                      | 99         |
| 4.3.1.1. nAChR activity provokes prolonged depolarisation of DA axonal membranes .....                                                                                 | 99         |
| <i>i. Single pulse stimulation provokes an axonal spike and prolonged depolarisation when nAChRs are activated, but only a spike when nAChRs are antagonised .....</i> | <i>99</i>  |
| <i>ii. The prolonged depolarisation is attenuated when ACh levels are reduced by oxotremorine ..</i>                                                                   | <i>104</i> |
| <i>iii. Probing occurrence of prolonged depolarisation .....</i>                                                                                                       | <i>106</i> |

|                                                                                                                                                                                  |            |
|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------|
| 4.3.1.2. nAChR activity limits successive population spike.....                                                                                                                  | 109        |
| 4.3.1.3. Forced opening of VGSCs restores prolonged depolarisation and supports population spike attenuation.....                                                                | 113        |
| 4.3.1.4. Partial conclusion .....                                                                                                                                                | 118        |
| 4.3.2. nAChR activation provokes Ca <sup>2+</sup> transient upon initial stimulation but prevents subsequent events despite further stimulation.....                             | 120        |
| 4.3.3. VGSC availability is limiting subsequent release when nAChRs are active.....                                                                                              | 127        |
| 4.3.3.1. Partial inhibition of VGSCs limits subsequent DA release when nAChRs are active.....                                                                                    | 127        |
| <i>i. Compromising the pool of VGSCs using low concentration of TTX disproportionately impairs release evoked by pulse-trains. ....</i>                                          | <i>128</i> |
| <i>ii. When nAChRs are antagonised by DH<math>\beta</math>E, TTX does not differently affect trains versus single pulse release.....</i>                                         | <i>130</i> |
| 4.3.3.2. Prolonging inactivation of VGSCs limits DA release when nAChRs are active.....                                                                                          | 131        |
| <i>i. When nAChRs are active, prolonging the inactivation of VGSCs reduces DA release evoked by a train more than for a single pulse.....</i>                                    | <i>131</i> |
| <i>ii. When nAChRs are antagonised by DH<math>\beta</math>E, prolonging inactivation of VGSCs does not differently affect DA release by single versus train pulses .....</i>     | <i>133</i> |
| 4.3.3.3. DAT-Cre animals have higher DA release levels as well as different VGSCs handling compared to WT C57BL6/J animals.....                                                  | 136        |
| <i>i. DAT-Cre animals present higher DA release levels, but blocking nAChRs using DH<math>\beta</math>E still produces the effects expected from WT experiments .....</i>        | <i>136</i> |
| <i>ii. In DAT-Cre animals, partially impeding the pool of VGSCs available for re-release using TTX does not disproportionately affect DA release evoked by pulse-trains.....</i> | <i>139</i> |
| <i>iii. In DAT-Cre animals, prolonging inactivation of VGSCs only affects the 4p/1p ratio in NAcC. ....</i>                                                                      | <i>143</i> |
| <i>iv. In NAcC of DAT-Cre animals, prolonging inactivation of VGSCs reduces height of fourth spike during pulse-train.....</i>                                                   | <i>145</i> |
| 4.3.4. VGSC blockers are not acting through differential impact on ACh release .....                                                                                             | 148        |
| 4.3.5. VGSC blockers affect DA release independently of GABA receptor control.....                                                                                               | 152        |
| 4.4. Conclusion and discussion.....                                                                                                                                              | 156        |
| 4.4.1. Synchronous population spikes evoked by electrical stimulation .....                                                                                                      | 157        |
| 4.4.2. The nAChR-mediated prolonged depolarisation at a population of DA axons: occurrence at each axon, or asynchronous population signal?.....                                 | 158        |
| 4.4.3. Amplitude of nAChR-mediated prolonged depolarisation .....                                                                                                                | 160        |
| 4.4.4. Regional differences in response to nAChR activation .....                                                                                                                | 161        |
| 4.4.5. Subsequent population spike during pulse-trains: deciphering actual membrane voltage changes from sensor dynamics.....                                                    | 162        |
| <b>5. Inter-specie approach: Exploring ACh-DA interaction in <i>Drosophila melanogaster</i> .....</b>                                                                            | <b>165</b> |
| 5.1. Introduction .....                                                                                                                                                          | 166        |
| 5.1.1. Function and anatomy of the fly's centre for associative learning and memory: the mushroom body (MB) .....                                                                | 166        |
| 5.1.2. Role of DANs in the MB.....                                                                                                                                               | 168        |
| 5.1.3. ACh-DA interactions within the MB .....                                                                                                                                   | 171        |
| 5.1.4. Comparison of nAChR subunit enrichment in DANs within the MB in flies and striatum in mice.....                                                                           | 172        |
| 5.1.5. Overarching aims of the inter-specie approach .....                                                                                                                       | 174        |
| 5.2. Methods .....                                                                                                                                                               | 175        |
| 5.2.1. Fly strains .....                                                                                                                                                         | 175        |
| 5.2.2. Live functional imaging in <i>Drosophila</i> .....                                                                                                                        | 176        |
| 5.2.3. Odour delivery and foot shock protocols used for functional imaging in <i>Drosophila</i> .....                                                                            | 179        |
| 5.2.4. Analysis of functional imaging in live flies.....                                                                                                                         | 181        |
| 5.2.5. Single cell data extraction and analysis.....                                                                                                                             | 182        |
| 5.3. Results .....                                                                                                                                                               | 183        |
| 5.3.1. Establishing ACh and DA release patterns from odour responsive Kenyon cells in behaving flies .....                                                                       | 183        |
| 5.3.1.1. Kenyon cells ACh release dynamics upon short odour presentation .....                                                                                                   | 183        |



|                                                                                                                                                             |            |
|-------------------------------------------------------------------------------------------------------------------------------------------------------------|------------|
| 5.3.1.2. Kenyon cells ACh release dynamics upon prolonged odour presentation .....                                                                          | 185        |
| 5.3.1.3. Odour presentation triggers DA release from DANs .....                                                                                             | 187        |
| 5.3.2. Exploring modalities for fine tuning of DA release from aversive DANs .....                                                                          | 189        |
| 5.3.2.1. DA release evoked by shocks is not impacted by consequent odour presentation.....                                                                  | 189        |
| 5.3.2.2. Internal state alters DA release evoked by foot shocks.....                                                                                        | 193        |
| <i>i. DA release evoked by foot shocks is heightened from baseline in starved animals .....</i>                                                             | <i>193</i> |
| <i>ii. In starved animals, DA release evoked by shocks is lowered when shocks are provided in coincidence with an odour stimulus.....</i>                   | <i>196</i> |
| 5.4. Conclusion and discussion.....                                                                                                                         | 200        |
| 5.4.1. ACh release plasticity in odour responses. ....                                                                                                      | 201        |
| 5.4.2. No difference in shock response in the presence or absence of ACh in the environment in fed flies: technical limitations or biological process?..... | 202        |
| <b>6. General discussion.....</b>                                                                                                                           | <b>204</b> |
| 6.1. Revisiting the involvement of VGKCs in the nAChR-mediated control of DA release .....                                                                  | 206        |
| 6.2. Potential <i>in vivo</i> implications of the prolonged depolarisation mediated by nAChRs activation at DA axons .....                                  | 207        |
| 6.2.1. The controversial role of nAChRs on DA release <i>in vivo</i> .....                                                                                  | 207        |
| 6.2.2. Time-course and kinetics of nAChR-mediated depolarisation at a population of striatal DA axons .....                                                 | 209        |
| 6.2.3. What can we infer about desensitisation of nAChRs? .....                                                                                             | 210        |
| 6.2.4. The involvement of VGSCs in nAChR-mediated STD of DA release could also restrict signal propagation at DA axons .....                                | 211        |
| 6.3. Dopamine and ACh in Parkinson's disease .....                                                                                                          | 214        |
| 6.4. Conclusion.....                                                                                                                                        | 215        |
| <b>References .....</b>                                                                                                                                     | <b>217</b> |

## **Statement of Authorship**

This thesis does not contain any material that has been accepted for the award of any other degree in any University or other institution. All data represented in this thesis are fully my own work, and where data has been contributed by others their contribution is stated below and in the main text.

## Conference Abstracts and Manuscripts

### Conference Abstracts

*Exploring the Role of Axonal Voltage-gated Sodium Channels in the Modulation of Dopamine Release by Presynaptic Acetylcholine Nicotinic Receptors*, **L. Duquenoy**, M. Khurram, B. O'Connor, Y. Hao, S. Lee, W. Wu, M.Z. Lin, K.R. Brimblecombe, S.J. Cragg.

**Poster presentation. Wellcome Trust Collaborators meeting, Oxford, UK, 2024**

*Exploring the Role of Axonal Voltage-gated Sodium Channels in the Modulation of Dopamine Release by Presynaptic Acetylcholine Nicotinic Receptors*, **L. Duquenoy**, M. Khurram, B. O'Connor, Y. Hao, S. Lee, W. Wu, M.Z. Lin, K.R. Brimblecombe, S.J. Cragg

**Poster presentation. FENS Federation for European Neuroscience Conference, Vienna, Austria, 2024**

*Compensatory enhancement of input maintains aversive dopaminergic reinforcement in hungry Drosophila*, E. Meschi, **L. Duquenoy**, N. Otto G. Dempsey and S. Waddell.

**Poster presentation. Asia-Pacific Drosophila Neuroscience Conference, Tokyo, Japan, 2024**

*Exploring how presynaptic cholinergic input onto dopaminergic axons impacts dopamine release in the striatum in mice*, **L. Duquenoy**

**Talk. Medical Science Doctoral Training Program Symposium, Oxford, UK, 2023**

*Exploring how presynaptic cholinergic input modulates dopamine release in mice and Drosophila melanogaster*, **L. Duquenoy**, S. Waddell, S.J. Cragg

**Poster presentation. Monitoring Molecules in Neurosciences, Lyon, France, 2022**

### Manuscripts

**L. Duquenoy**<sup>†</sup>, B. O'Connor<sup>†</sup>, S.J. Cragg, *Voltage sensor imaging in a population of dopaminergic axons in the mouse striatum*, **protocols.io, 2024**. <sup>†</sup>Authors contributed equally to the work.

E. Meschi, **L. Duquenoy**, N. Otto G. Dempsey and S. Waddell. *Compensatory enhancement of input maintains aversive dopaminergic reinforcement in hungry Drosophila*, **Neuron, 2024**.

K. Jovanovski, **L. Duquenoy**, J. Mitchell, I. Kapoor C. Treiber, V. Croset, S. Parepalli, P. Cognigni, J. Felsenberg, S. Waddell. *Dopaminergic systems create reward seeking despite adverse consequences*, **Nature, 2023**.

K.R. Brimblecombe, A. Harris, N. Bengoa-Vergniory, B. O'Connor, **L. Duquenoy**, R. Anand, E. Lopes, B.M. Roberts, L. Burgeno, M. Walton, S.J. Cragg. *Gabapentinoids increase striatal dopamine release and enhance behavioural transitions in a mouse model of Parkinson's. In preparation.*

## Acknowledgements

There are many people I would like to thank, who made this DPhil a unique and truly enriching experience. It was one of the most challenging and rewarding times of my life, and I am grateful to everyone who support me through it.

First I would like to thank my supervisors Professor Stephanie Cragg and Scott Waddell for giving the opportunity to work under their supervision and supporting me through the admission process. In particular, I would like to thank Professor Stephanie Cragg for allowing me to join her laboratory on a full-time basis and for the financial support receives during my 4<sup>th</sup> year. Her incredibly valuable mentorship throughout this DPhil as well as her drive and passion for science are truly inspiring and helped me grow scientifically.

I am grateful to the 1+3 neuroscience programme, the Medical Science Division, the Goodger and Schorstein Scholarship for funding me during my DPhil, and to St John's College for the outstanding support they provided throughout my degrees in Oxford.

Many thanks also to Professor Frédéric Marion-Poll and Professor John Carlson who fostered my early scientific career as an undergraduate and graduate researcher.

I would also like to thank Dr Eleonora Meschi, Dr Ashwin Miriyala, Olivia McGinnis and Lea Ballenberger for their experimental and moral support within the fly labs.

I would like to give an immense thank you to all members of the Cragg lab, past and present. I feel incredibly lucky to have found such a supportive, collaborative and respectful lab environment. I would like to thank Dr Katherine Brimblecombe for her invaluable advice, support, and for introducing me to funky British expressions. I would like to thank Bethan O'Connor, Jess Livesey and Shinil Raina for their incredible support throughout the DPhil, and in particular Bethan O'Connor who has been my partner in crime, and without whom I would not have made it this far.

To all my friends in Oxford, thank you for making this experience a wonderful one.

To all my family and my friends from home for their love and their support over the past few years. Thank you for allowing me to complain, thank you for coming to visit, thank you for being here for me, even when you can't be there physically.

To my parents, Véronique et Jean-Pierre, for their unconditional love and for your unwavering support throughout my entire academic path and DPhil. Merci! Je vous aime.

Last, but certainly not least, to the love of my life Ashwin. You have made each and every single day of this journey better and brighter. I am immensely grateful for your support and love, which helped me through the hardest times and gave me the confidence to finish this DPhil!

## Abstract

Mesostriatal dopamine (DA) transmission plays key roles in the functions of the basal ganglia such as reward prediction signalling and regulation of goal-directed behaviour. Dysregulation of DA release is implicated in numerous neurological and psychiatric disorders such as Parkinson's disease and addiction. DA signalling, in addition to being driven through action potential (AP) generated at the cell body, can be gated locally by neuromodulators present within the striatum. Elucidation of mechanisms modulating axonal DA release is crucial to further understand the various role for DA signalling within the basal ganglia, and could help uncover potential therapeutic targets.

In the mammalian striatum, acetylcholine (ACh) released from cholinergic interneurons (ChIs) is a modulator of DA release through nicotinic acetylcholine receptors (nAChRs) located on striatal DA axons. *Ex vivo* studies using fast-scan cyclic voltammetry (FCV) have shown that nAChRs can modify the frequency dependency of DA release. Indeed, nAChRs either promote DA release when stimulated at low frequencies or provoke a pronounced short-term depression (STD) of DA release when stimulated at higher frequencies. Although the downstream mechanism through which nAChRs mediate this dual effect on DA release remains unknown, recent work has suggested that nAChRs exert their effect through modification of DA axonal excitability. This thesis examines the potential role of voltage gated-potassium channels (VGKCs) and voltage-gated sodium channels (VGSCs) in the mechanisms involved in how nAChRs operate on striatal DA axons in mice.

In **chapter 3**, I investigate the potential role of VGKCs. I found in mouse *ex vivo* striatum that altering VGKCs function affected dopamine release in similar ways whether nAChRs were active or antagonised, suggesting that VGSCs are not the driving force behind the effects of nAChRs on DA release.

Next, in **chapter 4**, I examined the potential role of VGSCs. I describe the implementation of a new genetically encoded voltage indicator (GEVI), ASAP5, which allowed direct interrogation of the membrane voltage of populations of striatal DA axons. Using this technique, along with calcium imaging, I revealed that nAChR activation evoked a prolonged depolarisation of DA axons, which is accompanied by an intracellular  $\text{Ca}^{2+}$  transient. I also found that limited VGSC availability following nAChR-mediated depolarisation is a limiting factor for population spike height during pulse-trains. These smaller population spike also triggered smaller  $\text{Ca}^{2+}$  transients and consequently less DA release, providing an explanation for the nAChR-mediated STD of DA release.

Finally, in **chapter 5**, I streamlined a novel approach to study the ACh-DA interaction by investigating these potentially conserved mechanisms in the simpler brain of *Drosophila melanogaster*.

Data presented in this thesis provided novel insights into the nAChR modulation of DA release and suggests a potential mechanism involving VGSCs. Furthermore, the implementation of new voltage imaging in a population of axons opens a range of new hypothesis to be tested concerning DA axonal physiology.

## Abbreviations

|                       |                                                                   |
|-----------------------|-------------------------------------------------------------------|
| 1p                    | single pulse                                                      |
| 4p                    | train of 4 pulses                                                 |
| 4-AP                  | 4-aminopyridine                                                   |
| AAV                   | Adeno-associated virus                                            |
| ACh                   | Acetylcholine                                                     |
| AChE                  | Acetylcholinesterase                                              |
| AP                    | Action potential                                                  |
| aCSF                  | Artificial cerebrospinal fluid                                    |
| ASAP5f                | Accelerated Sensor of Action Potentials version 5 fast            |
| AUC                   | Area under the curve                                              |
| CFM                   | Carbon fibre microelectrode                                       |
| ChI                   | Cholinergic interneuron                                           |
| cpFP                  | Circularly permuted fluorescent protein                           |
| cpGFP                 | Circularly permuted green fluorescent protein                     |
| D1R                   | DA D1-receptor                                                    |
| D2R                   | DA D2-receptor                                                    |
| D5R                   | DA D5-receptor                                                    |
| DA                    | Dopamine                                                          |
| [DA] <sub>o</sub>     | Extracellular dopamine                                            |
| DAN                   | Dopaminergic neuron                                               |
| DAT                   | Dopamine transporter                                              |
| DH $\beta$ E          | Dihydro- $\beta$ -erythroidine                                    |
| DLS                   | Dorso-lateral striatum                                            |
| EPSPs                 | Excitatory postsynaptic potentials (EPSPs or axEPSPs when axonal) |
| FCV                   | Fast-scan cyclic voltammetry                                      |
| GABA                  | $\gamma$ -aminobutyric acid                                       |
| GABA <sub>A</sub> -Rs | $\gamma$ -aminobutyric acid A receptors                           |
| GABA <sub>B</sub> -Rs | $\gamma$ -aminobutyric acid B receptors                           |
| GCaMP8m               | intracellular calcium sensor version 8 medium                     |
| GECI                  | Genetically encoded calcium indicator                             |
| GEVI                  | Genetically encoded voltage indicator                             |

|                                |                                                                                                                              |
|--------------------------------|------------------------------------------------------------------------------------------------------------------------------|
| GPCR                           | G protein-coupled receptor                                                                                                   |
| GPe                            | External segment of the globus pallidus                                                                                      |
| GPi                            | Internal segment of the globus pallidus                                                                                      |
| GRAB                           | GPCR-activation-based sensor                                                                                                 |
| HPLC                           | High-performance liquid chromatography                                                                                       |
| IPI                            | Inter-pulse interval                                                                                                         |
| [K <sup>+</sup> ] <sub>o</sub> | Extracellular potassium channel                                                                                              |
| KCs                            | Kenyon Cells, intrinsic odour-responsive cholinergic neurons of the mushroom body                                            |
| LED                            | Light-emitting diode                                                                                                         |
| mAChR                          | Muscarinic acetylcholine receptor                                                                                            |
| MB                             | Mushroom Body, association and learning centre, comprised of KCs and divided into lobes and compartments.                    |
| MBONs                          | Mushroom Body Output Neurons, driving behavioural outcome.                                                                   |
| MCH                            | 4-methylcyclohexanol, odourant used for olfactory conditioning.                                                              |
| MSN                            | Medium spiny neuron                                                                                                          |
| NAc                            | Nucleus accumbens                                                                                                            |
| NAcC                           | Nucleus accumbens core                                                                                                       |
| nAChR                          | Nicotinic acetylcholine receptor                                                                                             |
| OCT                            | 3-Octanol, odourant used for olfactory conditioning                                                                          |
| Oxo-m                          | Oxotremorine                                                                                                                 |
| PAM                            | Protocerebral Anterior Medial, large cluster of > 100 mostly rewarding DANs innervating the horizontal lobes of the MB       |
| PD                             | Parkinson's disease                                                                                                          |
| PFn                            | Parafascicular nucleus                                                                                                       |
| PPL1                           | Protocerebral Anterior Lateral 1, cluster of 12 negatively reinforcing DANs innervating the heel and vertical lobe of the MB |
| PPN                            | Pedunculopontine nucleus                                                                                                     |
| PT                             | Pyramidal tract                                                                                                              |
| RPE                            | Reward prediction error                                                                                                      |
| Ruf                            | Rufinamide                                                                                                                   |
| ROI                            | Region of interest                                                                                                           |

|      |                                        |
|------|----------------------------------------|
| SEM  | Standard error of the mean             |
| SNc  | Substantia nigra pars compacta         |
| SNr  | Substantia nigra pars reticulata       |
| SST  | Somatostatin                           |
| STD  | Short-term depression                  |
| STN  | Subthalamic nucleus                    |
| TH   | Tyrosine hydroxylase                   |
| TTX  | Tetrodotoxin                           |
| VGKC | Voltage-gated K <sup>+</sup> channel   |
| VGSC | Voltage-gated Na <sup>+</sup> channel  |
| VGCC | Voltage-gated Ca <sup>2+</sup> channel |
| VTA  | Ventral tegmental area                 |
| WT   | Wild-type                              |



# 1

## General Introduction

## 1.1. Thesis overview

Mesostriatal dopamine (DA) transmission plays key roles in the reward prediction signalling and regulation of goal-directed behaviour. Dysregulation of DA signalling is implicated in multiple neurological and psychiatric disorders such as Parkinson's disease and addiction. Striatal DA release can be driven through action potential (AP) generated at the cell body but have also been shown be gated locally by neuromodulators present within the striatum (Sulzer et al. 2016). Elucidating mechanisms involved in the local modulation of striatal DA release could help uncover potential therapeutic targets and deepen our understanding of the behaviourally relevant roles for DA signalling.

The mammalian striatum is also particularly enriched in acetylcholine (ACh) released from striatal cholinergic interneurons (ChIs). Striatal DA and ACh reciprocally regulate each other's release with ACh modulating DA release through axonal nicotinic acetylcholine receptors (nAChRs) located on striatal DA axons. *Ex vivo* studies using fast-scan cyclic voltammetry (FCV) have shown that nAChRs can modify the frequency dependency of DA release (Rice and Cragg 2004; Zhang and Sulzer 2004). nAChRs can either promote DA release when stimulated at low frequencies or induce a pronounced short-term depression (STD) of DA release when stimulated at higher frequencies. nAChR promotes DA release by evoking release upon activation (Threlfell et al. 2012; Cachope et al. 2012; Wang et al. 2014b, a). However, despite few hypothesis – including vesicle pool depletion and changes to the probability of release (Cragg 2003; Wang et al. 2014b) – the mechanisms allowing nAChRs to also inhibit DA release are still unresolved.

Recent work has suggested that nAChRs exert their effect through modification of DA axonal excitability (Zhang et al. 2025). This thesis examines the potential role of voltage gated-potassium channels (VGKCs) (**chapter 3**) and voltage-gated sodium channels

(VGSCs) (**chapter 4**) in the mechanisms involved in how nAChRs exert their dual effects on striatal DA in mice. In **chapter 4**, I also describe the implementation of a new genetically encoded voltage indicator (GEVI), ASAP5f (Hao et al. 2024), which allowed direct interrogation of the membrane voltage of a population of striatal DA axons.

Finally, **chapter 5** is a standalone chapter in which I streamlined a novel approach to study the ACh-DA interaction by investigating these potentially conserved mechanisms in the brain of *Drosophila melanogaster*. The mushroom body (MB) is the centre for associative learning of the fly's brain, and exhibits functions similar to the striatum (Waddell 2010; Cognigni et al. 2018). Plus, the MB hosts relevant ACh-DA connections thought to be involved in aversive learning (Cervantes-Sandoval et al. 2017). The background information for this chapter will be covered in detail within **Chapter 5**, and this introduction chapter will solely focus on providing background concerning the mammalian striatum, as well as the origin, function and modulatory roles of DA and ACh within it.

## 1.2. Organisation and function of the striatum

### 1.2.1. The striatum is a major input nucleus from the basal ganglia

The mammalian basal ganglia is a highly evolutionary conserved and interconnected network of subcortical nuclei involved multiple behavioural processes such as reinforcement learning, habits formation (Yin and Knowlton 2006) and action selection (Graybiel et al. 1994a; Balleine et al. 2007). This structure is composed of three principal input nuclei: the striatum, the subthalamic nucleus (STN) and the substantia nigra pars Compacta (SNc) together with the ventral tegmental area (VTA); two output nuclei: the substantia nigra pars reticulata (SNr) and the internal globus pallidus (GPi); and finally, an

intrinsic nucleus: the external globus pallidus (GPe) (Redgrave and Costa 2021)(Simplified schematic of the basal ganglia connectivity in **Figure 1.1**).

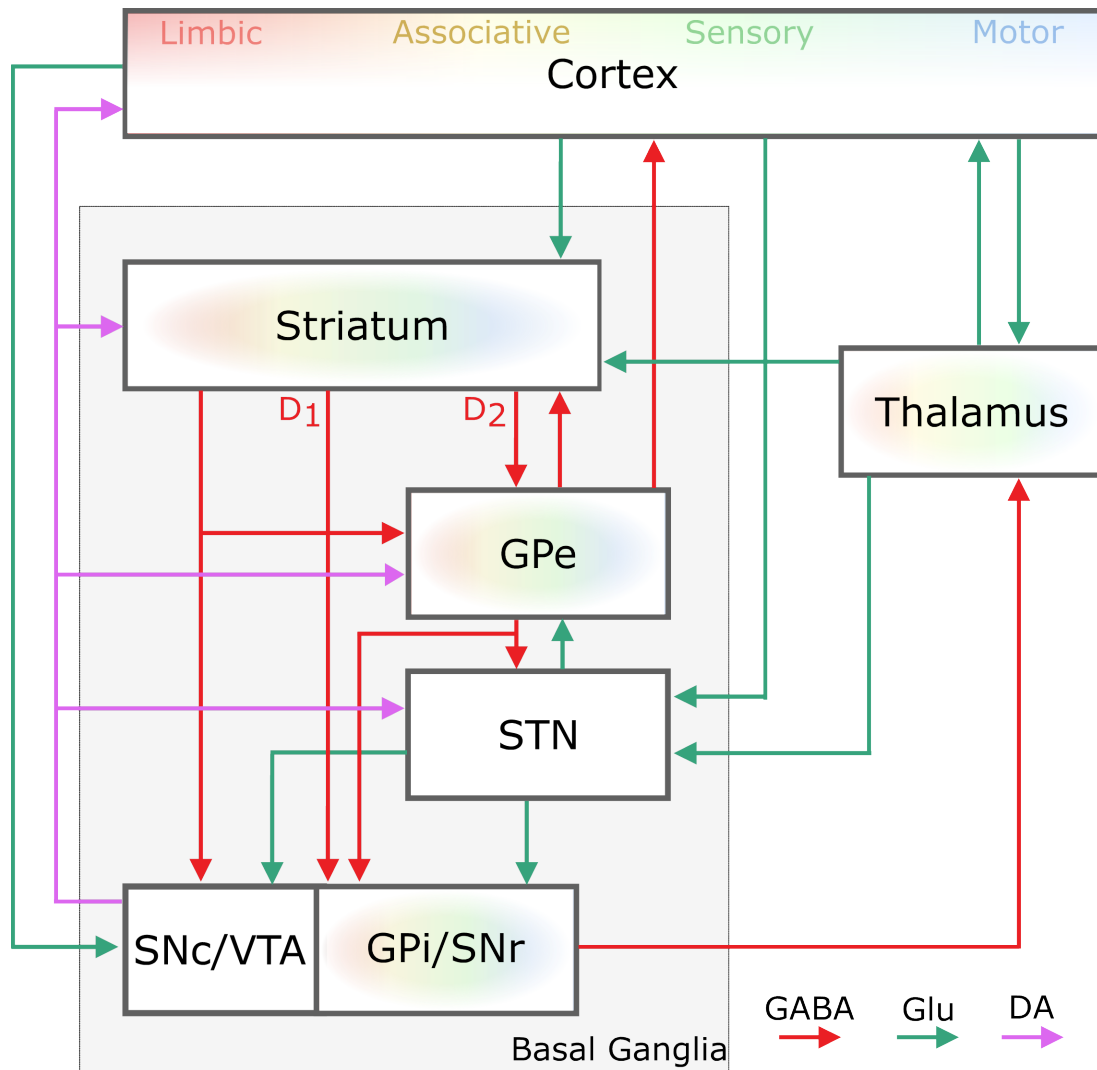
Numerous and diverse brain regions project to the basal ganglia input nuclei (Steiner and Tseng 2016; Redgrave and Costa 2021). The striatum – the largest nucleus of the basal ganglia – receives inputs varied in their provenance and in their nature. Indeed, it receives excitatory glutamatergic inputs from structures external to the basal ganglia including cortex, or thalamus, through which motivational, limbic and sensorimotor information are relayed; as well as inhibitory GABAergic inputs from the GPe, the intrinsic nucleus of the basal ganglia. The striatum receives projections from subcortical areas such as the brain stem, the amygdala and the hippocampus. It also receives critical modulatory inputs from dopaminergic neurons whose cell bodies are located in another input nucleus of the basal ganglia: the SNc/VTA in the midbrain. All of these inputs are computed by neurons intrinsic to the striatum and medium spiny neurons (MSNs) which send inhibitory GABAergic processes directly or indirectly to *all* other basal ganglia nuclei. The topography of the striatum will be further detailed in the next section **1.2.2** and **1.2.3**.

The STN is the second major input nuclei. Historically, it used to be considered mainly as a structure relaying indirectly to the striatum to the output nuclei. But recent studies (Bonnievie and Zaghloul 2019) have drawn a more complex picture of its connection within the basal ganglia and with external structures. It receives inputs from similar regions as the striatum but sends exclusively excitatory glutamatergic projections onto the output nuclei (SNr and GPi), the SNc and the intrinsic GPe (Kitai and Deniau 1981; Kita et al. 1983).

The third input nucleus from the basal ganglia is the SNc/VTA nucleus composed of dopaminergic neurons. They receive both inhibitory (from the striatum) and excitatory (from

the STN) inputs from within the basal ganglia. This region also takes inputs from multiple other brain regions such as cortex, brain stem and raphe nuclei (Floresco et al. 2003). SNc and VTA dopaminergic neurons project to the striatum where they provide crucial modulatory inputs (Watabe-Uchida et al. 2012; Poulin et al. 2018). More detailed description of their projections, physiological properties and role will be given in section **1.3**.

Finally, in brief, both the output nuclei the GPi and SNr project back to the thalamus through GABAergic inhibitory connections (Steiner and Tseng 2016). The GPe also send GABAergic projections to the cortex. Therefore, the basal ganglia main output projections are also where its main inputs arise from. The structure of the basal ganglia with its layered inhibitory connections associated with the excitatory re-entrant loops from cortex and thalamus enable inhibition or disinhibition of specific pathways resulting in the selection of appropriate behaviours. This selection process relies heavily on the computations occurring within the striatum.



**Figure 1.1: Simplified model of basal ganglia connectivity with cortex and thalamus.**

A simplified representation of the basal ganglia showing the between cortex, thalamus, and the basal ganglia nuclei (modified from Redgrave and Costa, 2021). Shaded colours at the back of compartments highlight the topographical continuum formed of cortical and thalamic inputs (limbic, associative, sensory and motor) that are conserved throughout the basal ganglia and form parallel re-entrant loops with the basal ganglia. Inhibitory GABAergic pathways are indicated in red, glutamatergic excitatory projections in green and dopaminergic in pink. GPe = external segment of the globus pallidus, STN = subthalamic nucleus, SNc = substantia nigra pars compacta, SNr = substantia nigra pars reticulata, GPi = internal segment of the globus pallidus, D1 = D1-like DA receptor (direct pathway from D1-positive striatal MSNs to output nuclei of basal ganglia), D2 = D2-like DA receptor (indirect pathway from D2-positive striatal MSNs to output nuclei of basal ganglia through GPe and STN).

### 1.2.2. The integrative function of the striatum is supported by its topography

As detailed above, the striatum is the main input nucleus of the basal ganglia and receives a wide range of inputs from sensory-motor, limbic and associative areas of the brain. These inputs are integrated within the striatum and then distributed to the output nuclei. Although

the striatum does not possess clear physical boundaries in rodents, it is subdivided into functionally different regions on the basis of their anatomical location, nature of their inputs and biochemical identities. The different regions of the cortex and thalamus project to the striatum in a limbic, associative and sensorimotor gradient along the ventromedial-dorsolateral axis (Voorn et al. 2004). This gradient overlaps with a dorso-ventral gradient of dopaminergic innervation with SNc innervating principally dorsal striatum and VTA innervating mainly the ventral striatum. Within each midbrain nuclei, a medio-lateral gradient also exists, with lateral parts of the SNc and VTA respectively innervating more lateral regions in the striatum (Farassat et al. 2019). This is a brief and simplified description of the highly complex topography and connectivity of the striatum, since studies also show that most lateral parts of SNc innervate the tail of the striatum along a rostro-caudal gradient (Poulin et al. 2018). Despite their varied inputs, these different territories share similar neuronal type composition (detailed in the next section), and the same striated aspect— from which the striatum gets its name. This suggests that while the inputs differ, the computations they go through within the striatum are likely to be similar, therefore creating highly parallel integrative pathways.

The dorsal striatum – containing the caudate nucleus and the putamen – has historically been associated with sensorimotor functions and action selection (Graybiel et al. 1994b; Knowlton et al. 1996; Yin and Knowlton 2006) while the ventral striatum – the nucleus accumbens (NAc) and olfactory tubercle – has traditionally been considered as a limbic-motor interface and as the seat of associative learning and motivation (McFarland et al. 2003; Sellings and Clarke 2003; Ikemoto 2007). Throughout this body of work, the dorso-lateral (DLS) and ventral (nucleus accumbens core, NAcC) striatum are investigated side-

by-side, and comparisons are drawn where disparities in responses to our manipulation arose.

### **1.2.3. The striatum consists of diverse neuronal populations**

The striatum is composed of four main types of cells. First, the main output neurons of the striatum – the medium spiny neurons (MSNs) – represent up to 95% of the striatal neurons. These GABAergic neurons are homogeneously distributed throughout the striatum. However, they also present a chemical heterogeneity which splits them into two populations based on the type of DA receptor they express, the neurotransmitter and peptides they can also release, and their projections. One population can release dynorphin and substance P on top of releasing GABA, expresses D1-like receptors at its membrane, and sends its inhibitory projections to GPi and SNr – the direct pathway projecting directly to output nuclei. The second population releases enkephalin and GABA, possesses D2-like receptors and projects to the GPe where it mostly inhibits its synaptic partners. This pathway is called the indirect pathway, as D2-expressing MSNs project to the GPe which then sends its own intrinsic neurons to the basal ganglia output nuclei. Although both of these populations receive similar inputs and send inhibitory output projections, the nature of the DA receptor they express dictate their role within the striatum and their target nuclei. Indeed, DA-receptors are G-protein coupled receptors, and their downstream pathway dictates their action on the cells. The D1-like receptor family (including D1 and D5-receptors) exert an excitatory effect on the cells that express them, while the D2-like receptor family (including D2, D3 and D4-receptors) are mainly inhibitory (Trantham-Davidson et al. 2004; Beaulieu and Gainetdinov 2011). Besides major differences in their modulation by dopamine, these two MSNs population have historically been segregated as two very distinct pathways: the



first one promoting movement (direct) while the other one is thought to suppress it (indirect) (Steiner and Tseng 2016). However, recent studies suggest a more nuanced approach as these apparently distinct pathways shared some downstream interconnectivity (D1-expressing MSNs sending collaterals to the GPe for instance **Figure 1.1**) with evidence both can, at appropriate times, signal for either start or stop of action sequences (Calabresi et al. 2014).

The remaining 5% of striatal neurons comprises various different types of interneurons (Tepper and Bolam 2004). Most striatal types of interneurons are GABAergic and are critically involved for the modulation of striatal output (Tepper et al. 2018). One type however is mainly cholinergic (although they can also partly co-transmit GABA and glutamate (Lozovaya et al. 2018). The cholinergic interneurons (ChIs) are large aspiny neurons which are tonically active and occupy 1-2% of the striatum making the striatum the brain region that is the most enriched in acetylcholine (ACh) (Contant et al. 1996). The major role of ChIs within the striatum, including their powerful modulatory role on DA axons, will be detailed in section **1.4** and **1.5**.

Finally, a cocktail of other neurotransmitters is present in the striatum such as serotonin – 5-hydroxytryptamine, 5-HT (Steinbusch 1981), noradrenaline, glycine, somatostatin etc. which all seem to modulate DA release, but for which the behavioural relevance is still unclear. Overall, the striatum possesses a complex neurotransmitter landscape with DA as main modulatory neurotransmitter playing a key role in dictating its output.

## **1.3. The meso-striatal dopaminergic system**

### **1.3.1. Midbrain dopaminergic neurons: akin yet distinct**

#### **1.3.1.1. Morphologically disparate projections but similar cell morphology**

The cell bodies of DA neurons innervating the striatum are located in the midbrain. These midbrain DA neurons also project to multiple other brain regions such as cortex, amygdala, hippocampus, lateral habenula, septum and sub-thalamic (Redgrave and Costa 2021). As mentioned previously (section **1.2.1**), SNc and VTA DA neurons whose cell bodies are located in the midbrain are the two main populations of DA neurons populating the striatum. In brief, SNc and VTA DA neurons innervate the striatum in a continuum from dorsal to ventral striatum respectively (Poulin et al. 2018). Within each regions of the midbrain, cell bodies located on a rostro-lateral axis can also innervate the striatum along a rostro-caudal gradient (Poulin et al. 2018). DA neurons present within the VTA and SNc can also be segregated in smaller according to the topography of their projections, their various biochemical and genetical markers and their potential behavioural relevance (Poulin et al. 2014; Anderegg et al. 2015; Avisati et al. 2024).

Midbrain DA neurons receive inputs from various areas of the brain including glutamatergic excitatory inputs from the subthalamic nucleus, inhibitory GABAergic inputs from the striatum and glutamatergic and cholinergic inputs from the pedunculopontine nucleus (Floresco et al. 2003) and the STN. The two midbrain DA neurons population also receive different inputs, and one study using retrograde tracing from midbrain DA neurons showed that DA neurons whose cell bodies are located in the SNc receive strong excitatory

inputs from the somatosensory and motor cortices, as well as subthalamic nucleus, and DA neurons whose cell bodies are located in the VTA receive more inputs from lateral hypothalamus (Watabe-Uchida et al. 2012). Finally, their activity is also regulated by GABAergic interneurons which also project to the striatum (Nair-Roberts et al. 2008).

Despite displaying different input and output connections, both populations share similar morphology in that they extend a single unmyelinated axon to the striatum, where they form colossal and extremely branched axonal arbours. SNc neurons sending their axons to the DLS might display a higher level of axonal branching points than VTA neurons projecting to the NAcC (Yetnikoff et al. 2014; Rodríguez-López et al. 2017). Nevertheless, it has been estimated that the axon of single DA neuron from a rat brain can occupy up to 5-6% of the total volume of the striatum (Matsuda et al. 2009), and form up to form  $\sim 10^5$  en passant varicosities (Matsuda et al. 2009; Pissadaki and Bolam 2013). DA neurons form a small number of classical synapses (Descarries et al. 1996; Bérubé-Carrière et al. 2012) and are thought to mostly signal through volume transmission (Cragg and Rice 2004; Bérubé-Carrière et al. 2012). Indeed, DA neurons form a small number of symmetrical synapses, with 60-70% of axonal varicosities being asynaptic (Descarries et al. 1996). Such large and branched axonal arbours have extremely high metabolic demands for the upkeep of their arborisations (Pissadaki and Bolam 2013), and it is therefore likely that this unique morphology serves a major role in striatal computations. Indeed, on top of relaying information from the midbrain, DA release from these largely branched axons have been shown to be heavily modulated locally within the striatum (Rice et al. 2011; Sulzer et al. 2016).

### 1.3.1.2. Physiological properties

*In vivo*, DA neurons from VTA and SNc both display tonic firing patterns driven by intrinsic pacemaking properties. In rats, tonic firing has been shown to range between 1 to 10 Hz (Grace and Bunney 1984a, b; Hyland et al. 2002). They also exhibit a switch from tonic to burst firing at 20-40 Hz following excitatory inputs, although higher intra-burst intervals up to 100 Hz have also been reported (Hyland et al. 2002). The action potential (AP) generated by midbrain DA neurons are generally relatively broad (>2ms) (Grace and Bunney 1984b; Lacey et al. 1989).

Midbrain DA neurons also display heterogeneity in their firing patterns. For instance, SNc neurons exhibit large spikes with a strong after-hyperpolarisation (AHP) while VTA neurons exhibit smaller spikes with shallower AHP, and a lower tonic firing rate (Lammel et al. 2008). These neurons also exhibit disparate vulnerability towards neurodegeneration, as only SNc DA neurons are affected by abnormal cell death in Parkinson's disease (PD) for instance. Additionally, within each region (SNc and VTA), several subgroups of DA neurons can be drawn based on firing patterns and gene expression patterns (Roeper 2013; Poulin et al. 2014, 2018), painting a complex map of finely tuned but widely distributed dopaminergic neuromodulation. In *ex vivo* striatal slices however, axons are severed from their cell bodies and do not exhibit tonic firing patterns.

### 1.3.2. Striatal DA transmission

In the mammalian striatum, dopamine transmission occurs primarily through volume transmission (Cragg and Rice 2004) at the  $\sim 10^5$  en passant varicosities formed by the extensive axonal arbours of each DA neuron (Matsuda et al. 2009; Pissadaki and Bolam 2013). Electron-microscopy studies have shown that these varicosities are mostly

asynaptic, *ie.* where vesicles are condensed in the pre-synaptic terminal, no clear post-synaptic density can be found in potential post-synaptic partners, which further supports the idea that DA exerts its effects on striatal networks mainly through volume transmission (Pickel et al. 1981; Bérubé-Carrière et al. 2012). Furthermore, studies have demonstrated that DA transporters (DAT) and DA receptors are mostly localised outside of striatal synapses (Yung et al. 1995; Caillé et al. 1996; Uchigashima et al. 2016) suggesting that DA can diffuse in the extracellular matrix. Release machinery at DA active zones is however thought to operate broadly similarly to those of classical synapses, with  $\text{Ca}^{2+}$ -dependent release of a readily releasable pool of DA-containing vesicles (Liu et al. 2021), although there are distinctions in the presynaptic molecular machinery required (Robinson et al. 2019; Delignat-Lavaud et al. 2023). However, only 30 % of these varicosities have been found to have active release sites with the necessary machinery for action potential evoked DA release (Liu et al. 2018). Finally, a study found that electrical stimulation of *ex vivo* striatal mouse brain slices only triggered release from approximately 20% of the varicosities at DA axons, despite most of them displaying similar  $\text{Ca}^{2+}$  event following the stimulation (Pereira et al. 2016). Some varicosities unresponsive to electrical stimulation did however display DA release when stimulated through KCl application, suggesting that some silent varicosities also possess release mechanisms (Pereira et al. 2016).

Dopamine exerts its effects on cells and afferents in the striatum through a range of G-protein coupled receptors with various affinities for DA, classified in two groups according to their downstream pathways. The D1-like receptors – comprising D1- and D5-receptors – couple to  $\text{G}_{\alpha_{s/olf}}$  family of G-proteins which stimulate cAMP production by adenylyl cyclase. They are exclusively localised on post-synaptic cells (such as MSNs form the direct pathway

for instance) and are generally thought to have an excitatory effect on the cells in which they are expressed. The D2-like receptors comprise the D2-, D3- and D4-receptors that activate the  $G_{\alpha_{i/o}}$  family of G-proteins which inhibit adenylyl cyclase and are thought to have a generally inhibitory effect. The D2-like receptors can be located post-synaptically (on MSNs forming the indirect pathway or ChIs for instance) but are also found on DA axons where they act as inhibitory auto-receptors.

### **1.3.3. Local modulation of DA release**

Generation of APs by midbrain dopaminergic cell bodies does not always faithfully translate to DA release in the striatum (Rice et al. 2011; Sulzer et al. 2016; Mohebi et al. 2023). The relay of APs from the midbrain to release sites of extensively branched DA axonal arbours located in the striatum is affected by the local modulation. DA release is under local regulation of a large range of neurotransmitters and neuromodulators present in the striatum (Rice et al. 2011) such as small molecules (for instance ATP, nitric oxide, hydrogen peroxide, glycine); peptides like substance P or somatostatin; monoamines including serotonin and noradrenaline; and neurotransmitters such as ACh, glutamate or GABA (Chesselet and Reisine 1983; Avshalumov et al. 2008; Brimblecombe and Cragg 2015; Lopes et al. 2019; Roberts et al. 2020).

### **1.3.4. Behavioural role of striatal DA release**

Seminal studies have highlighted a role for dopaminergic activity in reward learning. A characteristic of DA neurons is that they switch firing mode from tonic to phasic (15-100 Hz) upon presentation of a reward or of a cue previously associated with a reward, but on the contrary switch to a pause when an expected reward is omitted (Schultz 1986; Hyland et al.

2002; Morris et al. 2004). Furthermore, DA can encode for the salience of the reward (Morris et al. 2004). Studies have also involved DA in reward prediction error (RPE) which consists in predicting expected reward based on past-experiences and confront this expectation with the actual received reward (Schultz 2016). This process helps reinforcing behaviours that are appropriate to a specific context (Schultz 2016). DA neurons have been shown to respond to various types of reward such as food, water, sex (Miyasaka et al. 2025) but have also been shown to respond to aversive signals such as bitter taste (quinine), tail pinches or foot shocks (Lopez and Lerner 2025). DA is also reported to code the motivational state of the animal (Mohebi et al. 2023). In this context, disruption to the DA system can also lead to addiction and expression of abnormal motivational drive in humans (DSM-IVR 2013). In mice, self-administration of optogenetic activation of the midbrain DA neurons can also provoke compulsive behaviours (Pascoli et al. 2015).

Activity at DA neurons have also been shown play a role in movement. Historically, this has been discovered through the motor-symptoms of PD and the realisation that these symptoms are linked to the characteristic loss of DA neurons (Barbeau 1962; Hornykiewicz 1998). In PD, the loss of DA release in the DLS causes a reduced activation of direct pathway D1-expressing MSNs, and a loss of inhibition to the D2-expressing MSNs (indirect pathway), which results in abnormal firing rate across multiple basal ganglia nuclei and is thought to be the cause of motor defects in PD (McGregor and Nelson 2019). Studies have highlighted an increased phasic firing and DA release at movement onset (Dodson et al. 2016; Howe and Dombeck 2016), and in motor learning.

Finally, the behavioural relevance for tonic firing of DA neurons is not fully understood. Tonic activity is thought to provide higher occupancy at D2-like striatal receptors compared to occupancy of the D1-like receptors (Dreyer et al. 2010). A study in primates monitoring

DA release during a Pavlovian task suggest that tonic levels can help monitor the continuous changes in a reward value (Wang et al. 2021). A more recent study using RIM knock-out mice (a protein necessary for the organisation of an active release zone), which strongly reduces AP-driven DA release, suggests that AP-independent DA release is sufficient for movement initiation but not for reward-motivated behaviours (Cai et al. 2024). This study however does not provide further explanation concerning the mechanisms through which AP-independent release would occur, as both phasic and tonic release modes are thought to depend on AP generation either by burst firing for phasic release or through AP generated by the pace-marker properties of DA neurons for tonic firing (Grace and Bunney 1984a, b). Disentangling the behavioural role of tonic versus phasic DA is technically challenging, and often techniques with lower temporal resolution – such as microdialysis and high performance liquid chromatography – are thought to reflect tonic changes, but these can more difficultly be correlated with specific behaviours (Berke 2018). Finally, the role of tonic DA release could vary across regions within the striatum as different frequency of slow oscillations have been reported in behaving mice across dorsal and ventral striatum (Jørgensen et al. 2023). More studies would be needed to more finely dissect the role of tonic firing of DA neurons and tonic DA release.

## **1.4. The striatal cholinergic system**

### **1.4.1. Striatal cholinergic interneurons and connectivity**

ChIs represent only 1-2% of the overall striatal neuron population (Phelps et al. 1985; Contant et al. 1996). Their small number is however compensated by their large spineless dendritic trees and their extensive axonal arbours (Kawaguchi 1993). Although they can form



classical synapses with their downstream partners, ChIs also use volume transmission (Contant et al. 1996; Descarries et al. 1997) to exert a strong influence on striatal networks. Although their effect can be carried over a wide range of targets spread in a large volume, the presence of acetylcholine esterase (AChE) greatly limits the spread of ACh by breaking down the neurotransmitter at incredibly fast rates (Quinn 1987; Nosaka and Wickens 2022). AChE density is not uniform across striatum and delineates microdomains called striosome (lacking in AChE) and matrix (enriched in AChE) (reviewed in Brimblecombe and Cragg 2017). ChI distribution is not homogeneous throughout the striatum as ChIs exhibit a gradient of decreased density along the antero-posterior axis (Matamales et al. 2016).

Physiologically, ChIs can be characterised by their large spikes followed by strong hyperpolarising currents (Kawaguchi 1993). ChIs display multiple autonomous firing patterns including burst firing, irregular firing and tonic firing (Goldberg and Reynolds 2011). These spontaneous firing modes have little dependence on the inputs (Sharott et al. 2012), and ChIs can tonically discharge even in *ex vivo* brain slices (Kramer et al. 2022).

In mammals, ChIs receive glutamatergic inputs from the cortex and the thalamus (in particular from the parafascicular (Pf) nucleus in rat) mediated through both ionotropic and metabotropic receptors (Kawaguchi 1993). ChIs also receive GABAergic inputs through GABA<sub>A</sub>-receptors from striatal interneurons and MSNs (Szydlowski et al. 2013) and in the NAcC at least, ChIs also receive inputs from GABAergic interneurons located in the VTA (Brown et al. 2012). ChIs are responsive to 5-HT through metabotropic 5-HT<sub>2,6,7</sub> receptors (Bonsi et al. 2007). Finally, ChIs are also strongly modulated by dopaminergic inputs, mainly through D2-receptors and D5-receptors which are respectively inhibitory and excitatory GPCRs. In rodent striatal slices, DA acting at D2-receptors have been demonstrated to

inhibit Chl firing (Maurice et al. 2004; Deng et al. 2007), while other reports have indicated that bath application of DA can excites Chls through D5-receptors (Centonze et al. 2003).

One of the most notable characteristics of Chls is their *pause* (~200 ms decrease tonic firing) in response to a learned association between an external stimulus and a reward or a punishment (Kimura et al. 1984; Aosaki et al. 1994; Apicella et al. 1997; Morris et al. 2004; Szydlowski et al. 2013) This conditioned pause response can in some cases be preceded by a short increase in Chls activity and can eventually be followed by a rebound activity as well. This pause is also a hallmark of the DA-ACh interaction since it is synchronised to a burst of activity in DA neurons and DA is necessary for the pause to be acquired during learning (Aosaki et al. 1994). The characteristics of the pause – in amplitude or latency for instance – evolve depending on the stage of learning at which the recording occurs and the type of task animals are taught (Zhang and Cragg 2017). Most importantly the characteristics of the pause does not vary according to the same parameters as DA phasic events, indicating they both code for different aspects of the reward prediction error (Zhang and Cragg 2017). Although DA has been considered to be the driving force for this pause mainly through involvement of inhibitory D<sub>2</sub>-receptors on Chls, other candidates such as cortical and thalamic inputs to the striatum are considered as well. Indeed, stimulation of the thalamus or striatal Pf afferent has been shown to induce a pause in the striatum both *in vitro* and *in vivo* (Ding et al. 2010; Doig et al. 2014; Kosillo et al. 2016). Clarity on the mechanisms underlying the pause has yet to be put forward.

Overall, the behavioural role for striatal Chls is still incompletely resolved, but they are thought to be involved in behavioural flexibility, reward memory and memory extinction

(Morris et al. 2004; Schulz and Reynolds 2013; Aoki et al. 2015; Fleming et al. 2022; Bouabid et al. 2025).

### **1.4.2. Cholinergic receptors present in the striatum**

Although ChIs have been demonstrated to also co-release GABA (Lozovaya et al. 2018) and glutamate (Gras et al. 2002), ChIs exerts their postsynaptic effect mainly through ACh release, at muscarinic ACh receptors, which are modulatory G-protein coupled receptors (GPCRs) and nicotinic ACh receptors (nAChRs), which are fast ligand-gated cation channels (Hille 2001). These channels have been named after agonists – muscarine and nicotine – that mimic action of ACh upon binding to respectively mAChRs and nAChRs. Both channels are expressed on most cell types within the striatum (Poppi et al. 2021).

#### **1.4.2.1. mAChRs**

The mAChRs are a family of GPCRs consisting in five subtypes ( $M_1$ - to  $M_5$ -AChRs) divided into the  $M_1$ -like receptors ( $M_1$ -,  $M_3$ - and  $M_5$ -AChRs) which couple to  $G_{q/11}$  and are regarded as excitatory receptors, and the  $M_2$ -like receptors ( $M_2$ - and  $M_4$ -mAChRs) which couple to  $G_{i/o}$  with a generally inhibitory impact. These receptors operate a long-term modulatory role on the cells on which they are expressed, either enhancing or limiting their excitability (Lim et al. 2014).

The terminals of glutamatergic afferents innervating the striatum express  $M_2$ - and  $M_4$ -mAChRs through which glutamate release is attenuated, which in turn limits its excitatory drive, in particular on MSNs (Assous 2021).  $M_2$ - and  $M_4$ -mAChRs are also present on ChIs and act as autoinhibitory receptors to prevent excessive ACh release (Yan and Surmeier 1996; Ding et al. 2006).

ChIs form direct synapses with MSNs(Izzo and Bolam 1988), which express both  $M_1$ - (excitatory) and  $M_4$ -mAChRs (inhibitory) which exert opposing effects on their excitability (Assous 2021).

Finally,  $M_5$ -mAChRs mRNA is expressed by DA neurons (Vilaró et al. 1990; Weiner et al. 1990). However, the role  $M_5$ -mAChRs on DA axons and DA release is poorly characterised (Shin et al. 2015; Zell et al. 2023). However, auto-activation of inhibitory  $M_2$ - and  $M_4$ -mAChRs at ChIs, by lowering ACh release, can affect DA release through nAChRs located on DA axons (Threlfell et al. 2010).

#### 1.4.2.2. nAChRs

The nAChRs are part of a conserved family of pentameric ligand-gated ion-channels with similar architecture to glycine receptors or GABA-A receptors or 5-HT<sub>3</sub> receptors for instance (Hille 2001; Dani 2015). In mammals, at least 16 genes codes for homologous yet distinct nAChR-subunits which are divided into 5 subunit types: the  $\alpha$ ,  $\beta$ ,  $\delta$ ,  $\gamma$  or  $\epsilon$ -subunits. In the central nervous system, only a panel of 12  $\alpha$ - and  $\beta$ -subunits are present ( $\alpha 1$ -  $\alpha 7$  and  $\beta 2$  –  $\beta 4$ ) (Corringer et al. 2000) which form mainly heteromeric receptors. The different associations of subunits within a receptor provide them with different kinetics and affinities for their ligands, also conferring them different functions (Alkondon et al. 1999; Albuquerque et al. 2009; Dani 2015). For example, upon ligand binding, nAChRs are cation permeable. Their permeability is non-selective to cations however the gradient is mostly in favour of  $Na^+$  and  $Ca^{2+}$  as opposed to  $K^+$ . From the total current flowing through open nAChRs, only 2 to 5 % is  $Ca^{2+}$ , suggesting that most of the current is supported by  $Na^+$  influx (Vernino et al. 1994; Dani and Mayer 1995). However, different subunit composition have been demonstrated to be more or less permeable to specific cations, and in particular with strong  $Ca^{2+}$

conductance's being attributed to  $\alpha 7$ -containing receptors (Albuquerque et al. 2009). Upon ACh application,  $\alpha 7$ -containing nAChRs have been shown to elicit fast current in midbrain GABA and DA neurons, while neurons expressing non- $\alpha 7$ -containing nAChRs exhibit slower onset current in response to agonist presentation. Further, the  $\beta 2$ -subunit was necessary for any current to arise in these neurons (Klink et al. 2001).

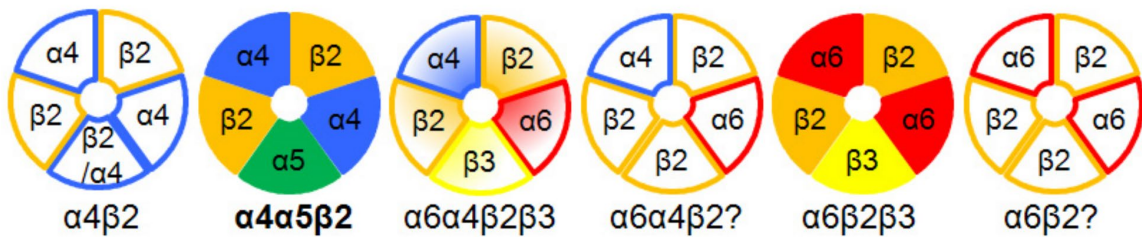
In general in the CNS, activity at nAChRs triggers  $\text{Na}^+$  (and potentially  $\text{Ca}^{2+}$ ) influx leading to membrane depolarization – such as through recruitment of voltage-gated sodium channels (VGSCs) – and consequently activation of voltage-gated  $\text{Ca}^{2+}$  channels (Albuquerque et al. 2009). The membrane depolarisation and consequent  $\text{Ca}^{2+}$  entry have been demonstrated to be ample enough to evoke neurotransmitter release, such as for example GABA release from CA1 interneurons in rat hippocampal slices (Alkondon et al. 1999) where nAChRs are likely to be located on periterminal sites of axons and in somatodendritic regions of CA1 interneurons. Presynaptic nAChR have been shown to modulate neurotransmitter release throughout the CNS, mostly using EPSCs in downstream neurons as a readout of neurotransmitter release and using pharmacology to determine the involvement of nAChRs. For example, presynaptic nAChRs have been reported to enhance glutamatergic release in several brain regions: in the VTA from glutamatergic afferent from the prefrontal cortex (Mansvelder and McGehee 2000; Mansvelder et al. 2002); in layer 5 of the prefrontal cortex from thalamocortical terminals (Lambe et al. 2003; Poorthuis et al. 2013); or in the striatum from glutamatergic PFn afferents (Tanimura et al. 2019). Another study found that activation of pyramidal tract fibres triggers a two-component EPSC on striatal MSNs, with the second peak of the signal caused by ACh release from ChIs excited by PT neurons, which leads to a ACh-dependent and nAChR-mediated glutamate release at the PT axons (Morgenstern et al. 2022). In the lateral geniculate nucleus, both glutamate and

GABA release seem to be enhanced by presynaptic nAChRs (Guo et al. 1998). Finally, in the striatum, synchronous activation ChI *ex vivo* drives 5-HT release through nAChRs present the axons of raphe nuclei afferents (Matityahu et al. 2024).

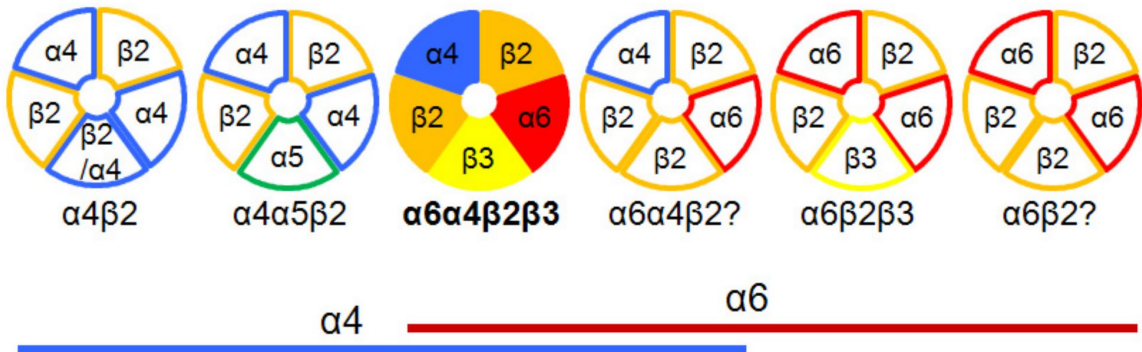
In the striatum, nAChRs can be found on most afferents and interneurons but do not seem to be expressed on MSNs (Assous 2021). Although the most abundant subunits in the striatum are the  $\alpha 4$  and  $\beta 2$ , cortex afferents have been demonstrated to express  $\alpha 7$ -containing nAChRs where they enhance glutamate release (Howe et al. 2016). Inputs to the ChIs from the PFn express nAChRs containing the  $\alpha 6$ - and  $\beta 2$ -subunits, which upon activation also enhance glutamate release from these terminals (Tanimura et al. 2019). Finally, dopaminergic afferents to the striatum express mainly  $\alpha 4\beta 2$ -containing nAChRs at their axonal membrane, but their subunit composition varies along the dorso-ventral gradient, with  $\alpha 4\alpha 5\beta 2$ -containing and  $\alpha 6\beta 2\beta 3$ -containing nAChRs being more prominent in DLS and more  $\alpha 4\alpha 6\beta 2\beta 3$ -containing nAChRs present in NAcC (Exley and Cragg 2008; Exley et al. 2008, 2011, 2012a, 2013) (**Figure 1.2**). Finally, some studies reported expression of  $\alpha 7$  subunit in DA neurons, however nothing indicates these are getting trafficked to the striatum and are thus more likely to be localised at the cell bodies of DA neurons in the midbrain. These  $\alpha 7$ -containing nAChRs are therefore unlikely to take part in the local modulation of DA release within the striatum. On brain slices *ex vivo*, varied effects of nAChR impact on DA release have been reported, notably depending on the experimental conditions and stimulation paradigms. Indeed, work on brain slices have demonstrated that nAChRs at DA axons can enhance DA release, but can also limit it (Rice and Cragg 2004; Zhang and Sulzer 2004). Modulation of DA release through nAChRs will be further explained in the next section.

Finally, the mechanisms underlying desensitisation of these receptors is not fully understood, but many studies have highlighted once again the crucial effect of the nAChRs subunit composition on the desensitisation parameters (Dani et al. 2000; Quick and Lester 2002; Giniatullin et al. 2005). For example, the high-affinity  $\alpha 4\beta 2$ -nAChRs desensitize with slower kinetics than the rapidly desensitizing  $\alpha 7$ -containing nAChRs. But even when receptors share the same subunits, their kinetics can differ greatly such as for the  $\alpha 4\alpha 5\beta 2$ -containing nAChRs which have higher  $EC_{50}$  and desensitization rate than the  $\alpha 4\beta 2$ -containing nAChRs.

## DLS



## NACc



**Figure 1.2: Subunit composition of nAChRs expressed on DA axons in the mouse striatum (schematic by Dr. R. Exley).** DA axons express nAChR-subunit composition varies along a dorso-ventral gradient in the striatum, with  $\alpha 4\alpha 5\beta 2$ -containing and  $\alpha 6\beta 2\beta 3$ -containing nAChRs being more prominent in DLS and  $\alpha 4\alpha 6\beta 2\beta 3$ -containing nAChRs being the major DA release modulators in NAcC.

## 1.5. ACh-DA interaction

Historically, the crosstalk between ACh and DA within the striatum has been seen as an antagonising one, from the realisation that motor symptoms in PD – characterised by a loss

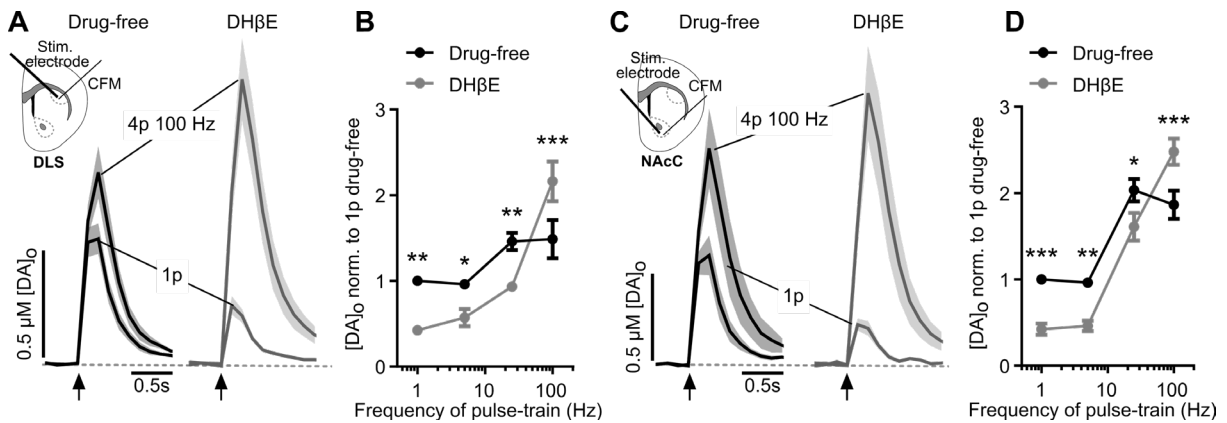
of DA neurons – can be treated by pro-dopaminergic drugs, or anti-cholinergic agents to partly restore striatal output to other basal ganglia nuclei (Barbeau 1962). This led to the DA-ACh imbalance theory, whereby DA was believed to mainly exert a powerful inhibitory effect on ChI – and henceforth ACh release. This would create strong disinhibition in PD, from the loss of DA neurons, leaving the striatum in a hypercholinergic state.

However, DA and ACh display much more complex interactions than previously thought. They reciprocally act on one another at a presynaptic level. DA can either promote or limit ACh release respectively through D1-like receptors or the D2-like receptors located on ChIs, and conversely, ACh can support or limit DA release solely through the dynamics control of nAChRs.

*In vivo*, the impact of nAChR on DA release is still unclear. Indeed, studies reports that nAChRs can support DA release (Mohebi et al. 2023; Matityahu et al. 2023), inhibit DA release (Cragg and Rice 2004; Zhang et al. 2025), or that nAChRs are not giving rise to phasic patterns of DA release since deletion of  $\beta 2$ -containing nAChRs does not disturb those patterns (Krok et al. 2023). On striatal brain slices *ex vivo* however, nAChRs on DA axons have revealed to be key modulators of DA release and have been reported to alter the frequency dependency of dopamine release (Rice and Cragg 2004; Zhang and Sulzer 2004). DA release measured using fast-scan cyclic voltammetry (FCV) evoked by low frequency electrical stimulation ( $< \sim 10$  Hz) is increased when nAChRs are active compared to when they are pharmacologically antagonised (Rice and Cragg 2004; Zhang and Sulzer 2004; Threlfell et al. 2012). However, at higher frequency stimulations, DA release was lower when nAChRs were active revealing a strong short-term depression (STD) of DA release. Plus, when nAChRs are active, the frequency dependency of DA release is low, *i.e.* a low frequency stimulation elicits a DA transient that is only marginally smaller than the one evoked by high-



frequency pulse trains but becomes more responsive to frequency when nAChRs are antagonised (**Figure 1.3**).



**Figure 1.3: nAChR activity reduces the frequency dependency of DA release.**

**A, C:** Data collected using FCV. Mean ( $\pm$  SEM, shaded area)  $[DA]_o$  transients in response to 1 pulse electrical stimulation (1p) and 4 pulses at 100 Hz (4p 100 Hz) in DLS (**A**) and NAcC (**C**), in drug-free condition in black after bath application of  $\beta 2$ -containing nAChR antagonist dihydro- $\beta$ -erythroidine hydrobromide (DH $\beta$ E) in grey. The arrow indicates the time of stimulation.  $n = 5$  slices in DLS and  $n = 6$  slices in NAcC.

**B, D:** Peak  $[DA]_o$  in response to different frequency of train-pulses (4p) normalise to the peak  $[DA]_o$  evoked by 1p stimulation in the drug-free condition in DLS (**B**) and NAcC (**D**). Two-way ANOVA multiple comparison test (in DLS (**B**), drug-effect:  $F(1, 32) = 5.101$ ,  $p = 0.0309$ , frequency effect:  $F(3, 32) = 32.34$ ,  $p < 0.0001$ , interaction:  $F(3, 32) = 10.67$ ,  $p < 0.0001$ ; in NAcC (**D**), drug-effect:  $F(1, 40) = 7.746$ ,  $p = 0.0082$ , frequency effect:  $F(3, 40) = 89.72$ ,  $p < 0.0001$ , interaction:  $F(3, 40) = 12.36$ ,  $p < 0.0001$ ) and Fisher's LSD test.

On one hand, investigation of how nAChRs can support DA release found, using amperometry, that electrically evoked DA release is composed of two phases occurring within milliseconds of one another (Wang et al. 2014a, b). The first phase relies on the direct activation of dopaminergic axons and creates a first release event. A few milliseconds later, a second release event is triggered upon activation by ACh of the nAChRs. In addition, optogenetically triggering ACh release through activation of ChIs elicits DA release which is abolished when nAChRs are antagonised by the  $\beta 2$ -containing nAChR antagonist dihydro- $\beta$ -erythroidine hydrobromide (DH $\beta$ E) (Threlfell et al. 2012; Cachope et al. 2012; Brimblecombe et al. 2018). Additionally, spontaneous or induced activity at striatal ChIs is thought to be able to drive ectopic APs at DA axons through nAChRs (Threlfell et al. 2012; Liu et al. 2022). These data indicate that nAChRs seem able to support DA release (at low frequency

stimulation) by provoking a release event following their activation, under these conditions at least.

On the other hand, investigations of the mechanisms by which activity at nAChRs can generate STD in DA release have not reached a consensus yet. An initial hypothesis suggested DA vesicle pool depletion (Wang et al. 2014b, a) to be the cause of the nAChR-mediated DA release STD, but other studies have shown otherwise (Cragg 2003; Zhang et al. 2025). In particular, findings from a recent study (Zhang et al. 2025) suggest that rather than depleting the readily releasable pool of vesicles (RRP), activity at nAChRs limits the excitability of the DA membrane for around 100 ms following a train-pulse stimulation, which in turns prevent subsequent release for any stimulation arriving within that time-window. This data therefore suggests that rather than modulating DA release depending on the frequency of stimulation *per se*, nAChRs limit release within a ~ 100 ms time-window after their activation. Unfortunately, the lack of temporal resolution arising both from the nature of the sensor used in this study and the absence of time-locked stimulus have prevented further investigation of the underlying mechanisms and cannot resolve specific threshold versus subthreshold depolarisation events.

To date, the intracellular mechanisms by which nAChRs can either promote or inhibit DA release still remain to be found. Despite providing partial explanation for the strong STD found at high-frequency (> 10 Hz) pulse-trains, these data do not allow for delineation of a potential intracellular mechanisms of action by which nAChRs act on DA release. It paves the way for new hypothesis, especially those exploring how the excitability of DA axonal membrane is modified by activation of nAChRs. Two main types of voltage-gated ion channels (potassium and sodium) are known to strongly regulate neuronal membrane excitability (Debanne 2004). Early on from studies on giant squid axon (Hodgkin and Huxley

1952a, b) have demonstrated the capacity of voltage-gated sodium channels (VGSCs) to depolarise cell membrane and the capacity of voltage-gated potassium channels (VGKCs) to repolarise the membrane after initial depolarisation.

In this study, we will test the potential involvement of VGKCs (**Chapter 3**) and VGSCs (**Chapter 4**) in the downstream mechanisms of nAChRs and further details on the hypothesis tested will be given in each chapter.

Due to the complexity of connectivity in the mammalian striatum, we also explored ACh-DA interactions in another model organism: *Drosophila melanogaster*. The use of the neuronal networks within the *Drosophila* Mushroom Body (MB, centre for olfactory learning) as a model to investigate ACh-DA interactions can be justified by the highly evolutionary conserved nature of the receptors and the targeted voltage-gated ion channels, as well as with the conserved morphological and functional roles of DA neurons in *Drosophila*. Plus, *Drosophila* allows for easier access to genes, and the simpler and well understood circuits provide an unrivalled mechanistic insight with behavioural relevance. Introduction to the fly's MB and relevant ACh-DA crosstalk within will be given more in details in **Chapter 5**.

# 2

## General Methods

## **2.1. Use of mouse as an animal model to investigate dopamine release mechanisms**

### **2.1.1. Ethical statement for the use of animals**

This body of work explore mechanisms through which presynaptic nAChRs on DA axons affect DA release within the mammalian adult striatum and within the *Drosophila melanogaster* brain. Since the entirety of the inter-specie approach of this thesis is concentrated in Chapter 6, introduction to the fly brain will be given then.

This thesis is based on decades of studies investigating ACh-DA interaction within the mammalian striatum we therefore inserted the current study in the same framework. Using real brain tissue which preserves the complexity of the striatal network was crucial for our understanding of ACh-DA interaction within the appropriate context. We used acute *ex vivo* striatal mouse brain slices which best allow for studies on local modulation of DA axons. The use of mice allows us to have access to wide range genetic modification such as to express specific sensors and reporters within a defined set of cells using readily available genetically modified animals. Most semantic studies interested in the local modulation of DA release within the striatum have used mice as a model, which allows us to compare the results in this study to wide range of literature. Furthermore, the present high degrees of homology to humans as much in the organisation and connections with their brain, as in the proteins expressed on their cells. This approach could therefore potentially uncover highly conserved.

All experiments were performed under the UK (Scientific Procedures) Act of 1986 (PPL P9371BF54 and PP8860348) following local ethical approval, and Home Office guidelines. Animals were housed in the Bioscience Medical Building at the University of

Oxford and kept on a C57BL/6 background. They were grouped housed, had access to food and water *ad libitum*, were kept on a 12h light-dark cycle, in a room with controlled temperature (~22°C) and relative humidity (40-60%).

### **2.1.2. Mouse strains used in this body of work**

Adult wild-type (WT) mouse (C57BL/6J) sourced from Charles River were used for fast-scan cyclic voltammetry and GRAB-imaging experiments. This strain is the most appropriate for the study of DA signalling as it possess a wild-type gene for  $\alpha$ -synuclein (Abeliovich et al. 2000) which is necessary for unaltered DA signalling, while this gene have been reported to be spontaneously deleted in some other WT C57BL/6 strains e.g. Harlan (Specht and Schoepfer 2001).

Experiments involving voltage and calcium sensors required these sensors (packaged in viruses) to be expressed exclusively in dopaminergic neurons (see intracranial surgeries, section 2.2). To this end, we were performed these experiments using transgenic mice (DAT-Cre) expressing Cre recombinase under the control of the promoter for the gene (*Slc6a3*) for the DAT (Bäckman et al.). Heterozygous (+/-) DAT-Cre mice were locally bred from homozygous DAT-Cre mice on a C57BL/6J background. DAT-Cre +/- were also used in fast-scan cyclic voltammetry experiments (Chapter 4), to control for any changes in DA transmission occurring because of the genetic manipulation they underwent.

## **2.2. Stereotaxic intracranial injections for viral transfection of genetically encoded fluorescent sensors for imaging experiments**

In this study, stereotaxic surgeries were performed to inject adeno associated viruses (AAVs) fused with fluorescent indicators of extracellular acetylcholine sensor in striatal neurons (see **section 2.6**), or voltage and calcium sensors in dopaminergic neurons (see Chapter 4). Use of genetically modified animal lines were not available for the specific virus that we used.

Throughout the stereotaxic intracranial surgeries, mice were anaesthetized with isoflurane. In the first instant the level of isoflurane would be set at 4% for induction of anaesthesia and was then adjusted between 1.5% to 3.5% during the surgery to assure maintenance of the appropriate level of anaesthesia, with a consistent breathing rate (minimum 1 breath per second) and the absence of pedal reflex. Temperature was monitored through the surgery using an external thermometer and maintained using a heating mat with feedback loop. All the surgeries were conducted following aseptic techniques.

Before safely and securely placing the animal's head in a stereotaxic frame (Kopf Instruments), the incision site was shaved and cleaned with ethanol (70%, diluted in sterile saline), followed by 6 swabs alternating sterile saline and chlorhexidine (HiBi scrub, at 4%). Before incision, mice were injected with 1 mL of sterile saline to prevent dehydration due to the anaesthesia, meloxicam (Metacam, 5mg/kg) to prevent post-operative pain or discomfort, and a local analgesic Marcaine (2mg/kg) was injected at the incision site.

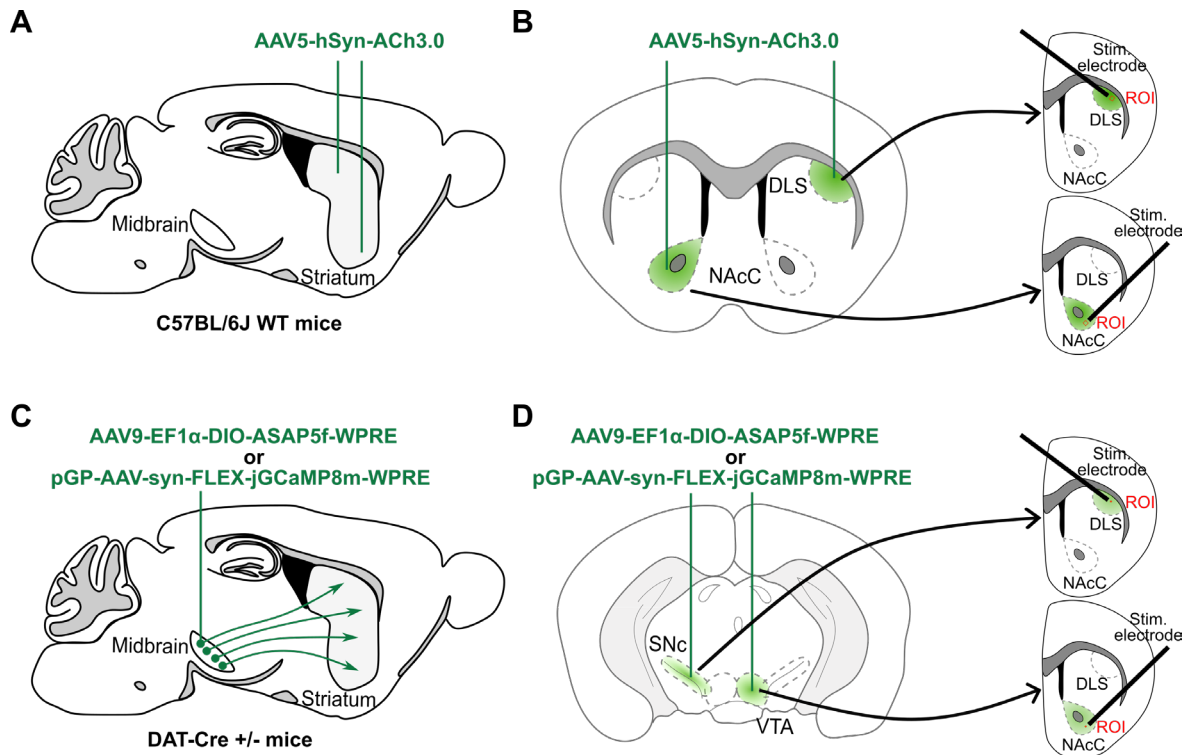
After adjusting the positioning of the skull in the stereotaxic frame, craniotomies were performed with a hand drill at different coordinates depending on the virus to be injected (**Figure 2.1**). Injections in the striatum (in Chapter 3 and 4) were made either unilaterally or bilaterally. Craniotomies were conducted at A/P: + 0.65, M/L: +/- 2, D/V: -2.6 to -2.4 to target viral expression in the Dorso-Lateral Striatum (DLS) and A/P: + 1.4, M/L: +/- 0.9, D/V: -3.7 to -3.4 to target viral expression in Nucleus Accumbens Core (NAcC).

For injections in the midbrain (in Chapter 4), the coordinates used to target the SNc for recordings in the DLS were A/P: -3, M/L: +/- 1.25, D/V: -4.25 to -4.2 and A/P: -3, M/L: +/- 1, D/V: -4.25 to -4.2 for the targeting of VTA for recording in the NAcC. We found that bilaterally injecting in the same midbrain sub-region was providing us with the best expression in the corresponding striatal region of interest, and therefore only performed bilateral injections (VTA/VTA or SNc/SNc).

Each site received an injection of 1  $\mu$ L of the viral solution at an infusion rate of 100 nL/min delivered with a 32-gauge Hamilton syringe (Hamilton Company). After the injection, the needle was left in place for 5 min before being withdrawn in order to allow for the viral solution to diffuse in the brain tissue.

Animals were then closed using monofilament suture and skin glue. Animals were then placed in a heated cage (35°C) to recover before being transfer in a post-surgical cage containing soft bedding to avoid any abrasion to the wound. During the following 7 days, animals were closely monitored closely, and weight daily. During the first 3 post-operative days, meloxicam (Metacam, 5 mg/kg) was provided in the form of a strawberry flavoured jelly, to alleviate any pain or discomfort should the mice experience it. Mice were then used for experiments 3 to 4 weeks post-surgery to allow for sufficient time viral expression.





**Figure 2.1 : Schematic of injection sites and their matching recording sites**

**A:** Injection site in the striatum (used for GRAB<sub>ACh3.0</sub> injection in WT (C57BL/6) mice in chapter 3 and 4) shown on a sagittal view of a mouse brain.

**B:** On the left, injection site in the striatum shown on a coronal striatum slice (used for GRAB<sub>ACh3.0</sub> in chapter 3 and 4). On the right, corresponding recording sites in hemisected coronal striatum slices.

**C:** Injection site in the midbrain (used for voltage sensor ASAP5f and jGCaMP8m in DAT-CRE mice in chapter 4) shown on a sagittal view of a mouse brain.

**D:** On the left, injection site in the midbrain (used for voltage sensor ASAP5f and jGCaMP8m in DAT-CRE mice in chapter 4) shown on a coronal slice of midbrain. Injections were made bilaterally in either VTA or SNc, but injection sites are here illustrated on the same slice. On the right, corresponding recording sites in hemisected coronal striatum slices. The recordings sites are located in the axonal projection arbours of the injected dopaminergic cell bodies.

## 2.3. Live mouse brain slices preparation and solutions

All experiments using mouse brain tissue (Chapter 3 & 4) were performed on coronal slices. Coronal striatal slices only contain DA axons, which allow to isolate local control of dopamine release in the striatum from cell-body generated activity.

Mice were sacrificed using cervical dislocation and the brain was quickly extracted after decapitation. Cervical dislocation was performed on unanaesthetised animals or animals that underwent a brief exposure (enough to lose the righting reflex) to isoflurane (4%, anaesthetic solution).

Brains were promptly extracted and placed in an ice-cold slicing solution. The slicing solution was equilibrated with oxygen (95% O<sub>2</sub>, 5% CO<sub>2</sub>) and contained: 85 mM NaCl, 25 mM NaHCO<sub>3</sub>, 2.5 mM KCl, 1.25 mM NaH<sub>2</sub>PO<sub>4</sub>, 0.5 mM CaCl<sub>2</sub>, 7 mM MgCl<sub>2</sub>, 10 mM glucose, 65 mM sucrose or 30 mM NaCl, 26 mM NaHCO<sub>3</sub>, 4.5 mM KCl, 1.2 mM NaH<sub>2</sub>PO<sub>4</sub>, 1 mM MgCl<sub>2</sub>, 10 mM D-glucose, 194 mM sucrose.

Brains were then placed into a Leica vibratome (VT1200 S, Leica) and sliced at 0.42 mm/s and 300 µm thick. I used slices from approximately +1.5 mm to +0.5 mm Bregma from the Franklin-Paxinos atlas to target the dorsal and ventral striatum. Slices were first transferred for 15 min to a 32°C ± 1°C bath of artificial cerebrospinal fluid (aCSF) containing 130 mM NaCl, 25 mM NaHCO<sub>3</sub>, 2.5 mM KCl, 1.25 mM NaH<sub>2</sub>PO<sub>4</sub>, 2.5 mM CaCl<sub>2</sub>, 2 mM MgCl<sub>2</sub>, 10 mM glucose equilibrated with carbogen too (95% O<sub>2</sub>, 5% CO<sub>2</sub>). This aCSF bath was then removed left at ambient temperature for the rest of the day.

During experiments, hemisected slices were submerged into a recording chamber superfused with aCSF heated to 32 ± 1°C and equilibrated with carbogen as explained above. The slices were held by pins.

## **2.4. Electrical stimulation on mouse brain slices**

In this body of work, all experiments performed on mouse brain slices use electrically evoked stimulation. The stimulating electrode used was a surface bipolar concentric Pt/Ir electrode (outer/inner diameter:125/25 µm; FHC) was used for all experiments. Electric pulses were given at an intensity of 650 mA and were 200 µs long. Stimulations were given

every 2.5 min for all experiments except voltage sensor experiments, for which the stimulations were given every 1 min. Frequency at which electric pulses are given is detailed for each experiment. For example, if 4 pulses are delivered at 100 Hz, the second pulse comes 10 ms after the end of the first one, and so on. Electrical stimulation composed of a single pulse are abbreviated “1p”, and several pulses are in a row they are abbreviated as “4p 100 Hz” for 4 pulses at 100 Hz.

During experiments, the stimulating electrode was placed on top of the tissue such as it makes enough contact with the tissue without altering the integrity of the slice. The electrical stimulation provided are thought to synchronously activate all cells in the vicinity of the electrode (Cragg 2003). Therefore, all intra-striatal circuits are activated during the electric stimulation such as cholinergic interneurons and GABA interneurons for example.

## **2.5. Electrochemical detection of dopamine *ex vivo* in live mouse brain slices using fast-scan cyclic voltammetry (FCV)**

### **2.5.1. FCV as a method to detect dopamine release in acute mouse brain slices**

In this body of work, fast scan cyclic voltammetry (FCV) with carbon fibre microelectrode (CFM) was used as a method to detect dopamine release in real-time in acute coronal mouse brain slices.

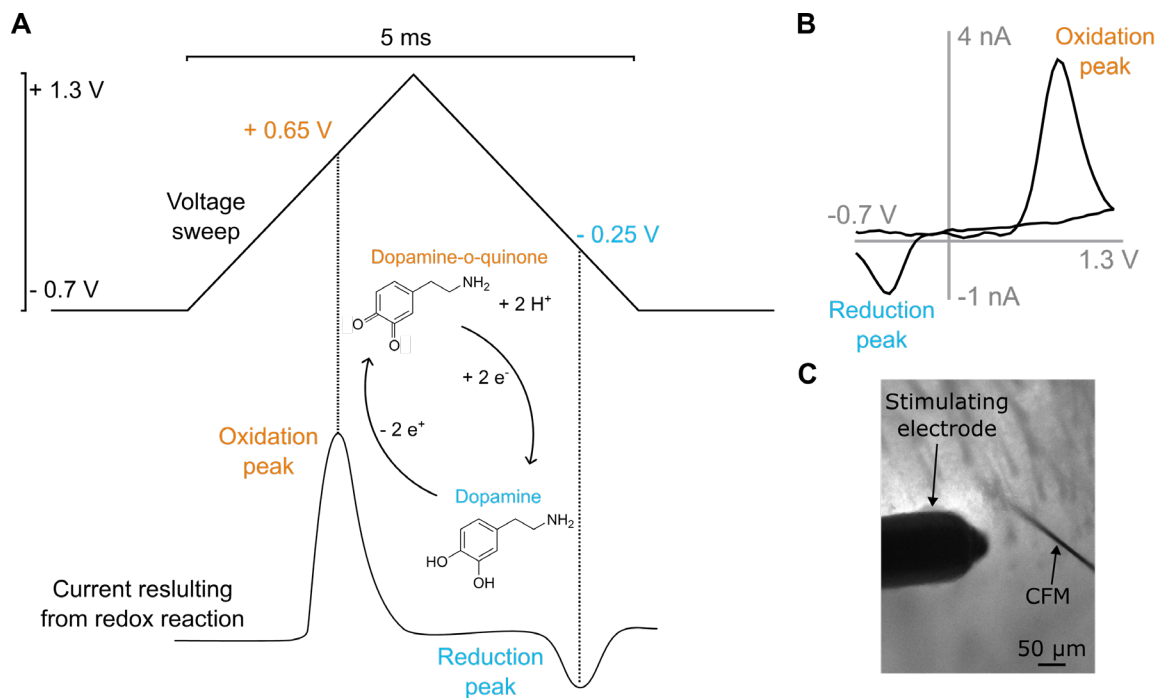
The FCV method is based on the electroactive properties of the monoamines such as dopamine, noradrenaline or serotonin. Dopamine possesses two hydroxyl groups that can

be oxidised to form dopamine-o-quinone and reduced back to dopamine (**Figure 2.2 A**). The switch from one state to the other can be induced and monitored through a CFM inserted in the tissue at a region of interest. A triangular shaped potential ( $-0.7\text{ V}$  to  $+1.3\text{ V}$ ) is then applied to this microelectrode to oxidise dopamine on the upward ramp at approximately  $+650\text{ mV}$  and reduced on the downward phase at approximately  $-250\text{ mV}$ . The redox reaction induced by the triangular voltage ramp creates a movement of ions that is recorded as a faradaic current against an Ag/AgCl reference electrode. The potential at which a molecule oxidises and reduces are specific to each molecule, and the shape of the faradaic current indicates the type of molecules that is being detected upon electrical stimulation. A voltammogram where the potential applied is plotted against the current recorded is useful to identify the molecule of interest (**Figure 2.2 B**). In our case, dopamine and serotonin possess very similar voltammograms, however, in the striatum, serotonin release is marginal compared to the amount of dopamine, and therefore our recordings report dopamine changes in the environment of the microfibre electrode. The faradaic current recorded reflects the actual dopamine concentration  $[\text{DA}]_0$  in the environment in real time.

In addition to the faradaic current, another main current detected at the CFM is called the charging current. This current varies on much longer time scales than the faradaic and occurs when charged particles around the electrode in the tissues moved or rearranged. We usually leave our electrode in the tissue at a region of interest for 45 min to 1 h before starting the recording for the environment of the CFM to have reorganised and therefore for the charging current variations to have stabilised. Throughout our experiments, this background current is monitored but subtracted to enable the sole recording of the faradaic current using a Millar Voltameter (Julian Millar, Barts and the London School of Medicine and Dentistry).

To achieve high temporal resolution, the triangular wave is applied in sweeps lasting 5 ms and repeated at a scanning rate of 8 Hz, therefore leading in measurement of dopamine concentration  $[DA]_o$  every 125 ms. This temporal resolution is appropriate to detect release events (Armstrong-James et al. 1980; Rice and Nicholson 1989). However, it is too slow to detect changes that occur on a millisecond basis such as the release events provoked by the opening of nicotinic acetylcholine receptors at dopaminergic axons. Higher sweep frequency could be implemented however it would have reduced the amplitude of the signal and therefore lowered the signal-to-noise ratio which is not desirable.

Other methods for dopamine detection are available that would have higher temporal resolution. For example, amperometry, which is a technique similar to voltammetry except that the potential is held at the oxidising potential of dopamine instead of the triangular waveform. Although using this method the scanning rate can reach, it provides much less specific chemical identification compared to voltammetry. Indeed, because the current is constant, we cannot plot a voltammogram and ensure that the signal recorded is originating solely from the oxidation of the targeted compound.



**Figure 2.2 : Fast-scan cyclic voltammetry (FCV)**

**A:** At the top, triangular voltage sweep applied to the CFM, at the bottom, current resulting of the dopamine redox reaction with the dopamine oxidation peak in orange at 0.65 V and its reduction peak in blue occurring at -0.25 V.

**B:** Example of a voltammogram where the resulting current is plotted against the voltage applied during the sweep.

**C:** Example of the positioning of the CFM and the stimulating electrode to perform FCV.

Another new technique that can be used to detect DA in acute mouse brain slices is imaging of new genetically encoded fluorescent indicators. These tools – for example GRAB<sub>DA</sub> (Sun et al. 2018, 2020) or dLight (Patriarchi et al. 2018) – are genetically encoded modified dopamine receptors linked to a circularly permuted GFP which emits fluorescence upon binding of dopamine. Although sampling at faster rates than 8 Hz is possible with these sensors, the limiting factors are the on and off kinetics of the sensor themselves which are not fast enough to faithfully report events occurring within milliseconds of one another. Furthermore, what FCV offers that fluorescent reporters do not is a pseudolinear sensitivity to dopamine (sensors are often non-linear), and the ability to calibrate electrodes to convert our signals into actual dopamine concentration detected.

Thus, the most appropriate methods to report  $[DA]_o$  in real-time in mouse brain slices remains for our purposes is the FCV method.

### **2.5.2. Carbon fibre microelectrode (CFM)**

CFMs were made in house using borosilicate capillary tubes (2.0 mm outside diameter, 1.16 mm inside diameter, Harvard Apparatus). Acetone was used to help threading the capillary tube with a single epoxy-free carbon fibre (7  $\mu\text{m}$  diameter, Goodfellow Cambridge Ltd.). The threaded capillary tube was then inserted in a PE-2 vertical microelectrode heated puller (Narishige, Japan) in order for the glass to seal with the carbon fibre. The tighter the seal the better the signal-to-noise ratio will be. The carbon fibre was then cut using a scalpel under a microscope at x100 magnification so that it is exposed after the seal tip for a length of 50 nm to 130 nm. This length allows desirable sensitivity to dopamine and enough surface area for our recordings. Finally, an insulated copper wire was stripped from the insulation and the copper wire was dipped into silver conductance paint and inserted at the back of the threaded and sealed capillary tube. The silver paint ensures good electrical contact between the carbon fibre and the copper wire. The electrode was then closed using cyanoacrylate glue to maintain the copper wire in place.

The carbon fibre diameter (7  $\mu\text{m}$ ) and length (50  $\mu\text{m}$  to 130  $\mu\text{m}$ ) allow for detection at multiple release site since it exceed the size of a synaptic cleft ( $\sim 20\text{-}30\text{ nm}$ ) and are therefore adapted to detect volume transmission such as DA transmission.

A single CFM was used per day as repeated exposure to brain tissue lowers the electrode's sensitivity to dopamine. Each electrode was calibrated using a 2  $\mu\text{M}$  DA solution

to allow conversion of the faradaic currents into a DA concentration. Only one concentration was required as electrode responses are linear over the range of concentrations of DA detected in brain tissue. The DA solution was stored as a 2.5 mM stock solution in 0.1 M  $\text{HClO}_4$  at 4°C and made up on the day to the final 2  $\mu\text{M}$  concentration by dilution in aCSF.

### 2.5.3. FCV recordings

Throughout this study, evoked dopamine transients were recorded using FCV and hand-made CFMs. Recordings were performed in DLS and NAcC.

Hemisected slices were put in a 32°C aCSF bath. The CFM was inserted into the tissue around 100  $\mu\text{m}$  away from the tip of the stimulating electrode at an angle of 21° and a depth of 100  $\mu\text{m}$  to 120  $\mu\text{m}$  (**Figure 2.2 C**). The signal gain was adjusted and the CFMs was only used if the signal gain was between 3 mV/nA and 12 mV/nA. Before starting the recordings, the charging current was left to equilibrate for 1h as explained in **section 2.5.1**. The signal gain was monitored and adjusted throughout the experiment. Stimulations were given every 2.5 min to allow the dopaminergic release to recover from the previous stimulation. Single pulses were given until the evoked  $[\text{DA}]_o$  stabilises around the same value ( $\pm 10\%$ ) for 4 to 5 stimulations in a row. Only once this stage is reached then we did proceed with the experiment, if stabilisation of  $[\text{DA}]_o$  couldn't be reached, we moved on to another site. Up to 3 sites per region were tried on one slice before changing the slice if none of the sites exhibited stable evoked  $[\text{DA}]_o$  to single pulse stimulations. Once the experiment had started if the  $[\text{DA}]_o$  to single pulse stimulation had dropped or increased more than 15% from the stable  $[\text{DA}]_o$  evoked by single pulses within 10 to 15 min, the experiment was later



excluded. At the end of the day, each CFM was calibrated as described in **section 2.5.2** and a calibration factor was extracted.

Data was acquired using a Clampex 10.7 (Molecular Devices) and custom-made Microsoft Excel macros (Visual Basic). Oxidation peaks extracted from the voltammograms acquired during the experiment were plotted over time, and these values were converted to  $[DA]_o$  using the calibration factor to create  $[DA]_o$  transients.

## **2.6. Using the genetic encoded GRAB<sub>ACh</sub> sensor to record evoked acetylcholine transients in live mouse brain slices**

### **2.6.1. About GRAB sensors and GRAB<sub>ACh</sub>**

G-protein-coupled receptor-activation-based sensors (GRAB sensors) are genetically encoded transmembrane fluorescent sensors, that allow detection of specific neurotransmitter or neuropeptide in the environment towards which the sensor is faced. GRAB sensors are based on modified receptors for a specific neurotransmitter or neuropeptide of interest that is linked to a circularly permuted green fluorescence protein (cpGFP). Upon binding of the molecule of interest to the modified receptor, a conformational change occurs and triggers the cpGFP to emit fluorescence. The sensor retains partly the properties of the receptors it is derived from such as sensitivity, however, it does not act as a channel and only negligibly initiate the G-protein coupled receptors (GPCR) downstream pathways the original receptor would have (Jing et al., 2018, 2020).

GRAB<sub>ACh</sub> sensors were developed by combining a circularly permuted green fluorescence protein (cpGFP) and a human M3-mAChR (Jing et al. 2018, 2020). When acetylcholine is released in the nearby environment of the sensor, it can bind to the modified receptor, which by conformational changes provokes the cpGFP to emit fluorescence. An increase in fluorescence translates an increase in extracellular concentration of ACh, however it is not possible to translate this increase in fluorescence to an actual concentration value. I used this technique to look at changes in evoked striatal acetylcholine release in different conditions (**Chapter 3 & 4**).

GRAB<sub>ACh</sub> imaging was also performed on live *Drosophila* using 2-photon imaging for which the methods and fly line used are described in Chapter 5.

### 2.6.2. Animals used for GRAB<sub>ACh</sub> imaging

All animals used for GRAB<sub>ACh3.0</sub> experiments were mixed-sex wild-type (C57BL/6) mice aged 7 to 14 weeks which had undergone the procedure described in **section 2.2** for intracranial injection in the striatum. We used the following viral solution from WZ Biosciences: AAV5-hSyn-ACh3.0(ACh4.3) (Cat #YL001003-AV5) at a titre ranging from  $3.78 \times 10^{12}$  vg/ml to  $4.07 \times 10^{12}$  vg/ml in saline.

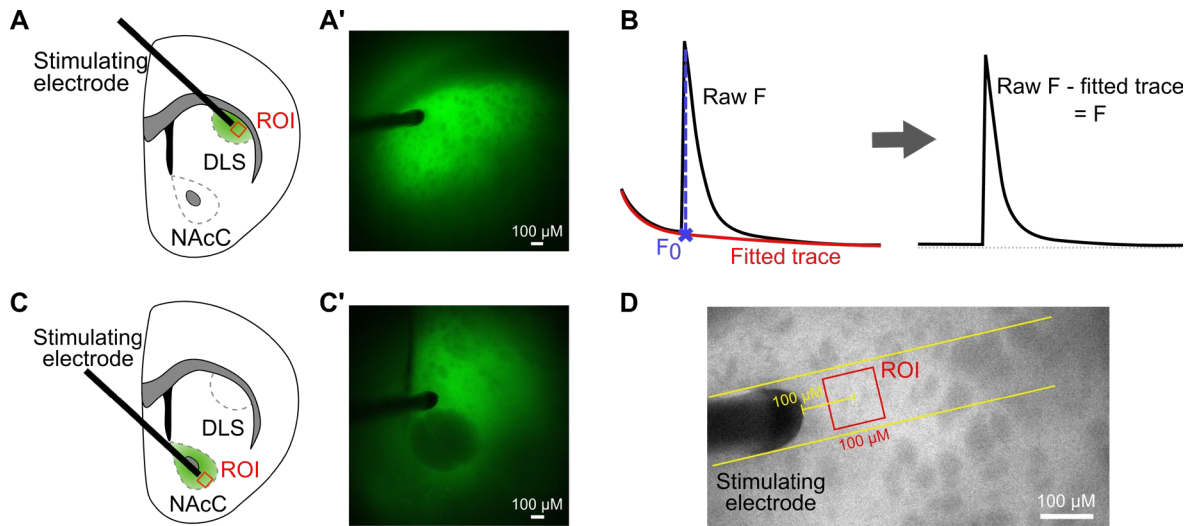
The virus was expressed non-specifically in neurons of the striatum for a readout of striatal ACh release. The human synapsin (hSyn) promoter therefore was adapted for expression in all neurons in the vicinity of the injection site (Kügler et al., 2003).

### 2.6.3. Imaging parameters to detect ACh release in the striatum using

#### GRAB<sub>ACh</sub>

We used a blue LED light ( $\lambda = 470$  nm) at 5 mW (measured under at 10x objective) to perform these experiments. Imaging of evoked ACh release using the GRAB<sub>ACh3.0</sub> (Jing et al. 2020) sensor was performed using an IRIS 9 CMOS camera (Teledyne Photometrics) under a 10x objective. The images were acquired using Fiji2 Micromanager software. The exposure length was set at 10 ms and the frame rate at 100 Hz. The field of view was selected to allow the camera to accommodate the 100 fps, and the images were binned 2x2 to allow the camera to acquire at the required frame rate. The field of view always included the tip of the stimulating electrode which allowed later positioning of the Region of Interest (ROI) for the subsequent analysis of the signal (**Figure 2.3 A, A', C, C' and D**). Images were acquired as TIF files.

The ROI was chosen to be located at the distance from the stimulating electrode that matched those used for FCV recording electrodes, so that GRAB signals are acquired from a region that is similar to the region in which FCV would be performed. The ROI was designed as a 100  $\mu\text{m}$  x 100  $\mu\text{m}$  square whose centre lies 100  $\mu\text{m}$  away from the tip of the stimulation electrode. The ROI is also tilted at the same angle as the angle at which the stimulating electrode is positioned onto the slice (**Figure 2.3 A, A', C, C' and D**). The slices were illuminated 400 ms or 1 s before the electrical stimulation was given. Each recording lasted in total 2 s to 3 s to allow the signal to return to baseline after stimulation.



**Figure 2.3 : Performing GRAB<sub>ACh3.0</sub> recording in coronal mouse brain slices.**

**A:** Schematic of the recording site in DLS. **A':** Picture of a slice with GRAB<sub>ACh3.0</sub> expression in the DLS and positioning of the stimulating electrode under a 10x objective.

**B:** Schematic of the analysis of the signal. The averaged fluorescence values are extracted from the ROI over time (raw F), an exponential curve is fitted to before and after the stimulation occurs. To correct for bleaching, the fitted curve is subtracted to the raw F to provide the fluorescence values F from which calculations such as peak  $\Delta F / F_0$  and AUC will be taken.  $F_0$  is taken as the value on the fitted curve that corresponds to the peak time.

**C:** Schematic of the recording site in NAcC. **C':** Picture of a slice with GRAB<sub>ACh3.0</sub> expression in the NAcC and positioning of the stimulating electrode under a 10x objective.

**D:** Picture showing an example of field of view during image acquisition under a 10x objective. The ROI is chosen a 100 μm square whose centre is 100 μm away from the tip of the electrode. The ROI is rotated to align with the stimulating electrode.

#### 2.6.4. Data extraction and analysis for GRAB<sub>ACh</sub> imaging

Average fluorescence within the ROI (described in 2.6.3) along with the frame number was extracted in ImageJ using a custom-made macro. Raw values of fluorescence over time were then transferred to a custom-made MatLab code (MATLAB R2024\_b, MathWorks). The frames number were converted into seconds. The fluorescence recorded followed an exponential decay throughout the recording. Therefore, we first fitted a 2-term exponential decay function ( $f(x) = abx + cdx$ ) to the pre-and post- stimulation trace (Figure 2.3 B). The post stimulation trace was set as the time at which the average fluorescence has gone back to baseline. This fit was then subtracted to the raw fluorescence to correct for the initial decay which provided us with the change in fluorescence  $\Delta F$ . Lastly, the  $\Delta F$  was then divided

by the fit to obtain the normalised transient  $\Delta F/F$ . The baseline fluorescence  $F_0$  was then calculated so it would be equal to the value on the fit curve at the time of peak in the raw fluorescence. Area under the curve (AUC) was calculated using the Trapz function on MatLab.

## 2.7. Statistical analyses

Statistical analyses were carried out using GraphPad Prism. All datasets were tested for normality using the Shapiro-Wilk test which compare the standard deviation to a normal distribution and, depending on if the  $n$  is sufficient, the D'Agostino & Pearson test which analyses the shape (skewness and kurtosis) of the distribution of the data by comparing it to an expected normal distribution. The subsequent statistical tests to compare different treatments were chosen accordingly: parametric tests were performed if the data had a normal distribution (for which the QQ and homoscedasticity plots were inspected when necessary), and un-parametric otherwise. For pilot data-sets with small number of repetition ( $n > 5$ ), tests were chosen depending on whether the variable measured is known to usually follow a normal distribution. For instance,  $[DA]_o$  measured by FCV usually follows a normal distribution, thus pilot data-sets with low number of repetitions were assumed to also follow a normal distribution and parametric tests were performed for these experiments. Repetition number  $n$  was chosen based on the repetition number traditionally used in the field to reflect meaningful differences, which usually falls between five to ten repetitions. In this body of work, a repetition reflects a single recording site per slice. Variables were considered dependent when the different treatments were applied to the same recording site within a slice or to the same fly within a single continuous recording, leading to the use of paired statistical tests. Where data is independent, i.e., when the groups compared were recorded

from different brain slices, from different sites within the slice or from different flies, the statistical tests chosen were un-paired. The threshold for statistical significance was  $p < 0.05$ . Statistical significance was reported as: ns  $p > 0.05$ ; \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ ; \*\*\*\*  $p < 0.0001$ .

## 2.8. Drugs

Drugs were diluted in either deionised water (dH<sub>2</sub>O) or dimethyl-sulfoxide (DMSO) to stock solutions and aliquots were stored at -20°C. Drugs were diluted to the final working concentration in carbonated (saturated with 95% O<sub>2</sub>/ 5% CO<sub>2</sub>) aCSF on the day of the experiment.

| Drug                                                       | Action                                | Supplier                | Solvent           | Concentration             | References                                   |
|------------------------------------------------------------|---------------------------------------|-------------------------|-------------------|---------------------------|----------------------------------------------|
| 4-aminopyridine (4-AP)                                     | VGKC blocker                          | Sigma-Aldrich           | dH <sub>2</sub> O | 100 $\mu$ M               | Condon et al., 2019                          |
| (+)-Bicuculline                                            | GABA <sub>A</sub> receptor antagonist | Cayman chemical company | DMSO              | 10 $\mu$ M (DMSO: 0.05%)  | Lopes et al., 2019                           |
| CGP 55845, HCl                                             | GABA <sub>B</sub> receptor antagonist | AbCam                   | DMSO              | 4 $\mu$ M (DMSO: 0.05%)   | Roberts et al., 2020                         |
| Cocaine                                                    | DAT inhibitor                         | Sigma-Aldrich           | dH <sub>2</sub> O | 5 $\mu$ M                 | Condon et al., 2019                          |
| Dihydro- $\beta$ -erythroidine hydrobromide (DH $\beta$ E) | $\beta$ 2-nAChR antagonist            | Tocris Bioscience       | dH <sub>2</sub> O | 1 $\mu$ M                 | Threlfell et al., 2012                       |
| Oxotremorine (Oxo-m)                                       | mAChR antagonist                      | Tocris Bioscience       | dH <sub>2</sub> O | 250 nM – 500 nM           | Threlfell et al., 2010                       |
| Rufinamide                                                 | Prolongs VGSCs inactivation           | Cayman chemical company | DMSO              | 100 $\mu$ M (DMSO: 0.25%) | (Otoom et al. 1998)                          |
| Tetrodotoxin (TTX)                                         | Broad VGSCs blocker                   | Bio-Techne              | dH <sub>2</sub> O | 4 nM – 24 nM or 1 $\mu$ M | Brody et al., 2000<br>Threlfell et al., 2012 |
| Veratridine                                                | Prolongs VGSCs opening                | Chem Cruz               | DMSO              | 2 $\mu$ M (DMSO: 0.05%)   | (Lin et al. 2022b)                           |

**Table 2.1: Drugs used in this thesis, including their mode of action and references to studies clarifying their use for our purposes and chosen concentrations.**

# 3

## The Role of Axonal Voltage-gated Potassium Channels in the Modulation of Dopamine Release by Nicotinic Acetylcholine Receptors

### 3.1. Introduction and initial hypothesis

In the mammalian striatum, ACh released from ChIs modulates DA release through nAChRs located on DA axons (Rice and Cragg 2004; Zhang and Sulzer 2004; Exley and Cragg 2008; Exley et al. 2008, 2012a; Threlfell et al. 2012). *Ex vivo* work on mouse brain slices have shown that the activation of nAChRs on DA axons changes the frequency dependency of dopamine release (Threlfell and Cragg 2011; Brimblecombe et al. 2018). Thus, when nAChRs are active, the frequency dependency of DA release is low, *i.e.* a low frequency stimulation elicits a DA transient that is only marginally smaller than the one evoked by high-frequency pulse trains. However, when nAChRs are pharmacologically antagonised by the specific  $\beta 2$ -containing nAChR antagonist dihydro- $\beta$ -erythroidine hydrobromide (DH $\beta$ E), the frequency dependency of DA release increases greatly and release at low frequency stimulation decreases while train-pulses evoke more release compared to when nAChRs are active. Therefore, nAChRs seem to support DA release at low frequency stimulations, while they hinder release at higher frequencies.

Electrically evoked dopamine (DA) release is composed of two phases occurring within milliseconds of one another (Wang et al. 2014a, b): the first relies on the direct activation of dopaminergic axons, and second is triggered by activation by ACh of the nAChRs. Therefore, nAChRs support release at low frequency stimulation by provoking a release event upon activation. However, nAChRs can also drastically limit DA release when a pulse-train follows, and mechanisms for that dual-action on DA release remains unknown.

DA vesicle pool depletion (Wang et al. 2014b, a) has been put forward as an explanation to the nAChR-mediated STD in DA release, but other studies have contradicting



results (Cragg 2003; Zhang et al. 2025). In particular, a recent study (Zhang et al. 2025) uses new genetically-encoded voltage indicators to suggest that nAChR activation decreases the excitability of DA axons by limiting subsequent axonal reactivation. However, the lack in temporal resolution arising both from the nature of the sensor itself and the absence of time-locked stimulus have prevented further investigation of the underlying mechanisms.

To date, the intra-cellular mechanisms by which nAChRs can either promote or inhibit DA release still remains to be found. From the dynamic effect of nAChRs on DA release, as well as the recent data suggesting they modify axonal excitability, we delved into the potential key roles in the mechanisms downstream of nAChR activation of two main types of voltage-gated ion channels (potassium and sodium) which are known to strongly regulate neuronal membrane excitability (Debanne 2004). Both voltage gated ion-channels types have been highlighted early on from studies on giant squid axon (Hodgkin and Huxley 1952a, b) for their capacity to depolarise (VGSCs) and repolarise (VGKCs) the membrane after initial depolarisation. This chapter will focus solely on the potential involvement of voltage-gated potassium channels (VGKCs) and work on voltage-gated sodium channels (VGSCs) will be presented in the next chapter.

VGKCs form a large evolutionarily conserved family. VGKCs are mostly homotetramers – although they can also heterotetramerize – channels composed of Kv  $\alpha$ -subunits. Each  $\alpha$ -subunit is formed by 6 transmembrane segments comprising a voltage-sensing domain (segment 1 to 4) and an extremely potassium-specific pore domain (segments 5 and 6) (González et al. 2012; Kim and Nimigean 2016). These tetramers can also bind to  $\beta$ -subunits which regulate the trafficking but also the gating and kinetics of the channels (González et al. 2012; Kim and Nimigean 2016).

Although their roles are diverse, VGKCs in excitable cells such as neurons can modulate the waveform of an action potential (AP) and general firing patterns (Debanne 2004; Rama et al. 2018). Their wide range of intrinsic properties and of voltage dependence for their activation and inactivation kinetics provide these channels with the ability to finely modulate AP shape and ensures the reset of the membrane after depolarisation. For example, the inactivation kinetics can affect AP duration, as demonstrated in mossy fibres boutons where heteromers of fast-inactivation type-A VGKCs (Kv1.4) and delayed rectifiers VGKCs (Kv1.1) broaden spikes upon repeated stimulation (Geiger and Jonas 2000). VGKCs blockage using the broad VGKCs blocker 4-aminopyridine (4-AP) (Yeh et al. 1976; Choquet and Korn 1992; McCormack et al. 1994) can also widens spikes (Bostock et al. 1981) leading to enhanced neurotransmitter release through increased calcium entry (Katz and Miledi 1967; Llinás et al. 1976; Augustine 1990; Geiger and Jonas 2000).

Therefore, inactivation of VGKCs upon nAChR activation – which widens spikes – could provide an explanation for the heightened dopamine release in response to single-pulse stimulations observed when nAChRs are active compared to when they are antagonised. Following this first stimulation, we hypothesised that the VGKCs inactivation due to nAChR activation is particularly pronounced and slowing the repolarisation of the membrane of the axons so much so that it prevents subsequent action potentials despite further stimulation (during pulse-trains), potentially impairing the ability of VGSCs to be activated too. The absence or greater difficulty of the axon to produce spikes following initial activation would then prevent further release.

In contrast, antagonising nAChRs would prevent the initial fast inactivation of VGKCs, therefore creating an initial spike that is narrower than when nAChRs are active. Subsequent

pulses would thus also be able to generate spikes, leading to an increase in dopamine release associated with each electrical pulse.

Although little is known about the DA axons composition in VGKCs subunits, it has been previously shown – through varying external potassium concentration ( $[K^+]_o$ ) and 4-AP (Condon et al. 2019) in the absence of nAChR control – that  $K^+$ -dependent mechanisms are key to govern axonal short-term plasticity and calcium entry in striatal mouse brain slices.

In this chapter, we will explore a potential role of VGKCs in the nAChR-driven modulation of DA release mainly using FCV, application of a broad VGKCs blocker (4-AP) and varying  $[K^+]_o$ . We also confirmed, using novel GRAB<sub>ACh</sub> sensor, that potential changes in ACh release due to 4-AP wash was not a confounding effect of our FCV data.

## 3.2. Methods

Experiments described in this chapter were carried out as described in Chapter 2. Specifics of this chapter are detailed in this section.

### 3.2.1. Slice preparation

Acute ex vivo coronal striatum slices were prepared for FCV and GRAB<sub>ACh3.0</sub> as described in **section 2.3**.

The number of slices used for each experiment is noted “n”. At maximum, 2 slices from the same animal have been used for each experiment, and a minimum of 6 animals were used per experiments. All experimental procedures involving the use of animals were carried out according to institutional guidelines and conformed to the UK Animals (Scientific Procedures) Act 1986.

### 3.2.2. FCV recordings

Animals used for FCV experiments were mixed gender (7 to 14 weeks of age) C57BL/6J mice. FCV recordings were performed as described **sections 2.4** and **2.5**.

### 3.2.3. GRAB<sub>ACh</sub> imaging

Animals used for GRAB<sub>ACh</sub> experiments were mixed gender (9 to 14 weeks of age) C57BL/6J mice. GRAB imaging was performed as described in **sections 2.6.2** on animals who have undergone intracranial surgeries in the striatum (described in **section 2.2**) and injected with AAV5-hSyn-ACh3.0(ACh4.3) (Cat #YL001003-AV5) at a concentration of  $3.78 \times 10^{12}$  vg/ml diluted in sterile saline, between 3 and 4 weeks prior to the experiment.

### 3.2.4. Data acquisition and analysis

FCV data was acquired and analysed as described in **section 2.5**. Imaging data was acquired and analysed as described in **section 2.6.3** and **2.6.4** respectively. Statistical analyses were carried out using GraphPad Prism as described in **section 2.7**. and the threshold for statistical significance was  $p < 0.05$ . Statistical significance was reported as: ns  $p > 0.05$ ; \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ ; \*\*\*\*  $p < 0.0001$ .

### 3.2.5. Drugs

Drugs were diluted into aCSF (saturated with 95% O<sub>2</sub> / 5% CO<sub>2</sub>) to their final working concentration (**Table 3-1**) on the day of experiment from concentrated aliquots diluted in dH<sub>2</sub>O and kept at -20°C. The aCSF-drug solution was washed on for 10 to 20 min before probing effect of the drug on DA release.

| Drug                                                       | Action                     | Supplier          | Solvent           | Concentration | References             |
|------------------------------------------------------------|----------------------------|-------------------|-------------------|---------------|------------------------|
| 4-aminopyridine (4-AP)                                     | Broad VGKC blocker         | Sigma-Aldrich     | dH <sub>2</sub> O | 100 $\mu$ M   | Condon et al., 2019    |
| Dihydro- $\beta$ -erythroidine hydrobromide (DH $\beta$ E) | $\beta$ 2-nAChR antagonist | Tocris Bioscience | dH <sub>2</sub> O | 1 $\mu$ M     | Threlfell et al., 2012 |

**Table 3-1: Drugs used in Chapter 3, including their mode of action and references to studies clarifying their use for our purposes and chosen concentrations.**

### 3.3. Results

#### 3.3.1. VGKC inhibition only partially substitutes for nAChR activation

##### when nAChRs are inhibited by DH $\beta$ E

As explained in **section 3.1** when nAChRs are active, extracellular dopamine ([DA]<sub>o</sub>) evoked by single pulse is higher compared to when nAChRs are antagonised, as opposed to [DA]<sub>o</sub> evoked by high frequency train-pulses which is hindered compared to when nAChRs are active. This stimulation-frequency dependent change in DA release is often probed using a ratio of the [DA]<sub>o</sub> evoked by pulse-trains over [DA]<sub>o</sub> evoked by 1p, here the 4p/1p ratio. This ratio is much lower when nAChRs are active contrary to when they are antagonised. We hypothesised that upon nAChR activation, VGKCs quickly inactivate triggering wider spikes and thus larger release events (Katz and Miledi 1967; Llinás et al. 1976; Augustine 1990), but that this inactivation is such that it incapacitates the membrane to produce more spikes during train pulses, thereby preventing further release. As a corollary, we hypothesise that when nAChRs are antagonised, VGKCs are available and able to repolarise the membrane in due time past the first stimulation allow any further stimulation to initiate spikes triggering release events.

Using this hypothesis as a starting point, we first tested whether blocking of VGKCs when nAChRs are antagonised could substitute for nAChR activation. Although blocking VGKCs will increase release in general, we expect a greater relative increase of [DA]<sub>o</sub> after a single pulse than for pulse-train stimulation. Therefore, we tested in particular whether VGKC inhibition could mimic nAChR activation by increasing [DA]<sub>o</sub> evoked by single stimuli pulse and increase 4p/1p ratio while inhibiting nAChRs.

We first bath applied DH $\beta$ E, a  $\beta$ 2-containing nAChR antagonist, following by an application of 100  $\mu$ M 4-AP, a broad VGKC blocker. We performed these successive bath applications in the two regions of the striatum (**section 1.3**) DLS and NAcC which receive inputs from SNc DA neurons and VTA DA neurons respectively, in order to have an overview of the potential disparities between these distinct populations.

Both in DLS and NAcC, blockage of nAChRs through wash on of 1  $\mu$ M DH $\beta$ E had the expected effect of decreasing the [DA]<sub>o</sub> evoked by 1p stimulation (in DLS **Figure 3.1 A and B**, p-value < 0.0001 ; in NAcC **Figure 3.1 E and F**, p-value = 0.0004). However, here [DA]<sub>o</sub> evoked by 4p 100 Hz stimulation seemed unaffected by nAChRs antagonism (in DLS **Figure 3.1 A and C**, p-value = 0.2746 ; in NAcC **Figure 3.1 E and F**, p-value = 0.1503). Consequently, addition of DH $\beta$ E increased greatly the 4p/1p ratio (in DLS **Figure 3.1 A and D**, p-value < 0.0002 ; in NAcC **Figure 3.1 E and H**, p-value = 0.0002).

Next, we tested whether addition of a the VGKCs blocker 4-AP would mimic the opening of nAChRs when nAChRs are pharmacologically antagonised by DHBE. We found that in both striatal regions, [DA]<sub>o</sub> evoked by 1p stimulation was increased by additional blockage of VGKCs using 4-AP (DH $\beta$ E + 4-AP condition) compared to [DA]<sub>o</sub> evoked by 1p stimulation when on the nAChRs are antagonised in the presence of DH $\beta$ E only (in DLS **Figure 3.1 A and B**, p-value = 0.0073 ; in NAcC **Figure 3.1 E and F** p-value = 0.0184).

We then compared the [DA]<sub>o</sub> levels evoked by 1p when nAChRs and VGKCs are blocked (DH $\beta$ E + 4-AP condition) and in drug-free condition when nAChRs are active to assess whether VGKCs blockage is fully substituting for nAChR activation, in which case release levels should be similar in both aforementioned conditions. However, [DA]<sub>o</sub> evoked

by 1p stimulation in the presence of DH $\beta$ E and 4-AP did not reach the initial release levels from the drug-free condition (in DLS **Figure 3.1 A and B**, p-value = 0.0218; in NAcC **Figure 3.1 E and F** p-value < 0.0001).

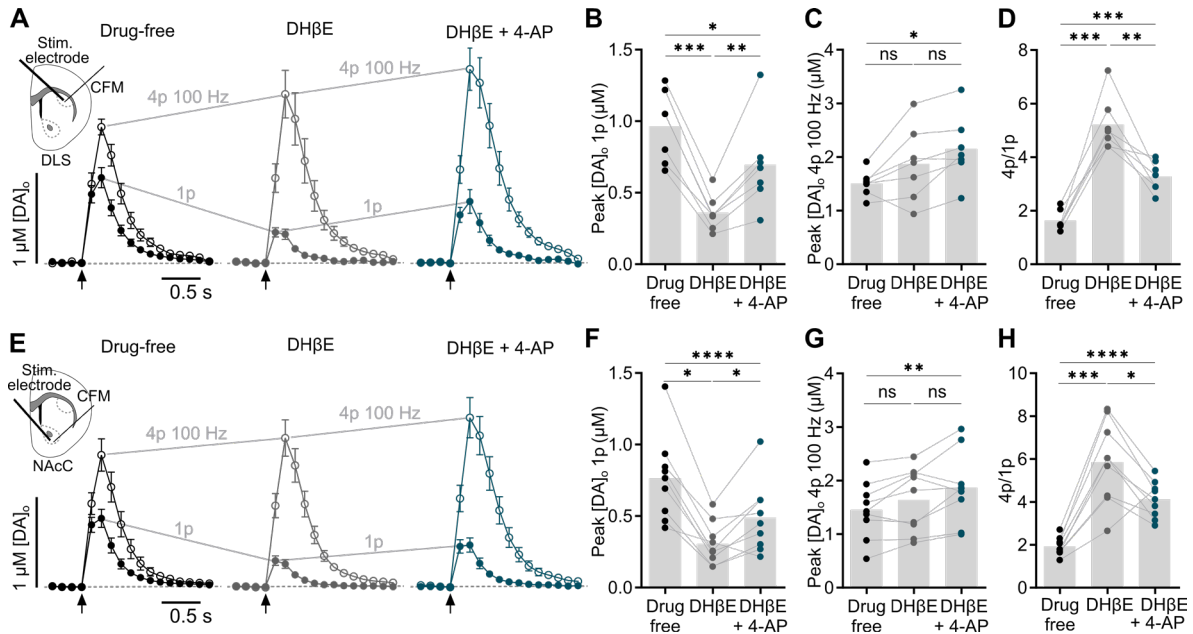
Therefore, concerning release evoked by 1p stimulation, blocking VGKCs when nAChRs are already antagonised seem to partially substitute for nAChR opening, since release is increased upon VGKCs blockage when nAChR are antagonised, but these release levels do not yet reach similar levels than in drug-free condition where nAChRs are active.

Next, we looked at release evoked by 4p 100 Hz stimulation. In DLS, when both nAChRs and VGKCs are blocked (DH $\beta$ E + 4-AP condition) release evoked by 4p 100 Hz was only slightly increased compared to when only nAChRs (DH $\beta$ E condition) are antagonised (**Figure 3.1 A and C**, p-value = 0.0468). However, in NAcC, release evoked by 4p was unchanged after VGKCs blockage when nAChRs are already inhibited (DH $\beta$ E + 4-AP condition) compared to when only nAChRs are antagonised by DH $\beta$ E (**Figure 3.1 E and G** p-value = 0.1428).

We then compared [DA]<sub>o</sub> evoked by 4p when nAChRs and VGKCs are blocked (DH $\beta$ E + 4-AP condition) and the [DA]<sub>o</sub> in drug-free condition when nAChRs are active. In both regions, [DA]<sub>o</sub> evoked by pulse-trains (4p 100 Hz) was significantly higher than in drug-free condition (in DLS **Figure 3.1 A and C**, p-value = 0.0210 ; in NAcC **Figure 3.1 E and G**, p-value = 0.0052). This increase was expected since 4-AP is known to widen spikes which consequently increases neurotransmitter release (Katz and Miledi 1967; Llinás et al. 1976; Augustine 1990). However, even if blocking VGKCs elevates release across stimulation paradigms (1p and 4p), release might not be increased uniformly. Indeed, nAChR activation increases release evoked 1p more but seems to hinder release at 4p consequently greatly



decreasing the 4p/1p ratio. Therefore, if blocking VGKCs when nAChRs are antagonised mimics the opening of nAChRs, then it should affect release at 1p more than release at 4p and diminish the 4p/1p.



Thus, we next tested whether the 4p/1p ratio was affected comparing first the DH $\beta$ E + 4-AP condition and the DH $\beta$ E only condition. In both regions, the 4p/1p ratio decreased upon VGKCs blockage when nAChRs are antagonised (DH $\beta$ E + 4-AP condition) compared to when only nAChRs (DH $\beta$ E condition) are antagonised (in DLS **Figure 3.1 A and D**,  $p$ -value = 0.0079; in NAcC **Figure 3.1 E and H**  $p$ -value = 0.0230). Therefore, release at 1p was increased more than release at 4p upon blockage of VGKCs, which seems to suggest VGKCs

did substitute for nAChR activation. Comparing the ratio in the DH $\beta$ E + 4-AP condition and the in the drug-free condition will allow us to determine whether this substitution was a complete substitution or a partial one. In both regions, 4p/1p ratio found when both VGKCs and nAChRs are antagonised were significantly lower to the 4p/1p ratio found in drug-free condition when nAChRs are active (in DLS **Figure 3.1 A** and **D**, p-value = 0.0008; in NAcC **Figure 3.1 E** and **H** p-value < 0.0001), suggesting the substitution was not a full substitution.

Altogether, this data suggests blocking VGKCs when nAChRs are already antagonised only partially substitute the opening of nAChRs. Indeed, VGKCs blockage mimicked the increased [DA]<sub>o</sub> evoked by single pulse and decreased 4p/1p ratio, but none reach the same values as in drug free condition when nAChRs are active.

### **3.3.2. VGKC inhibition partially prevents the nAChR-mediated STD of DA release**

Since our initial hypothesis is that following nAChR activation, VGKCs rapidly inactivate causing larger release events. However, VGKCs inactivation would be such that the membrane would be unable to produce more spikes during train pulses, therefore not allowing further release. We therefore wondered whether all VGKCs were thus inactivated when nAChRs are active. Using FCV to monitor  $[DA]_o$  in real-time, we investigated how blocking of VGKCs would affect  $[DA]_o$  by washing on the broad VGKCs blocker 4-AP (100  $\mu$ M) from drug-free conditions when nAChRs are active. If most VGKCs are indeed inactivated by nAChR activation, we would expect no increase, or eventually only a marginal increase in evoked  $[DA]_o$  following 4-AP wash on for all stimulation paradigms. The eventual small increase that we could expect to see could be due to few VGKCs types that are not blocked by 4-AP.

Further, we wondered whether the blockage of VGKCs would prevent the effects that antagonising nAChRs has on DA release. Indeed, if all VGKCs are already pharmacologically blocked, it would therefore render them unavailable, whether or not nAChRs are active, hence preventing the expected appearance of frequency-dependency of DA release. We thus tested whether adding nAChR antagonist DH $\beta$ E (1  $\mu$ M) when VGKCs are already blocked would decrease 1p release and increase 4p release like it does when starting from drug-free conditions, or if these effects would be attenuated or even absent since VGKCs are already blocked.

We therefore first applied 4-AP to assess the impact of 4-AP on DA release when nAChRs are active and compare it to drug-free condition. Next, we tested whether prior inhibition of VGKCs would prevent the effects of an nAChR antagonist by washing on DH $\beta$ E.

First, we compared the evoked [DA]<sub>o</sub> before (drug-free condition) and after 4-AP (100  $\mu$ M) wash on in order to assess the effect of blocking VGKCs on DA release when nAChRs are active. In DLS, the application of 4-AP increased [DA]<sub>o</sub> evoked by 1p (**Figure 3.2 A and B**, p-value = 0.0053) and by 4p 100 Hz stimulation (**Figure 3.2 A and C**, p-value = 0.0152) compared with [DA]<sub>o</sub> in drug-free conditions (**Figure 3.2 D**, p-value = 0.0375).

In NAcC, although after 4-AP wash on there is a trend towards the increase compared to drug-free condition of [DA]<sub>o</sub> evoked by 1p (**Figure 3.2 E and F**, p-value = 0.0664) and evoked by 4p 100 Hz (**Figure 3.2 E and G**, p-value = 0.5543) no statistical test revealed to be significant. Nevertheless, the 4p/1p ratio decreased upon bath application of 4-AP compared to drug-free condition (**Figure 3.2 H**, p-value = 0.0053), suggesting 4-AP elevated release evoked by 1p more than release evoked by pulse-trains.

These results seem to indicate that VGKCs are not fully inhibited in drug-free condition as our hypothesis suggested. Indeed, the trend towards an increase in [DA]<sub>o</sub> evoked by single stimulation suggest that spike width can be further increased, even during pulse-trains in DLS.

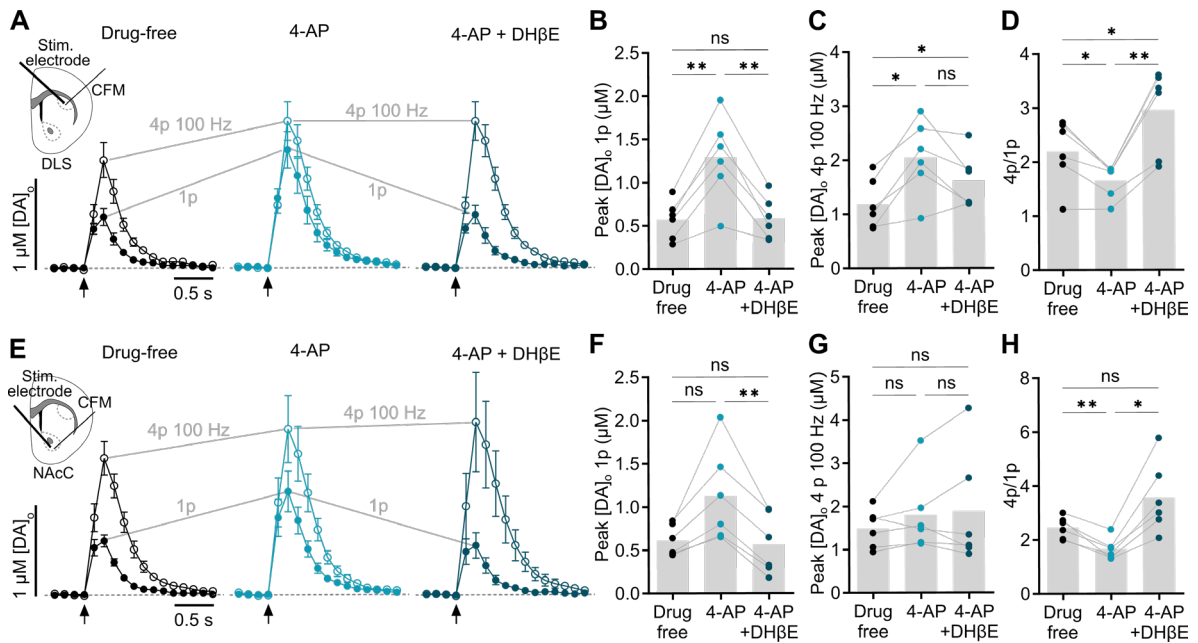
Next, we assessed whether prior inhibition of VGKCs prevented the effects of an nAChR antagonist. If it did, we expect no or only marginal changes to the [DA]<sub>o</sub> and in particular no significant changes for the 4p/1p ratio compared to the drug-free condition and to the 4-AP condition. However, a drop 1p-evoked release and a rise in 4p-evoked release or

an increased 4p/1p ratio would suggest that also blocking VGKCs seem to partially mimic nAChR activation, it is likely to not be downstream of the nAChR activation.

In both regions, application of DH $\beta$ E when VGKCs are blocked (4-AP + DH $\beta$ E condition) reduced [DA]<sub>o</sub> evoked by 1p compared to when only VGKCs are blocked (in DLS, **Figure 3.2 A and B**, p-value = 0.0088; in NAcC, **Figure 3.2 E and F**, p-value = 0.0082). Plus, the [DA]<sub>o</sub> evoked 1p in 4-AP + DH $\beta$ E condition was not statistically different from the drug-free condition (in DLS, **Figure 3.2 A and B**, p-value = 0.9627; in NAcC, **Figure 3.2 E and F**, p-value = 0.9124). This suggests that prior blocking of VGKCs did not prevent at all the effects of antagonising nAChRs on DA release evoked by 1p stimulation.

Antagonising nAChRs when VGKCs are blocked (4-AP + DH $\beta$ E condition) did not increase [DA]<sub>o</sub> release evoked by pulse trains further compared to when only VGKCs are blocked (in DLS, **Figure 3.2 A and C**, p-value = 0.2131; in NAcC, **Figure 3.2 E and G**, p-value = 0.9763). However, when compared to the drug-free condition, [DA]<sub>o</sub> evoked by 4p 100 Hz – when both VGKCs and nAChRs are inhibited – increased in DLS (**Figure 3.2 A and C**, p-value = 0.0152) but not in NAcC (**Figure 3.2 E and G**, p-value = 0.7573).

These results suggest that blocking of VGKCs can partially prevent the effects of antagonising nAChRs on DA release evoked by 4p 100 Hz stimulation, as it can prevent the DA increase compared to 4-AP condition in both regions, but can only prevent this increase in NAcC when compared to drug-free condition.



**Figure 3.2 : VGKCs are not fully blocked on drug-free condition and blocking them does not prevent the effect of antagonising nAChRs on DA release.**

**A, E:** Mean ( $\pm$  SEM)  $[DA]_0$  transients in response to 1 pulse electrical stimulation (1p) and 4 pulses at 100 Hz (4p 100 Hz) in DLS (**A**) and NAcC (**E**), in drug-free condition (left), after bath application of 100  $\mu$ M 4-AP (middle) and after addition of 1  $\mu$ M DH $\beta$ E (right). The arrow indicates the time of stimulation.  $n = 5$  in both regions.

**B, F:** Peak  $[DA]_0$  evoked by 1p stimulation in DLS (**B**) and NAcC (**F**). Each dot corresponds to a slice. Grey bars indicate the mean. One-way ANOVA (in DLS (**B**),  $F(1.077, 5.383) = 31.62$ ,  $p$ -value = 0.0018; in NAcC (**F**),  $F(1.129, 5.645) = 14.29$ ,  $p$ -value = 0.0094) and Šídák's multiple comparisons test.

**C, G:** Peak  $[DA]_0$  evoked by 4p 100 Hz stimulation in DLS (**C**) and NAcC (**G**). Each dot corresponds to a slice. Grey bars indicate the mean. One-way ANOVA (in DLS (**C**),  $F(1.488, 7.442) = 13.37$ ,  $p$ -value = 0.0047; in NAcC (**G**),  $F(1.128, 5.639) = 0.9845$ ,  $p$ -value = 0.3743) and Šídák's multiple comparisons test.

**D, H:** Ratio of peak  $[DA]_0$  released by 4p 100 Hz over 1p in DLS (**D**) and NAcC (**H**). Each dot corresponds to a slice. Grey bars indicate the mean. One-way ANOVA (in DLS (**D**),  $F(1.747, 8.734) = 28.28$ ,  $p$ -value = 0.0002; in NAcC (**G**),  $F(1.086, 5.431) = 13.85$ ,  $p$ -value = 0.0111) and Šídák's multiple comparisons test.

Mainly as a consequence of the drop in release evoked by 1p stimulation, the 4p/1p ratio increased in both regions when comparing 4-AP and 4-AP + DH $\beta$ E conditions (in DLS, **Figure 3.2 D**,  $p$ -value = 0.0039; in NAcC, **Figure 3.2 H**,  $p$ -value = 0.0141). In DLS, the ratio when both VGKCs and nAChRs are antagonised increased to higher than the ratio in drug-free conditions when nAChRs are active (**Figure 3.2 D**,  $p$ -value = 0.0215), while in NAcC, the ratio in 4-AP + DH $\beta$ E condition rose from the 4-AP condition only to matched its initial drug-free level (**Figure 3.2 H**,  $p$ -value = 0.1894).

Inhibition of nAChRs when VGKCs are already blocked did not prevent the usual effects antagonising nAChRs has on DA release which are to decrease  $[DA]_o$  following single pulse stimulation and increase in 4p/1p ratios. It however did prevent increasing of  $[DA]_o$  following pulse trains.

In conclusion, our initial hypothesis does not seem to hold and VGKCs inactivation seems unlikely to be downstream of the profound effects nAChR activation has on DA release. However, VGKCs blockage can still partially mimic nAChR activation (section 3.3) and partially prevent increase in  $[DA]_o$  evoked by 4p 100 Hz stimulation occurring when nAChRs are subsequently antagonised (section **3.4**), implying it might still play a role in the mechanisms downstream of nAChRs even if not the main driving force.

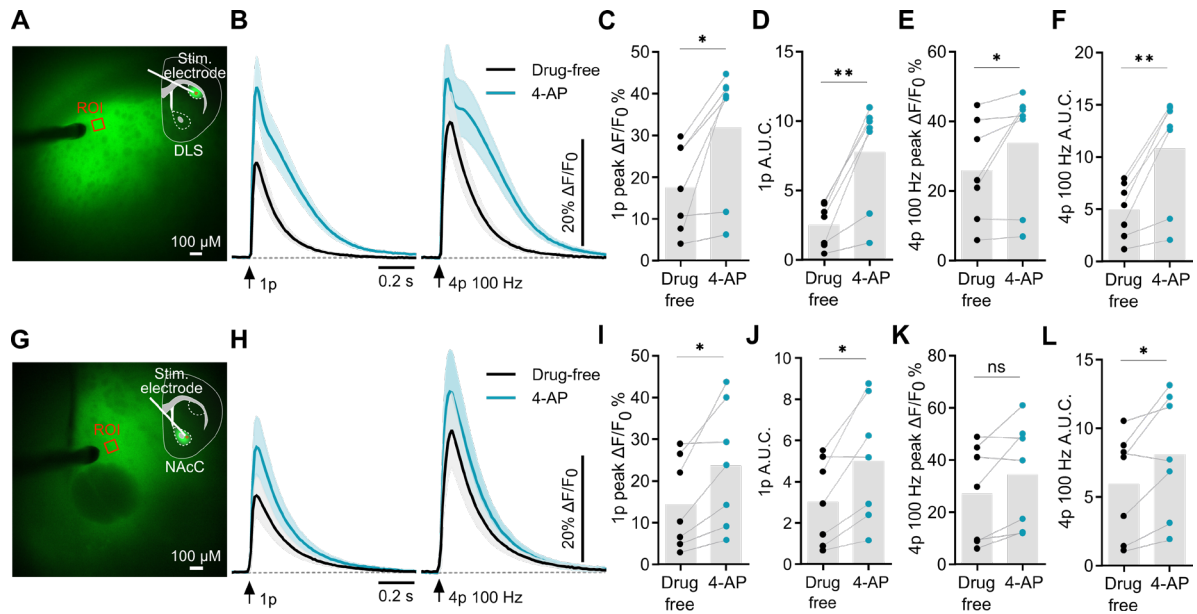
### **3.3.3. Effects of VGKCs blockade on DA release are not due to the change in ACh levels**

A confounding factor of electrical stimulations is that they activate all neurons and other brains cells in the vicinity of the stimulating electrode. In addition, our drug application is not targeted to specific cell types or regions, and exogenous application of pharmacological agents affect all circuits within the striatum.

Since our study is investigating the mechanisms downstream of activation of nAChRs at DA axons, varying ACh release levels could modify their occupancy levels and therefore their effect on DA release.

Previous unpublished data from the lab has shown that DH $\beta$ E does change ACh release when monitored with GRAB<sub>ACh</sub> (Vietti-Michelina 2022). Thus, we only tested whether 4-AP also increases ACh release. In order to detect extracellular levels of ACh in the striatum, we used the GRAB<sub>ACh3.0</sub> sensor (Jing et al. 2020). The GRAB<sub>ACh3.0</sub> sensor is a modified mAChR receptor fused with a cpGFP, which emits fluorescence upon binding of ACh. The viral construct (AAV5-hSyn-ACh3.0) for the sensor was injected directly in the striatum for expression in all neurons.





**Figure 3.3 : Blocking VGKCs using 4-AP (100  $\mu$ M) enhances ACh release.**

**A, G:** Example slices with expression of GRAB<sub>ACh3.0</sub> in DLS (**A**) and in NAcC (**G**) with positioning of the stimulation electrode (in block from the left) and where ROI would be positioned during analysis. Pictures taken under a 10x objective.

**B, H:** Mean ( $\pm$  SEM)  $\Delta F/F_0$  transients in response to 1 pulse electrical stimulation (1p) and 4 pulses at 100 Hz (4p 100 Hz) in DLS (**B**) and NAcC (**H**), in drug-free condition (black) and after bath application of 100  $\mu$ M 4-AP (blue). The arrow indicates the time of stimulation.  $n = 7$  in DLS and  $n = 7$  in NAcC.

**C, I:** Peak  $\Delta F/F_0$  evoked by 1p stimulation in DLS (**C**) and NAcC (**I**). Each dot corresponds to a slice. Grey bars indicate the mean. Paired t-test, in DLS (**C**),  $p$ -value = 0.0158; in NAcC (**I**),  $p$ -value = 0.0179.

**D, J:** AUC evoked by 1p stimulation in DLS (**D**) and NAcC (**J**). Grey bars indicate the mean. In DLS (**D**), Wilcoxon matched-pairs test,  $p$ -value = 0.0312; in NAcC (**J**), paired t-test  $p$ -value = 0.0548.

**E, K:** Peak  $\Delta F/F_0$  evoked by 4p 100 Hz stimulation in DLS (**E**) and NAcC (**K**). Each dot corresponds to a slice. Grey bars indicate the mean. In DLS (**E**), Wilcoxon matched-pairs test,  $p$ -value = 0.0156; in NAcC (**K**), paired t-test,  $p$ -value = 0.0148.

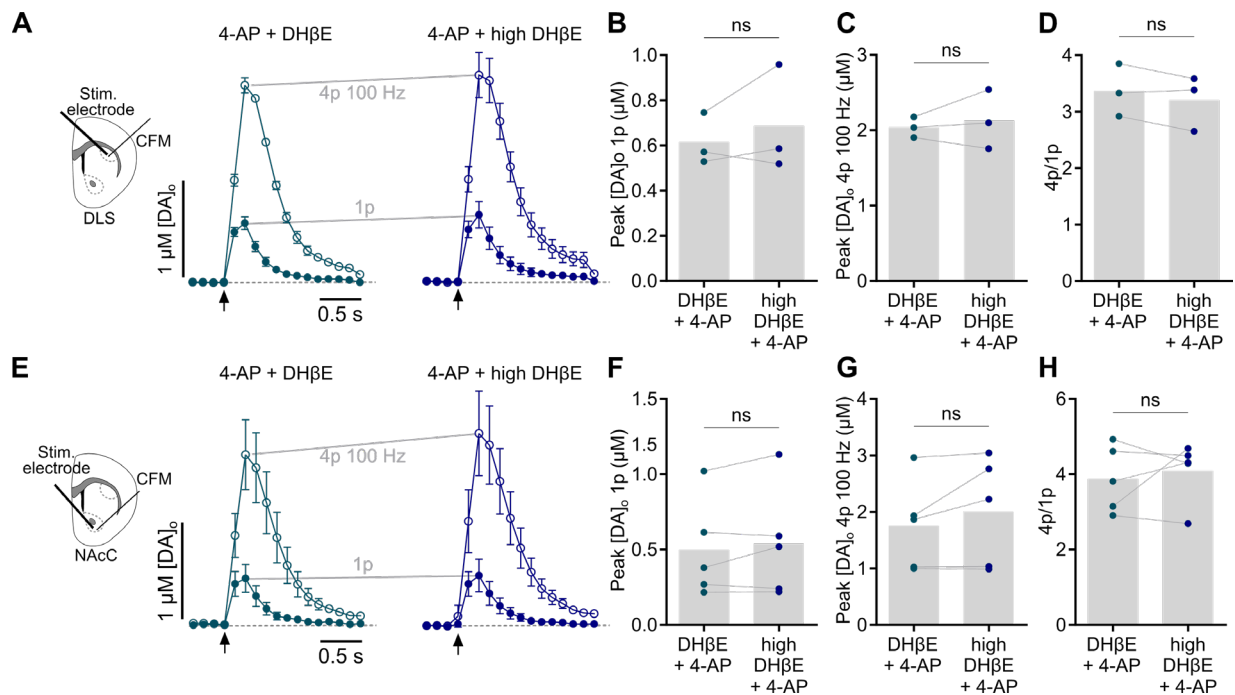
**F, L:** AUC. evoked by 4p 100 Hz stimulation in DLS (**F**) and NAcC (**L**). Each dot corresponds to a slice. Grey bars indicate the mean. In DLS (**F**), Wilcoxon matched-pairs test,  $p$ -value = 0.0156; in NAcC (**L**), paired t-test,  $p$ -value = 0.0238.

Blocking VGKCs increased the peak  $\Delta F/F_0$  evoked by 1p in both regions (**Figure 3.3 B, C, H, I**). 4-AP wash on increased peak  $\Delta F/F_0$  evoked by 4p 100 Hz stimulation only in DLS (**Figure 3.3 B, E**), but not in NAcC (**Figure 3.3 H, K**) despite a trend towards an increase too. However, peak  $\Delta F/F_0$  might not always reflect smaller changes in ACh release levels; studies from the lab (Vietti-Michelina 2022) have found that AUC might be more reflective of finer changes in external ACh concentrations. Hence, we also used the AUC of the transients as an approximation for changes in extracellular ACh concentration. In both regions, blocking

VGKCs using 4-AP increased the AUC of ACh transients evoked by 1p stimulation (**Figure 3.3 B, D, H and J**), and by 4p 100 Hz stimulation (**Figure 3.3 B, F, H and L**).

These results show that extracellular ACh concentration is increased by exogenous applications of the broad VGKCs blocker 4-AP. Since we do not expect changes in the efficacy of the AChE, this data suggest that the increase might be solely due to more ACh being released from ChIs.

Since DH $\beta$ E is a competitive inhibitor of the nAChRs, the elevated ACh release when 4-AP is present could affect the efficacy of DH $\beta$ E on nAChRs at DA axons. Therefore, with more ACh in the vicinity of nAChRs, it is possible that the DH $\beta$ E concentration we initially used did not fully occlude nAChR activity.



**Figure 3.4: Blocking VGKCs only partially mimics opening of nAChRs.**

**A, E:** Mean ( $\pm$  SEM) [DA]<sub>0</sub> transients in response to 1 pulse electrical stimulation (1p) and 4 pulses at 100 Hz (4p 100 Hz) in DLS (**A**) and NAcC (**E**), in 4-AP (100  $\mu$ M) and DH $\beta$ E (1  $\mu$ M) followed by bath application of higher concentration of DH $\beta$ E (10  $\mu$ M) still in the presence of 4-AP (100  $\mu$ M).  $n = 3$  in DLS and  $n = 5$  in NAcC.

**B, F:** Peak [DA]<sub>0</sub> evoked by 1p stimulation in DLS (**B**) and NAcC (**F**). Each dot corresponds to a slice. Grey bars indicate the mean. Paired t-test, in DLS (**B**),  $p$ -value = 0.4456; in NAcC (**F**),  $p$ -value = 0.3206.

**C, G:** Peak [DA]<sub>0</sub> evoked by 4p 100 Hz stimulation in DLS (**C**) and NAcC (**G**). Each dot corresponds to a slice. Grey bars indicate the mean. Paired t-test, in DLS (**C**),  $p$ -value = 0.5945; in NAcC (**G**),  $p$ -value = 0.1828.

**D, H:** Ratio of peak [DA]<sub>0</sub> in response to 4p 100 Hz over 1p in DLS (**D**) and NAcC (**H**). Each dot corresponds to a slice. Grey bars indicate the mean. Paired t-test, in DLS (**D**),  $p$ -value = 0.2711; in NAcC (**H**),  $p$ -value = 0.6017.

Our initial concentration of 1  $\mu\text{M}$  DH $\beta$ E has been shown to be largely inhibiting all  $\beta_2$ -containing nAChRs in drug-free condition (Clements 2008). Therefore, we chose to raise this concentration to 10  $\mu\text{M}$  DH $\beta$ E, as a 10-fold increase from the initial concentration should be sufficient to counterbalance the rise in ACh levels.

In order to test whether the concentration of DH $\beta$ E used in **section 3.3** and **3.4** was insufficient to fully occlude nAChRs effect on DA release, we tested whether increasing the concentration of DH $\beta$ E from 1  $\mu\text{M}$  to 10  $\mu\text{M}$  in the presence of 4-AP would reveal different effect of 4-AP on DA release when all nAChRs are antagonised.

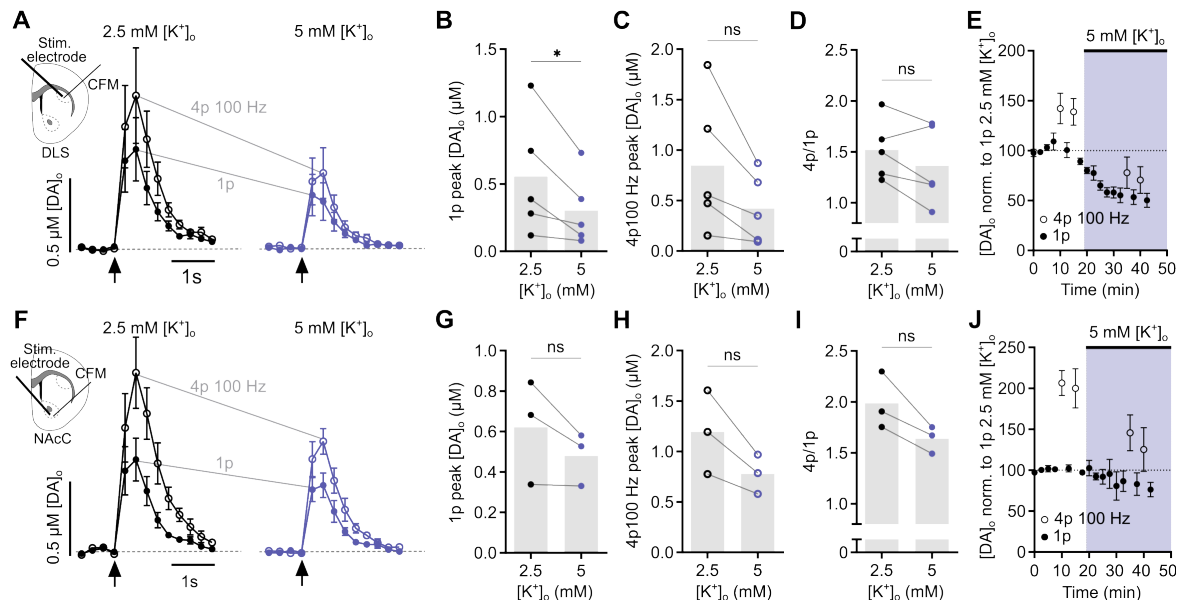
In both regions, neither release evoked by 1p or 4p was significantly modified after washing on DH $\beta$ E at a higher concentration (10  $\mu\text{M}$ ) compared to in the initial 1  $\mu\text{M}$  DH $\beta$ E used (**Figure 3.4 A, B, C, E, F and G**). The resulting 4p/1p ratios remain stable in both regions (**Figure 3.4 D and H**).

In conclusion, increasing further the DH $\beta$ E concentration does not change the effect of blocking VGKCs on DA release suggesting the initial concentration of DH $\beta$ E was sufficient to fully block nAChRs in experiments presented in **section 3.3** and **3.4** despite the increase in ACh release shown in **Figure 3.3**.

### 3.3.4. Higher external potassium seems to lower 4p/1p

Previous work (Threlfell et al. 2012; Condon et al. 2019) from the lab used a different aCSF solution containing HEPES acid and different salt concentrations (in mM): 120 NaCl, 5 KCl, 20 NaHCO<sub>3</sub>, 2 CaCl<sub>2</sub>, 2 MgSO<sub>4</sub>, 3.3 HEPES salt and 1.2 KH<sub>2</sub>PO<sub>4</sub>, as opposed to the solution used throughout this body of work which contains the following salt concentrations (in mM): 130 NaCl, 2.5 KCl, 26 NaHCO<sub>3</sub>, 2 CaCl<sub>2</sub>, 2 MgCl<sub>2</sub>, 1.25 NaHCl, 1.25 NaH<sub>2</sub>PO<sub>4</sub>. In particular, previous recordings undertaken by the lab used an external solution contained higher potassium concentration ( $[K^+]_o > 5$  mM) to the current aCSF used in this study (2.5 mM) which has taken a potassium concentration closer to the *in vivo* CSF composition (Czarniak et al. 2023). Upon comparing this current body of work with previous studies, we realised that it seems that the 4p/1p ratio using the higher concentration potassium solution was lower than in experiments performed in 2.5 mM  $[K^+]_o$  solution.

Although experiments in 3.3 and 3.4 seemed to suggest that VGKCs were not acting directly downstream of nAChR activation in the modulation of DA by ACh, it is possible that the pharmacological agent we used to block VGKCs, although defined as a broad VGKCs blocker, was not blocking the right VGKCs type. Therefore, we decided to test whether changing  $[K^+]_o$  in the external solution would affect the potential role VGKCs play in the nAChR modulation of DA release. Indeed, increasing  $[K^+]_o$  changes the Nernst equilibrium of the membrane thereby lowering the driving force for K<sup>+</sup> ions outward movements through the membrane, as well as impact the inactivation state of VGKCs themselves (Baukrowitz and Yellen 1995).



**Figure 3.5 : Increasing external potassium concentration in aCSF seems to reduce DA release and 4p/1p ratio.**

**A, F:** Mean ( $\pm$  SEM)  $[DA]_0$  transients in response to 1 pulse electrical stimulation (1p) and 4 pulses at 100 Hz (4p 100 Hz) in DLS (**A**) and NAcC (**F**), in aCSF containing 2.5 mM  $[K^+]_0$  following by aCSF containing 5 mM  $[K^+]_0$ .  $n = 5$  in DLS and  $n = 3$  in NAcC.

**B, G:** Peak  $[DA]_0$  evoked by 1p stimulation in DLS (**B**) and NAcC (**G**). Each dot corresponds to a slice. Grey bars indicate the mean. Paired t-test, in DLS (**B**),  $p$ -value = 0.0428; in NAcC (**G**),  $p$ -value = 0.1955.

**C, H:** Peak  $[DA]_0$  evoked by 4p 100 Hz stimulation in DLS (**C**) and NAcC (**H**). Each dot corresponds to a slice. Grey bars indicate the mean. Paired t-test, in DLS (**C**),  $p$ -value = 0.0538; in NAcC (**H**),  $p$ -value = 0.0823.

**D, I:** Ratio of peak  $[DA]_0$  in response to 4p 100 Hz over 1p in DLS (**D**) and NAcC (**I**). Each dot corresponds to a slice. Grey bars indicate the mean. Paired t-test, in DLS (**D**),  $p$ -value = 0.1299; in NAcC (**I**),  $p$ -value = 0.0730.

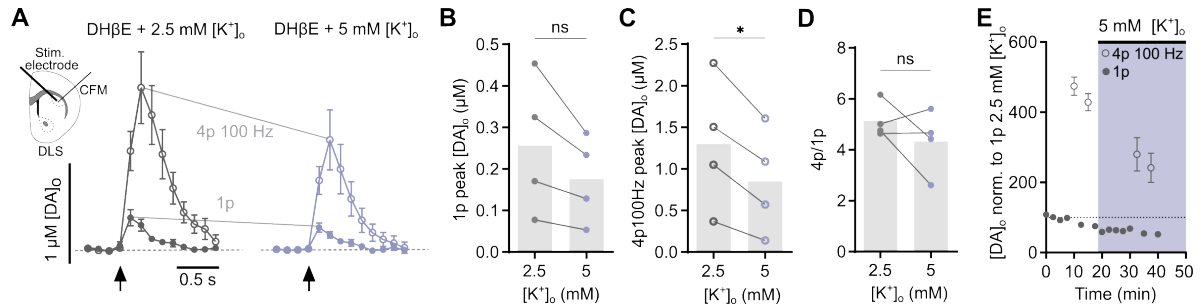
**E, J:** Mean peak  $[DA]_0$  over time in DLS (**E**) and NAcC (**J**) starting in 2.5 mM  $[K^+]_0$  aCSF and followed by a wash on of 5 mM  $[K^+]_0$  aCSF. Data is normalised to the average of the four first  $[DA]_0$  values evoked by 1p shown on the graph. The mean peak for release evoked by single pulse in **B** and **G** are the values flanking the  $[DA]_0$  evoked by 4p 100 Hz.

Data acquired in this panel have been collected by Mahnoor Khurram and me.

We therefore performed pilot experiments to assess how higher external potassium concentration in the aCSF could the effect release and 4p/1p ratio in the presence or absence of nAChR antagonist. To do this, we started in the aCSF solution used throughout this study containing 2.5 mM  $[K^+]_0$ , and washed on the same solution but with the potassium concentration increased to with 5 mM  $[K^+]_0$ . We first compared release and 4p/1p ratio between the different  $[K^+]_0$  conditions and in the absence of nAChR antagonist.

In both regions and for both 1p and 4p 100 Hz stimulations, we notice a trend towards lowered  $[DA]_0$  when external levels of potassium are increased to 5 mM compared to when they are equal to 2.5 mM (**Figure 3.5**). For both regions, the 4p/1p ratios tend towards a

decrease (**Figure 3.5 D and J**) which suggests that the  $[DA]_o$  evoked by pulse-trains is diminished more strongly by the changes in  $[K^+]_o$  than the  $[DA]_o$  evoked by 1p. The decrease in  $[DA]_o$  evoked by 4p and the decrease in 4p/1p ratio seems to partially mimics further activation of nAChRs.



**Figure 3.6 : Increasing external potassium concentration in aCSF seems to reduce DA release even when nAChRs are antagonised using DH $\beta$ E.**

**A:** Mean ( $\pm$  SEM)  $[DA]_o$  transients in response to 1 pulse electrical stimulation (1p) and 4 pulses at 100 Hz (4p 100 Hz), in aCSF containing 2.5 mM  $[K^+]_o$  following by aCSF containing 5 mM  $[K^+]_o$  in DLS. The whole experiment is conducted in the presence of DH $\beta$ E, nAChR blocker.  $n = 4$ .

**B:** Peak  $[DA]_o$  evoked by 1p stimulation. Grey bars indicate the mean. Paired t-test,  $p$ -value = 0.0851.

**C:** Peak  $[DA]_o$  evoked by 4p 100 Hz stimulation. Grey bars indicate the mean. Paired t-test,  $p$ -value = 0.0157.

**D:** Ratio of peak  $[DA]_o$  in response to 4p 100 Hz over 1p. Paired t-test,  $p$ -value = 0.3073.

**E:** Mean peak  $[DA]_o$  ( $\pm$  SEM) over time. The mean peak for release evoked by single pulse in **B** are the values flanking the  $[DA]_o$  evoked by 4p 100 Hz.

Data acquired in this panel have been collected by me and Mahnoor Khurram.

Since increasing  $[K^+]_o$  seems to partially mimic further opening of nAChRs when nAChRs are already active, we wondered whether the axons are affected differently by changes in external  $[K^+]_o$  when nAChRs are closed. We performed pilot experiments in DLS where nAChRs are antagonised using DH $\beta$ E throughout and starting in aCSF containing 2.5 mM  $[K^+]_o$  and later increased to 5 mM.

We found that  $[DA]_o$  evoked by 4p 100 Hz in 5 mM  $[K^+]_o$  aCSF was significantly lowered compared to release in 2.5 mM  $[K^+]_o$  aCSF (**Figure 3.6 A and C**). A trend towards a decrease was also observed for release evoked by single pulse (**Figure 3.6 A and B**). However, no clear trend was observed for the 4p/1p ratio signifying the general decrease in

evoked  $[DA]_o$  observed in 5 mM  $[K^+]_o$  aCSF may be uniform across stimulation paradigms. However, more repetitions would be needed to confirm this.

In conclusion, changes in external  $[K^+]_o$  do affect dopamine release whether or not nAChRs are active. This confirms that the aCSF used in this body of work, *i.e.* the aCSF containing a 2.5 mM  $[K^+]_o$ , seems better at reflecting the frequency dependency of DA release, by increasing release and 4p/1p ratio, allowing for wider working range especially when manipulations tend to reduce release levels or the ratio.

### 3.4. Conclusion and discussion

In this chapter, we investigated the potential role for VGKCs in the mechanisms downstream of nAChR activation at DA axons. We used FCV to monitor  $[DA]_o$  in real time and bath applied a broad VGKCs blocker 4-AP to test whether activation of nAChRs at DA axons provoked a prolonged inactivation of VGKCs that incapacitated the membrane to produce subsequent spikes leaving an apparent inhibition in DA release. We found that blocking VGKCs only partially mimic opening of nAChRs when nAChRs are already antagonised by DH $\beta$ E, but that blocking VGKCs does not prevent the effects produced by antagonising nAChRs. Altogether these results suggest the VGKCs effect on DA release is likely not downstream of nAChR activation.

However, although slice work with electrical stimulation gives us better control over the circuits involved and stimulus provided than *in vivo* work while keeping the complexity of the circuits to a certain extent as opposed to cell culture, this technique does not allow us to fully isolate only the two neuronal populations of interest within the striatal circuits. Therefore, it is likely that the VGK blocker also affected other cells and circuits within the striatum that might have had an impact on  $[DA]_o$ . We controlled for the main other neurotransmitter involved in the mechanism at interest here, ACh, and found that although ACh release level increase, this did not affect the result of our experiments regarding DA release. However, other more subtle changes in the circuit could have affected the DA axons in a way that changes their behaviour in response to some stimulation paradigms or other neurotransmitters. These potential confounds have not been tested here.



Secondly, we used 4-AP for its ability to block a broad range of VGKCs (Khammy et al. 2018) because little is known about what VGKCs are expressed on DA axons. Nevertheless, it is also possible that our choice of pharmacological agent was not targeted at the right types of VGKCs, and that 4-AP was too broad of a blocker and disrupted the membrane behaviour entirely masking potential more effects of nAChRs. These observations could however not be verified without a better access to the membrane voltage of the axons themselves.

Finally, the work in this chapter leads us to measure even more the importance for the field to gain easier access to the membrane behaviour of dopaminergic axons in order to measure the direct effect of receptor and channel activation as opposed to an indirect read-out of release. In the next chapter, in conjunction with FCV, novel approaches using state of the art voltage sensor imaging and ultra-fast calcium imaging at a population of dopaminergic axons allowed us to more finely dissect the potential mechanisms by which nAChRs modulate DA release. Although this chapter suggests that VGKCs are not the main actors in nAChR modulation of DA release, the next chapter (**chapter 4**) builds up on the hypothesis that nAChR activation still modifies the excitability of the axons, but this time focusing on the role of VGSCs which key players in action potential upstroke as well as in signal propagation within a neuron.

# 4

## The Role of Axonal Voltage-gated Sodium Channels in the Modulation of Dopamine Release by Nicotinic Acetylcholine Receptors

## 4.1. Introduction and initial hypothesis

In the striatum, the highly branched DA axons receive local inputs from ChIs. *Ex vivo* studies on brain slices have shown that ACh release from ChIs influences DA release through nAChRs located on DA axons (Rice and Cragg 2004; Zhang and Sulzer 2004; Exley and Cragg 2008; Exley et al. 2008, 2012a; Threlfell et al. 2012). These studies highlight that nAChRs can affect DA release in opposite ways, since nAChRs can both enhance or limit DA release depending on the type of stimulation.

Firstly, on the one hand, DA release can be supported by nAChRs when the striatum is stimulated with single electrical pulses or low frequency trains (< ~10 Hz). DA release detected with amperometry in these scenarios has been shown to be composed of two phases separated only by few milliseconds (Wang et al. 2014a, b): an initial direct DA release event independent from nAChR action and a subsequent DA release event ~10 ms later dependent upon ACh released in response to the electrical pulse. Targetted optogenetic stimulation of ChIs correspondingly evokes DA release from striatal DA axons measured with FCV (Threlfell and Cragg 2011; Brimblecombe et al. 2018).

Secondly, nAChRs can limit DA release. This is particularly evident during pulse-trains stimulations at higher frequencies (> 10 Hz). Antagonism of nAChRs using a specific  $\beta 2$ -containing nAChR antagonist DH $\beta$ E increases release level during high frequency pulse trains compared to when nAChRs are active, appearing to relieve a short-term depression (STD) and to promote frequency dependence of DA release.

However, the nAChR-mediated mechanisms that drive the STD of DA release that limits its apparent frequency-dependency are still unknown. The STD mediated by nAChRs following pulse-trains has been hypothesised previously to be due to DA vesicle depletion

(Wang et al. 2014b, a) however other studies find alternative explanations (Cragg 2003; Zhang et al. 2025). A recent study (Zhang et al. 2025) suggest that nAChR activation limits the excitability of the DA membrane for around 100 ms afterwards, effectively limiting DA release by subsequent stimuli arriving within this time frame (i.e. frequencies greater than 10 Hz). This finding provides a partial explanation for the previously reported frequency-dependence of DA release, which will arise not from a frequency dependence *per se*, but will be a function of the interval between activation of nAChRs and new incoming activity in DA axons. To date, no studies have provided an underlying mechanism though which nAChRs can support and limit DA release. However, a recent study did identify that nAChR activation can drive ectopic axonal action potential (AP) (Liu et al. 2022) and so there are likely to be ensuing changes to axonal excitability as a result. We explored how the excitability of DA axonal membrane is modified by activation of nAChRs, and candidate underlying mechanisms.

Several candidate mechanisms exist, including voltage-dependent modulation or changes to availability of VGKCs or VGSCs. In Chapter 3, we investigated the potential role of VGKCs in the mechanisms downstream of nAChR activation but did not find that changes in VGKC handling offered an explanation for how nAChRs modify the excitability of DA axons. In this chapter, we explore an alternative explanation through the potential role of VGSCs in nAChR modulation of DA release.

VGSCs play key roles in local AP generation as they are responsible for the upstroke of AP allowing the membrane to depolarise. VGSC genes are a highly conserved family branching from a single common ancestor, conferring all VGSCs with similar structures

(Hille 2001). VGSCs are formed by a single functional  $\alpha$ -subunit constituted of four domains, each comprising six transmembrane segments including a voltage sensing domain on the fourth segments. They can also be accompanied by auxiliary or regulatory  $\beta$ -subunits oriented towards the extracellular matrix (Hille 2001) chapter 3). In a series of seminal experiments, Hodgkin, Katz and Huxley demonstrated that VGSCs are responsible for the upstroke of action potential (Hodgkin and Katz 1949), and that although they can activate rapidly, their inactivation also occurs extremely quickly during the depolarisation (Hodgkin and Huxley 1952c, a, d; Hodgkin 1958). VGSCs then remain inactivated unless the membrane is repolarised or hyperpolarised, often by VGKCs. Using patch-clamp techniques, it has been shown that raising the resting membrane voltage (to -60 mV from -67.5 mV) can inactivate enough VGSCs to reduce subsequent  $\text{Na}^+$  currents evoked by a short depolarising voltage-step and, conversely, that hyperpolarising the membrane prior to stimulation increases  $\text{Na}^+$  conductance (Dodge 1963; Hille 2001).

**We thus hypothesised that the participation of VGSCs in DA axon membrane depolarisation when nAChRs are active limits the pool of VGSCs available during ensuing pulse trains and thereby limits the subsequent generation of action potentials in DA axons.**

Until now, the techniques available to study the impact of nAChRs on DA axons have been indirect, *ie.* measuring DA release using FCV or measuring cytosolic  $\text{Ca}^{2+}$  using GCaMP sensors. Although these techniques have allowed us to understand how nAChRs shape DA release, they do not possess the resolution to define the mechanisms through which nAChRs

modulate DA release through potential changes in the excitability of the membrane of DA axons.

Recent advances in optogenetic tools with the arrival of ultra-fast genetically encoded voltage indicators (GEVIs) have allowed us to access a whole new set of questions previously answerable, such as how nAChRs impact the membrane voltage of DA axons directly? The study by Zhang et al. (2025) used an early generation GEVI (Accelerated Sensor of Action Potentials - ASAP3) that does not have the temporal resolution or sensitivity to resolve specific threshold versus subthreshold depolarisation events, or their time course. For the first time, we implemented and optimised (**section 4.4.2.4**) (Duquenoy and O'Connor 2024) the novel GEVI ASAP5f (Hao et al. 2024) for recording membrane voltage variations akin to action potentials and other depolarisation events in a population of DA axons. We injected AAV9-EF1 $\alpha$ -DIO-ASAP5-WPRE in the midbrain of DAT-Cre mice to record from a population of axons of DA neurons in the striatum (**section 4.4.2.4**). We used ultra-fast sampling of 600 Hz in order to achieve appropriate near-millisecond temporal resolution for the signal, given that changes in membrane potential occur at a millisecond scale. With unprecedented sub-millisecond time resolution, this technique revealed identifiable population spikes and subthreshold events (**section 4.2.4.5**) in response to electrical stimulation. Using this GEVI, we directly investigated the effect of nAChR activation on DA axonal membrane following electrical stimulation. We found that nAChR activation provokes a **prolonged depolarisation** that **limits the amplitude of subsequent population spikes** during a pulse-train. Next, we assessed whether, the limited amplitude of the population spikes following nAChR activation could be the cause for the strong nAChR-mediated STD of DA release observed in studies using FCV. With the aim of bridging existing data on DA release dynamics (acquired

through FCV) and membrane voltage dynamics (acquired using ASAP5f), we set up ultra-fast jRCaMP8m imaging that allowed us to distinguish axonal  $\text{Ca}^{2+}$  events with high temporal resolution. Using this technique, we first found that nAChR activation elicits a strong axonal  $\text{Ca}^{2+}$  event, and confirmed that any subsequent electrical pulses only elicit very limited  $\text{Ca}^{2+}$  levels. Finally, using FCV, we tested whether **limited VGSC availability following nAChR activation**, potentially due to inactivation provoked by the prolonged depolarisation, was the cause for the limited spike height and consequently the strong STD of DA release. Our data supported our hypothesis that inactivation of VGSCs is the limiting factor in spike formation following nAChR activation. Finally, using a combination of GRAB<sub>ACh</sub> and FCV we ruled out the involvement of other neurotransmitters from the striatal circuit.

## 4.2. Methods

Experiments described in this chapter were carried out as described in Chapter 2. Specifics of this chapter – such as voltage imaging and ultra-fast jGCaMP8m imaging – are detailed in this section.

### 4.2.1. Slice preparation

Acute ex vivo coronal striatum slices were prepared for FCV, ASAP5f, jGCaMP8m and GRAB<sub>ACh3.0</sub> experiments as described in section **2.3**.

The number of slices used for each experiment is noted “n”. At maximum, 2 slices from the same animal have been used for each experiment. All experimental procedures involving the use of animals were carried out according to institutional guidelines and conformed to the UK Animals (Scientific Procedures) Act 1986.

### 4.2.2. FCV recordings

Animals used for FCV experiments were mixed gender (7 to 13 weeks of age) C57BL/6J mice heterozygous DAT-Cre (Slc6c3 heterozygous on a C57BL6 background). FCV recordings were performed as described **sections 2.4.** and **2.5**. FCV data was acquired and analysed as described in **section 2.5**. Imaging data was acquired and analysed as described in **section 2.6.3** and **2.6.4** respectively. Statistical analyses were carried out using GraphPad Prism as described in **section 2.7.** and the threshold for statistical significance was  $p < 0.05$ . Statistical significance was reported as: ns  $p > 0.05$ ; \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ ; \*\*\*\*  $p < 0.0001$ .



### 4.2.3. GRAB<sub>ACh</sub> imaging

Animals used for FCV experiments were mixed gender (9 to 13 weeks of age) C57BL/6J mice. GRAB imaging was performed as described in **sections 2.6.2** on animals who have undergone intracranial surgeries in the striatum (described in **section 2.2**) and injected with AAV5-hSyn-ACh3.0(ACh4.3) (Cat #YL001003-AV5) at a concentration of  $3.78 \times 10^{12}$  vg/ml diluted in sterile saline, between 3 and 4 weeks prior to the experiment. FCV data was acquired and analysed as described in **section 2.5**. Imaging data was acquired and analysed as described in **section 2.6.3** and **2.6.4** respectively. Statistical analyses were carried out using GraphPad Prism as described in **section 2.7**. and the threshold for statistical significance was  $p < 0.05$ . Statistical significance was reported as: ns  $p > 0.05$ ; \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ ; \*\*\*\*  $p < 0.0001$ .

### 4.2.4. Monitoring membrane voltage changes population of striatal dopaminergic axons using fluorescent voltage sensor

#### 4.2.4.1. About voltage sensors and ASAP5f

Roles of nAChRs on striatal dopaminergic axons have been probed for decades. However, the lack of access of these axons and the complexity of the striatal network has made it a difficult enterprise. Indeed, although patching is possible in some cases (Kramer et al. 2020, 2022; Xiao et al. 2021), it is only doable on the main unbranched axon of the medial fibre bundle and therefore would not answer the question of local modulation in the context of very branched axons within the striatum. Being able to match our data to years of studies on DA release in specific regions of the striatum was crucial for us to infer a link between the

behaviour of the membrane in response to an electrical stimulation and the well-known DA release dynamics.

Although there are many options available for voltage sensing fluorescent reporters (fluorescent dyes, rhodopsin-based or FRET-based), the best tool that allowed us to have a sensor specifically expressed in dopaminergic neurons, fast enough to resolve single action potentials and that would have good expression outside the soma for recordings on axons only were the GEVIs ASAP from the Lin lab (St-Pierre et al. 2014; Chamberland et al. 2017; Villette et al. 2019; Evans et al. 2023; Hao et al. 2024). We therefore decided to use these exciting new optogenetic with the aim of investigating the behaviour of the membranes of the dopaminergic axons in response to various stimuli and in different drug conditions.

The ASAP sensors are composed of 4 transmembrane helices that act as voltage-sensing domains (VSDs) thanks the intrinsic properties of charged amino-acids expressed within their bundles. A cpGFP protein is attached to extracellular loop between helix 3 and 4. Upon depolarisation, the VSD reorganises its 3D conformation, and the loop between the 3<sup>rd</sup> and 4<sup>th</sup> helix is particularly altered, which leads to changes in the conformation of the cpGFP allowing it to increase its fluorescence – if it is a upward reporting sensor (Evans et al. 2023) – or dim its fluorescence – for downward deflecting sensors (St-Pierre et al. 2014; Chamberland et al. 2017; Villette et al. 2019; Hao et al. 2024).

Upon discussion with the Lin lab, we decided to use the newly developed ASAP5f sensor can promptly switch from supporting to limiting DA release which has been designed to be faster and brighter than previous versions of GEVIs, and which is also sensitive to single action potentials and even subthreshold events at the membrane (Hao et al. 2024).

#### 4.2.4.2. Animals used for voltage sensor experiments

All animals used for voltage sensor experiments were mixed-sex DAT-Cre heterozygous (Slc6c3 heterozygous on a C57BL6 background) mice aged 9 to 13 weeks which had undergone the procedure described in **Chapter 2 section 2.2** for intracranial injection in the midbrain (**Figure 4.1 A**). We used the following viral solution provided by the Lin Lab: AAV9-EF1 $\alpha$ -DIO-ASAP5-WPRE, diluted in sterile saline to final concentration of  $1.92 \times 10^{12}$  vg/mL. Recordings were made in the region of the striatum in which the targeted midbrain neurons project, and therefore exclusively in axons.

#### 4.2.4.3. Imaging parameters allowing the detection of membrane voltage changes in a population of dopaminergic axons in the striatum

The detailed protocol for voltage sensor imaging in a population of dopaminergic axons in the striatum is available at (Duquenoy and O'Connor 2024).

We used a blue LED light ( $\lambda = 470$  nm) at 10 mW measured under at 10x objective, or approximately 3 mW measured under a 60x objective to perform these experiments.

The use of the Andor iXon Ultra 888 camera for its high speed and high sensitivity was crucial to our recordings. In order for the camera to reach very high frame rates, we used it in combination with an Optomask which prevents any photons to reach the sensor of the camera outside of a chosen field of view (148 pixels high x 496 pixels long). Images were binned 4x4 and acquired at 599.92 Hz (600 Hz) with an exposure time of 1.5870 ms. We chose an acquisition rate of 600 Hz to maximise the signal to noise ratio without compromising the time resolution of our signal since events at the membrane can occur in the range of milliseconds.

We used a main magnification of 1.25x in order to reduce vignetting effect throughout our experiments. We first used a the 10x objective to position the stimulating electrode and then switched the 60x objective and for our recordings (**Figure 4.1 A**). Once at 60x, we first located the tip of the electrode and then navigated to ~100  $\mu\text{m}$  away from the tip to set our field of view. Our ROI was defined by a 20  $\mu\text{m}$  x 20  $\mu\text{m}$  square located approximately 150  $\mu\text{m}$  from the tip of the stim (**Figure 4.1 A and B**).

The illumination and recording were synchronised and lasted a total of 3.5 s to 5 s depending on the experiments. Illumination was kept as short as possible after the stimulation in order to avoid unnecessary bleaching. The stimulations were automatically triggered 1.4s after the start of the illumination.

#### 4.2.4.4. Data extraction and analysis for voltage sensor experiments

Average fluorescence within the ROI (described in previous subsection) along with the frame number was extracted in ImageJ using a custom-made macro. Raw values of fluorescence over time were then transferred to a custom-made MatLab code (MATLAB R2024\_b, MathWorks).

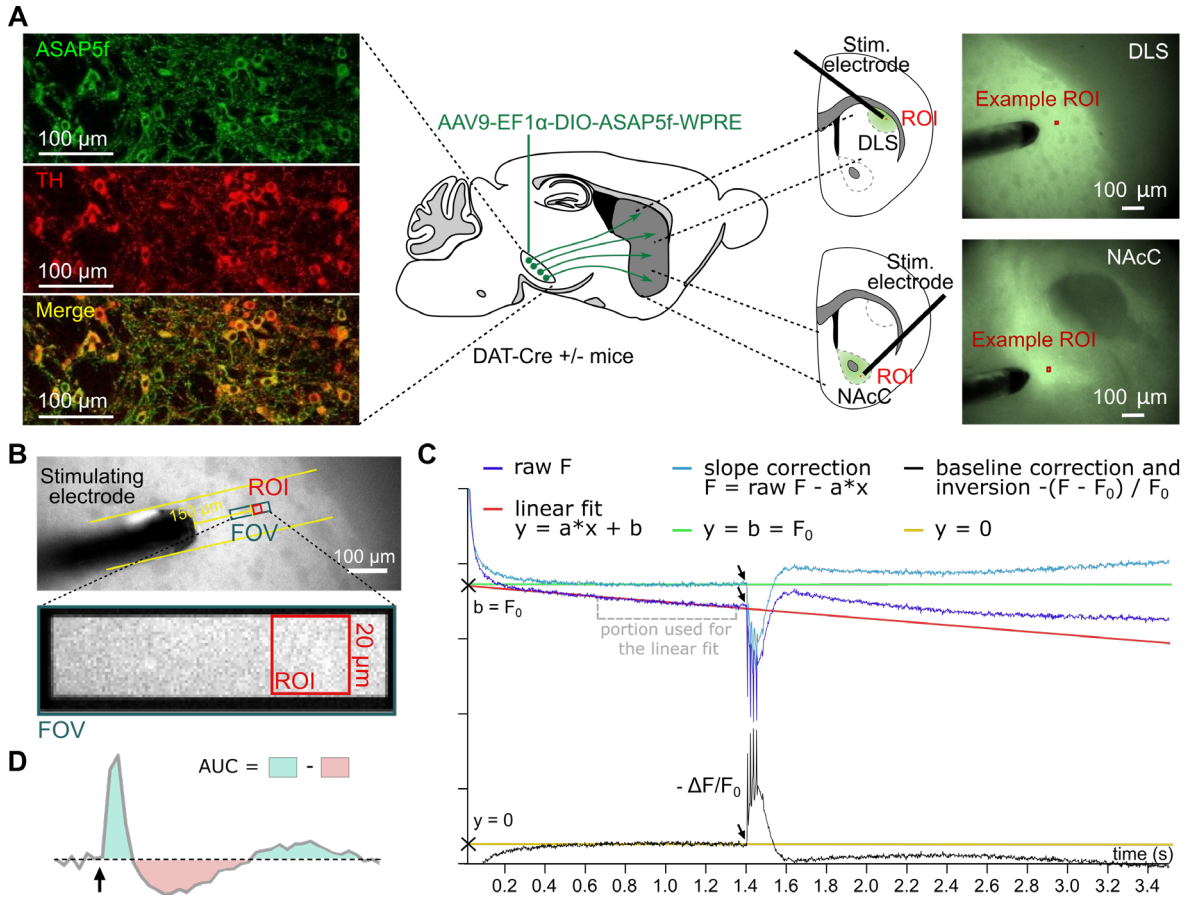
The frames number were converted into seconds. The raw fluorescence traces were fitted with linear function ( $f(x) = a * x + b$ ) to 660 ms and 1330 ms, for a total of 670 ms (400 frames) before the stimulation (Figure 4.1 C). The baseline fluorescence  $F_0$  was defined as the factor b of the linear fit. The factor a corresponding to the slope of the linear fit was multiplied to the frame number and subtracted to the raw fluorescence in order to correct for the decay caused by bleaching during individual recordings. Since ASAP5f deflect downwards upon depolarisation, the variation over time of the slope-corrected fluorescence

value ( $F$ ) was then calculated in the following way:  $-(F-F_0)/F_0 = \Delta F/F_0$  (**Figure 4.1 C**). It was then converted in percentage by multiplying by 100.

We used the area under the curve (AUC) as an unbiased approach to describe our signal. The total AUC was obtained using the Trapz function in MatLab from stimulation time until 100 ms post-stimulation. Example of AUC calculation shown in panel D in **Figure 4.1**. For spike analysis, timings of spikes were determined by the type of stimulation given. Each spike was re-baselined using an average of the 2 points before the time of stimulation, or 1 point before the time of stimulation for 100 Hz stimulations. The maximum (later called *height*) was taken from the re-baselined traces of the spikes using the max function in MatLab. The AUC for each spike is calculated on re-baselined trace of the spike from the moment of the stimulation and calculated using the Trapz over the next 5 points corresponding in 8 ms after the stimulation. Other calculations were made using Excel.

For analysis of the isolated nAChR-mediated depolarisation, we subtracted traces evoked by 1p stimulation from DH $\beta$ E condition (when nAChRs are antagonised) to the traces from drug-free condition (when nAChRs are active), all traces were normalised to the size of their initial spike. From these normalised subtracted traces, we recalculated the pre-stimulation baseline average and standard deviation of the mean for 100 frames before the stimulation onset. To calculate the duration of the nAChR-mediated prolonged depolarisation, we first fitted an exponential curve to the repolarisation phase of the event (using fit exp1 in MatLab) from one frame after the maximum until 110 frames (~ 183 ms) after the stimulation initiation. Next, we found the time point for which the fitted curve is lower than the averaged baseline + the standard deviation of the mean. This time point was reported as the “end time” of the nAChR-mediated depolarisation.

Statistical analyses were carried out using GraphPad Prism as described in **section 2.7.** and the threshold for statistical significance was  $p < 0.05$ . Statistical significance was reported as: \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ ; \*\*\*\*  $p < 0.0001$ .



**Figure 4.1 : Methods for ASAP5f experiments.**

**A:** The AAV9-packaged ASAP5f was injected in the midbrain of DAT-Cre +/- mice. On the left, example pictures of midbrain cell bodies labelled infected with ASAP5f (top), labelled by anti-TH (tyrosine hydroxylase) antibodies (middle) and co-labelled by both. On the right, example pictures of recording sites in the DLS (top) and in the NAcC (bottom).

**B:** Top: Example of field of view (x10 objective) with placement of the stimulating electrode and FOV during recording. Bottom: example of FOV (x60 objective) chosen for data acquisition with 20  $\mu\text{m}$  x 20  $\mu\text{m}$  ROI used for data collection.

**C:** Example raw trace (purple) along with calculations to slope-correct (blue), baseline and flip (black) the signal so depolarisations are seen upwards. Arrows indicate time of stimulation, here 4 pulses at 100 Hz.

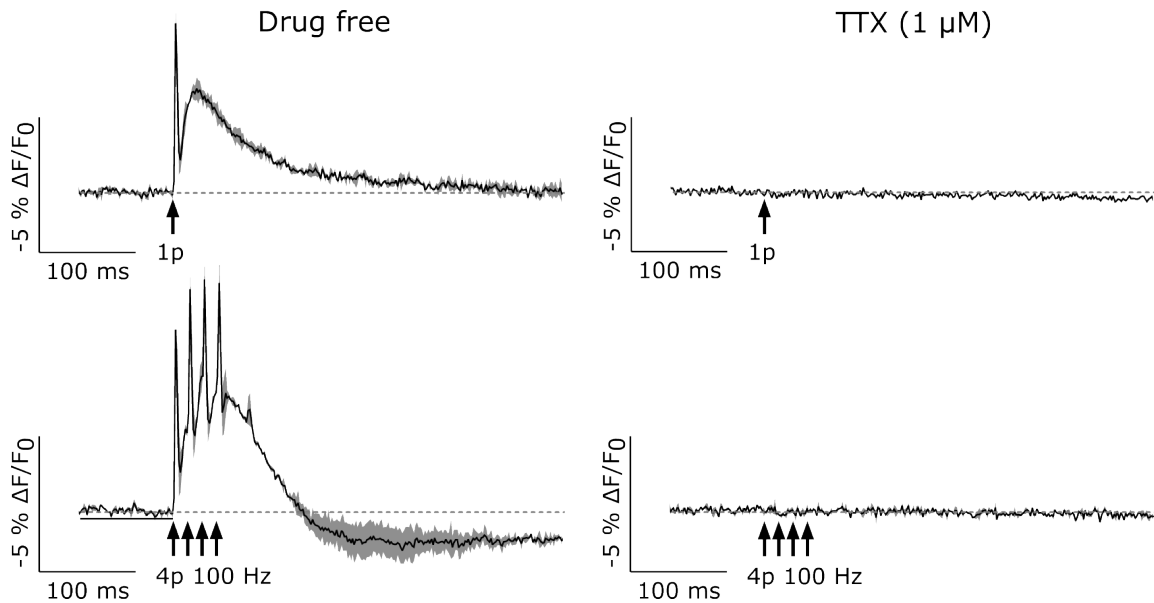
**D:** Example trace (from single electrical pulse stimulation in DH $\beta$ E) after slope correction, baselining and flipping demonstrating how AUC is calculated. Areas below 0 are subtracted to areas above zero.

#### 4.2.4.5. Voltage sensor characterisation for recording in a population of axons

##### *i. ASAP5f signal reflects changes in membrane voltage*

We first validated that evoked signals reported by ASAP5f were due to VGSC-mediated depolarisation rather than to an artefact, using voltage-gated sodium channel (VGSC) blocker tetrodotoxin (TTX) at 1  $\mu$ M, a concentration that usually abolishes all action potential and subthreshold events in patch-clamp experiments (Marshall et al. 1996; Khaliq and Bean 2010). Indeed, since VGSCs are responsible for the upstroke of the action potential, blocking them prevents the formation of spikes, and their propagation along the axons. Therefore, if any part of our signal remains after washing on TTX, it is likely to be a stimulation or movement artefact as opposed to a voltage change happening at the membrane membranes.

We tested a wide range of stimulation frequencies and found for all of them that the signal is completely abolished in the presence of 1  $\mu$ M TTX (**Figure 4.2**). We therefore concluded with confidence that our signal is reflecting actual changes at the membranes of the dopaminergic axons located within our ROI and not an artefact. No residual TTX-insensitive components were discernible.



**Figure 4.2: TTX abolishes ASAP5f signal evoked by single electrical pulse and train pulse.** On the **left**, ASAP5f signal from a population of DA axons evoked by single pulse (**top**) and 4p 100 Hz (**bottom**) in drug free condition. On the **right**, ASAP5f signal evoked by single pulse (**top**) and 4 p 100 Hz (**bottom**) after TTX (1  $\mu$ M) wash on (n = 2).

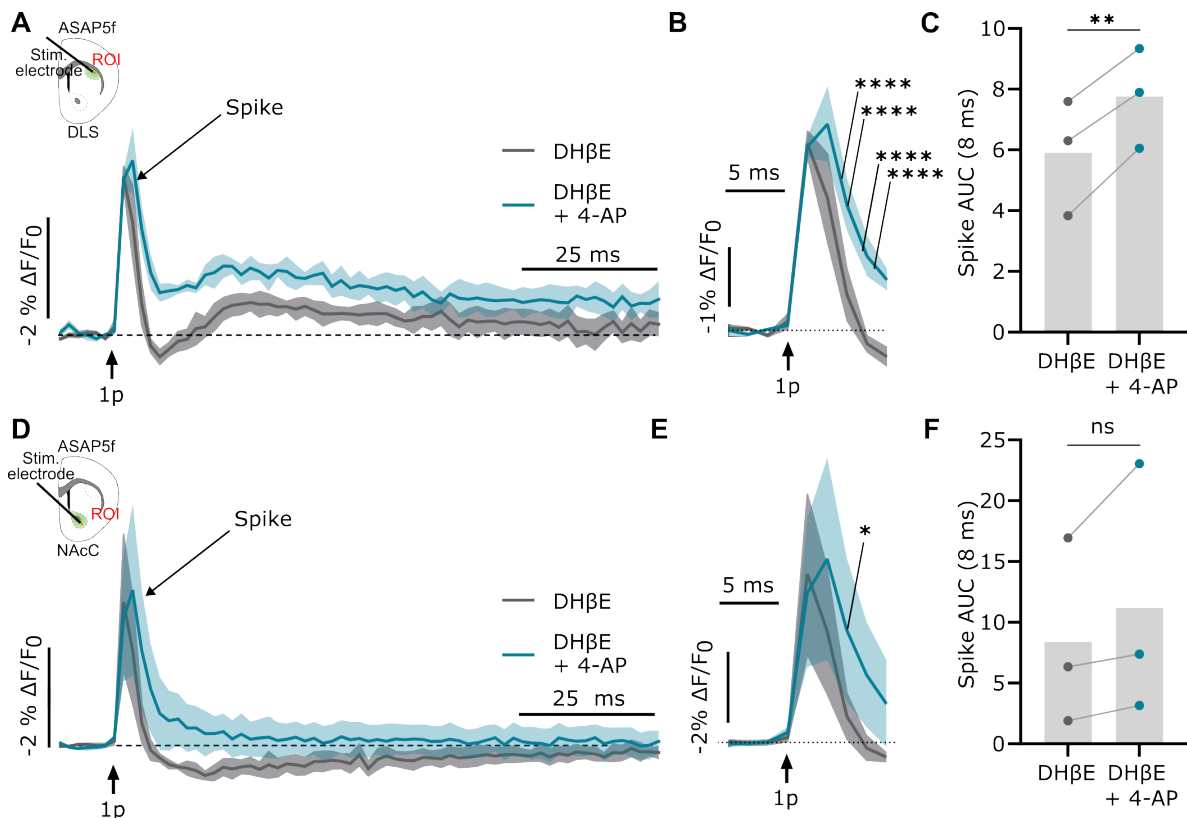
## *ii. Axonal transients are widened by VGKCs block*

Since it has been shown that ASAP5f can report individual spikes (Hao et al. 2024) and the initial fast depolarising and fast repolarising event evoked by an electrical pulse is reminiscent of a spike shape, therefore, we tested whether our signals could reflect synchronous spikes over the whole population of DA axons present within our ROI. We hypothesised that if these fast events were indeed spikes, then a voltage-gated potassium channel (VGKCs) blocker such as 4-aminopyridine (4-AP) should prolong these spikes, because blocking VGKCs slows down the repolarisation phase.

In order to have a better resolution for the spikes, we used a nAChR antagonist (DH $\beta$ E) to prevent the second slower event (described in **section 4.3.1** and **4.3.2**) to obstruct the repolarising phase of the first event (**Figure 4.3**). In the DLS, we found that the width of the signal (initial spike) was increased in the presence of 4-AP (**Figure 4.3 A, B**), as well as the area under the curve of the spike (**Figure 4.3 C**). In NAcC, the width of the signal (initial



spike) was also increased in the presence of 4-AP (**Figure 4.3 D, E**). In NAcC, although there is a trend towards an increase in the AUC in the presence of 4-AP, no clear measurement was found statistically significant from the control trace (in DH $\beta$ E only, **Figure 4.3 F**). The signal is enlarged in both regions, which confirms our hypothesis that those events reflect an action potential synchronised across the axonal population.



**Figure 4.3: Blocking VGKCs with 4-AP widens DA axon spikes.**

**A, D:** Mean ( $\pm$  SEM)  $-\Delta F/F_0$  (ASAP5f) response to 1 pulse electrical stimulation (1p) in DLS (**A**) and NAcC (**D**). We started the experiment in nAChR antagonist DH $\beta$ E (1  $\mu$ M) and added through bath application 4-AP (100  $\mu$ M). The arrow indicates the time of stimulation.  $n = 3$  in DLS and  $n = 3$  in NAcC.

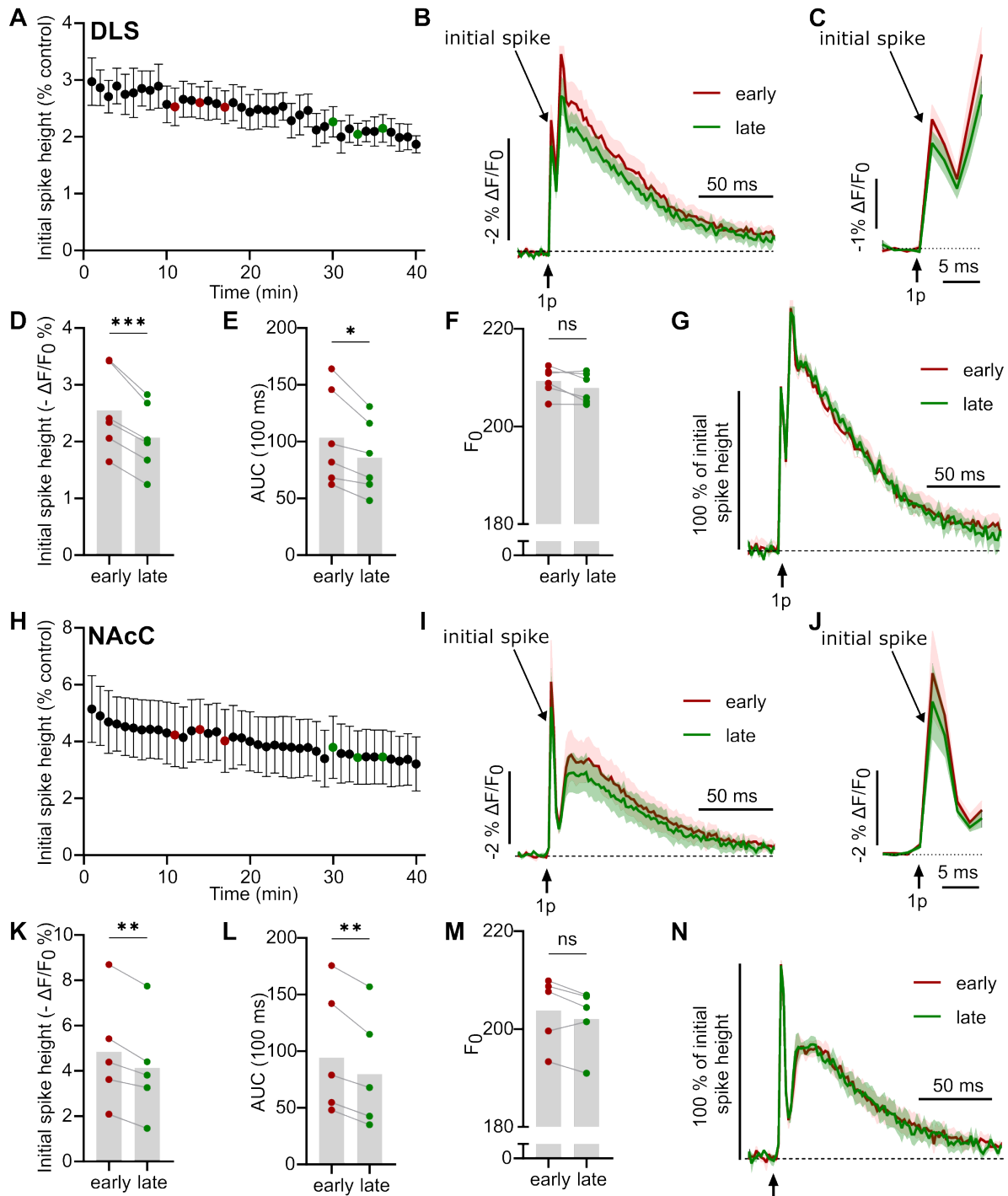
**B, E:** Zoom on initial spike shown in traces **A** and **D**. Mean ( $\pm$  SEM)  $\Delta F/F_0$  (ASAP5f) evoked by 1 pulse electrical stimulation (1p) in DLS (**B**) and NAcC (**E**). Two-way ANOVA in DLS (**B**) drug effect, ,time effect:  $F(8, 16) = 25.96$ ,  $P < 0.0001$ , interaction:  $F(8, 16) = 35.23$ ,  $p < 0.0001$ ; in NAcC (**E**), drug effect:  $F(1, 2) = 2.674$ ,  $p = 0.2436$ , time effect:  $F(8, 16) = 3.828$ ,  $p = 0.0107$ , interaction  $F(8, 16) = 2.963$ ,  $p = 0.0307$ .

**C, F:** AUC of spike from stimulation until end of trace **B** in DLS and **E** in NAcC. Paired t-test, in DLS (**C**)  $p = 0.0099$ , in NAcC (**F**)  $p = 0.2310$ .

*iii. The shape of the signal is stable over time despite overall  $\Delta F/F_0$  attenuation*

Fluorescence-based sensors can bleach over time, and therefore we tested the stability of the signal over the length of an experiment. We stimulated the slices every minute with a single electric pulse. We plotted the initial spike height of each stimulation over time (**Figure 4.4 A and H**). We averaged three stimulations at the beginning of the experiment ('early') correspond to the timings of the single pulses that would be given in most of our wash on experiments. The 'late' condition corresponds to the timings of the single pulse stimulations after the drug wash-on in most experiments. Stimulations used for those 'early' and 'late' averages are highlighted on **Figure 4.4 A and H**.

We assessed decay of initial spike peak height and AUC (taken over 100 ms after stimulation) over the time-course of the experiment. We found that the initial spike height and the AUC both are smaller in 'late' timepoints compared to 'early' timepoints (**Figure 4.4 B to E and I to L**). In both regions, we found a decay of approximately ~15-20% that was similar for both initial spike height and AUC. However, we found that the  $F_0$  seems to remain stable over time (**Figure 4.4 F and M**). Finally, and most importantly, the shape of the traces remains the same over the course of the experiments, as when the traces were normalised to their own spikes, the 'early' and 'late' traces overlap completely (**Figure 4.4 G and N**).



**Figure 4.4: The shape of evoked ASAP5f signal is stable over time despite overall  $\Delta F/F_0$  attenuation.**

**A, H:** Mean initial spike height ( $\pm$  SEM)  $-\Delta F/F_0$  in response mixed stimulation protocols (drug free throughout) in DLS (**A**) and in NAcC (**H**). In DLS,  $n=6$ ; in NAcC  $n=5$ .

**B, I:** Traces (Mean  $\pm$  SEM)  $-\Delta F/F_0$  of responses to single pulse electrical in 'early' (red) and 'late' (green) conditions in DLS (**B**) and in NAcC (**I**).

**C, J:** Zoom on initial spike from traces (Mean  $\pm$  SEM)  $-\Delta F/F_0$  of responses to single pulse electrical in 'early' (red) and 'late' (green) conditions in DLS (**C**) and in NAcC (**J**).

**D, K:** Initial spike height ( $-\Delta F/F_0$ ) of 'early' versus 'late' single pulse stimulations in DLS (**D**) and in NAcC (**K**). Each dot represents a separate slice. The grey bars represent the mean. Paired-t-test, in DLS (**D**)  $p = 0.0007$  and in NAcC (**K**)  $p = 0.0047$ .

**E, L:** AUC taken over 100 ms following the stimulation of traces in **B** and **I** respectively of ‘early’ versus ‘late’ single pulse stimulations in DLS (**E**) and in NAcC (**L**). Each dot represents a separate slice. The grey bars represent the mean. Paired-t-test, in DLS (**E**)  $p = 0.0059$  and in NAcC (**L**)  $p = 0.0034$ .

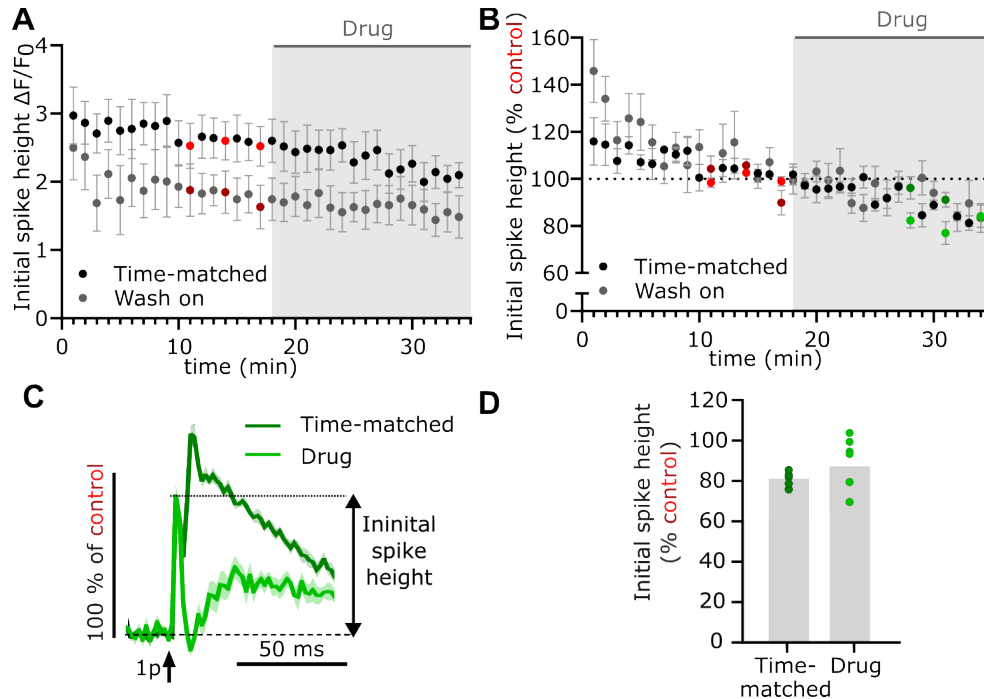
**F, M:**  $F_0$  calculated from traces of ‘early’ versus ‘late’ single pulse stimulations in DLS (**F**) and in NAcC (**M**). Each dot represents a separate slice. The grey bars represent the mean. Paired-t-test, in DLS (**F**)  $p = 0.0913$  and in NAcC (**M**)  $p = 0.1368$ .

**G, N:** Traces (Mean  $\pm$  SEM) of responses to single pulse electrical in ‘early’ (red) and ‘late’ (green) conditions normalised to their own initial spike to highlight that the shape of the signal remains the same throughout the experiment, in DLS (**G**) and in NAcC (**N**).

---

Thus, in order to quantitatively assess the differences in the signal shape caused by drugs application for example (**Figure 4.5**), we need to correct for the  $\Delta F/F_0$  decay observed during the characterisation of the sensor for in axonal population (**Figure 4.4**). Plus, different datasets can have an inherent variation in signal amplitude as demonstrated in an example drug application data set below (**Figure 4.5 A**). Therefore, to correct for this, we normalised each dataset within themselves for each slice. We first average three stimulations corresponding to single pulse stimulations at in the wash on data set (in dark red on the graphs) to calculate our pre-drug baseline. Then we normalised the whole wash on dataset to this pre-drug baseline (**Figure 4.5 B**). Next, we used the same calculation for the time-matched data, using an average of the time matched single pulse stimulations (in bright red on the graph) after the start of the experiment and normalised the whole time-matched dataset to this ‘early’ baseline (**Figure 4.5 B**). Now, both datasets are normalised to their own controls (pre-drug for the wash on dataset or ‘early’ for the time-matched dataset) and are comparable (**Figure 4.5 B**). To assess whether the wash-on has affected the initial spike height evoked by single pulse, we next averaged the initial spike height (normalised to the relevant control) evoked by single pulse after the drug has washed on for 10 min (in dark green on the graph, **Figure 4.5 B**). We also averaged of the corresponding initial spike height values taken from the time-matched dataset (in dark green on the graph, **Figure 4.5 B**). We then compared the spike height from the drug wash on and the time-matched dataset, both

normalised to their respective controls (Figure 4.5 C and D). We used the same calculation described for the height to normalise the AUC of the signals (not shown here). We used this data normalisation for all panels investigating the effect of a wash on single pulse release, unless otherwise stated. Specific differences in the calculations will be mentioned with each figure.



**Figure 4.5: Details of normalisation and baseline used for description of data collected using voltage-indicator ASAP5f using an example drug application dataset.**

**A:** Initial spike height (Mean  $\pm$  SEM)  $-\Delta F/F_0$  of initial spike in response to mixed stimulation protocols for a time-matched experiment (drug free throughout) in black and for an example wash on data set starting in drug-free condition in grey (named of the drug removed for clarity but data discussed in the result section). In different shades of red are the point that will be used to normalise the data set shown in **B**. Time-matched  $n = 6$ ; wash on  $n = 6$ .

**B:** Initial spike height (Mean  $\pm$  SEM)  $-\Delta F/F_0$  in response electrical stimulation as a percentage of their respective control initial spike height (in dark red for time-matched dataset and in bright red for wash on data set). In green are the points used for averaging to compare the spike height from time-matched dataset and the drug wash on data set.

**C:** Mean  $\pm$  SEM)  $-\Delta F/F_0$  of traces evoked by single pulse stimulations normalised to their respective initial spike height control (dark red for time-matched dataset and bright red for drug wash on).

**D:** Example of graph comparing the initial spike height normalised to their respective controls. Each dot represents a separate slice. The grey bars represent the mean. In this example the drug has not affected the first spike height compared to time-matched control.

## **4.2.5. Reporting $\text{Ca}^{2+}$ dynamics from a population of striatal dopaminergic axons using ultra-fast $\text{Ca}^{2+}$ imaging**

### **4.2.5.1. About jGCaMP8m sensor**

Genetically encoded  $\text{Ca}^{2+}$  indicators (GECIs) – and in particular GCaMP a fusion of cpGFP, calmodulin and M13 peptide – have been extensively used in the past decades as a proxy measure of neuronal activation. They report variations in intracellular  $\text{Ca}^{2+}$  concentration by conformational changes when  $\text{Ca}^{2+}$  binds to the calmodulin.

Although previous GCaMP sensors were reporting  $\text{Ca}^{2+}$  at much slower time scale than neural activity actually occurs, the newer version jGCaMP8 is more sensitive and possess faster kinetics. Work from Brill-Weil et al. (Brill-Weil et al. 2024) using jGCaMP8m indicates that it is now possible to disentangle  $\text{Ca}^{2+}$  events due to direct activation of DA axons from  $\text{Ca}^{2+}$  events due to activation of nAChRs in slices using electrical stimulation.

These improved kinetics are rendering jGCaMP8 the best tool for us to probe  $\text{Ca}^{2+}$  transients at a millisecond scale, which was impossible until now. In order to make sure there would be sufficient time resolution and brightness to be detected from the sensor, we chose jGCaMP8m which is fast enough to detect events in the ranges of milliseconds and brighter than the fastest sensor. Since we will image at ultra-fast frame rates, making sure the sensor remains reliably detectable is crucial.

In this body of work, preliminary jGCaMP8m experiments are used to test hypotheses led by our work with voltage sensor.

#### 4.2.5.2. Animals used to detect ultra-fast $\text{Ca}^{2+}$ transient in a population of dopaminergic axons in the striatum

Animal used for jGCaMP8m experiments were DAT-Cre heterozygous (Slc6c3 het) mice, two females aged from 12 to 12.6 weeks and one male aged 11.9 weeks which had undergone the procedure described in **section 2.2** for intracranial viral injection in the midbrain. We used the following viral solution from AddGene: AAV9 packaged pGP-AAV-syn-FLEX-jGCaMP8m-WPRE (162378-AAV9) diluted in sterile saline to final concentration of  $1 \times 10^{12}$  vg/ML (**Figure 4.6 A**).

#### 4.2.5.3. Imaging parameters for ultra-fast jGCaMP8m experiments

To perform these experiments, we used a blue LED light ( $\lambda = 470$  nm) at 5 mW measured under at 10x objective, or approximatively 3 mW measured under a 60x objective. The use of the Andor iXon Ultra 888 camera for its high speed and high sensitivity was crucial to our recordings (**section 4.2.4.2**). In order for the camera to reach very high frame rates, we used it in combination with an Optomask which prevents any photos to reach the sensor of the camera outside of a chosen field of view (148 pixels high x 392 pixels long). Images were binned 4x4 and acquired at 993.05 Hz (1000 Hz) with an exposure time of 0.917 ms. We chose an acquisition rate of 1000 Hz since to maximise our chances to see sub-second events.

For the placement of the ROI, I used the same method as of the voltage sensor experiments (**section 4.2.4.3**) for a final ROI size of  $20 \mu\text{m} \times 20 \mu\text{m}$  located around  $150 \mu\text{m}$  from the tip of the stimulating electrode (**Figure 4.6 B**).

The illumination and recording were synchronised and lasted a 6 s per recording to allow for signal to return to baseline after the stimulation. The stimulations were automatically triggered 1.4s after the start of the illumination.

#### 4.2.5.4. Data extraction and analysis for the ultra-fast jGCaMP8m imaging

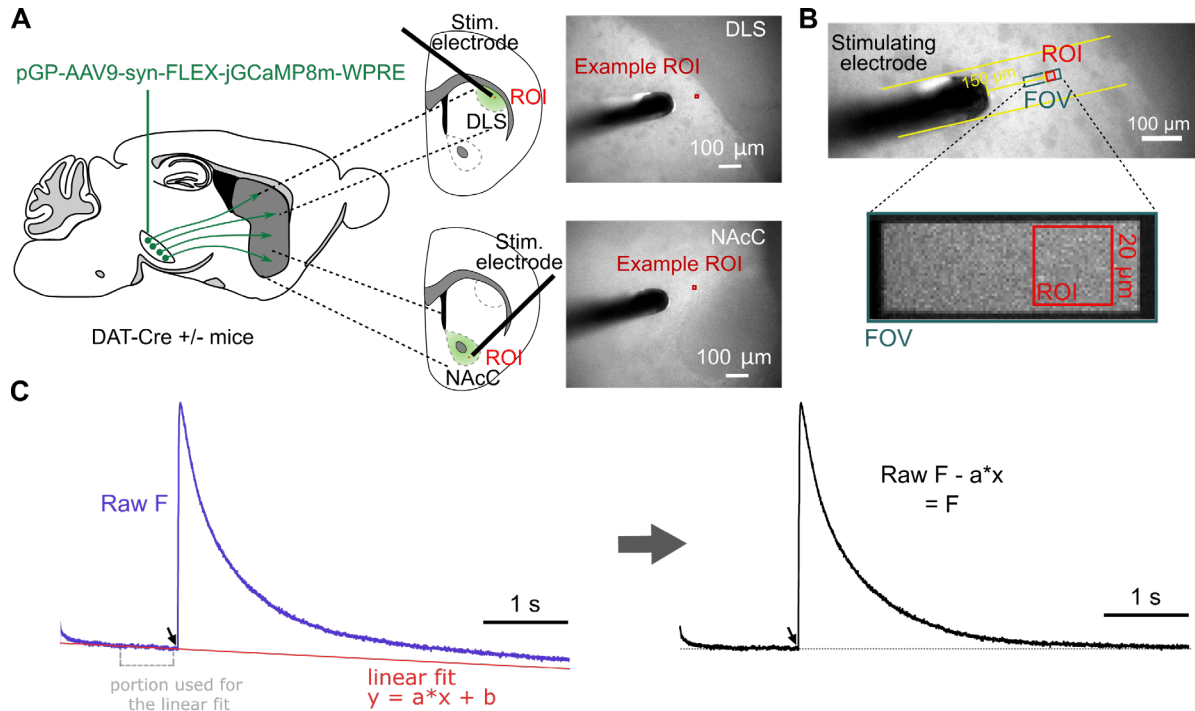
Average fluorescence within the ROI (described in previous subsection) along with the frame number was extracted in ImageJ using a custom-made macro. Raw values of fluorescence over time were then transferred to a custom-made MatLab code (MATLAB R2024\_b, MathWorks).

The frames number were converted into seconds. The raw fluorescence traces were fitted with linear function ( $f(x) = a \cdot x + b$ ) to the 500 ms before the stimulation (**Figure 4.6 C**). The baseline fluorescence  $F_0$  was defined as the factor  $b$  of the linear fit. The factor  $a$  corresponding to the slope of the linear fit was multiplied to the frame number and subtracted to the raw fluorescence in order to correct for the decay caused by bleaching during individual recordings. The variation over time of the slope-corrected fluorescence value ( $F$ ) was then calculated in the following way:  $(F - F_0) / F_0 = \Delta F / F_0$ . It was then converted in percentage by multiplying by 100.

The maximum peak fluorescence of the traces was found using the max function in MatLab, from the time of stimulation until 1.6 s after the stimulation. The area under the curve was obtained using the Trapz function in MatLab from stimulation time until 1.6 s post-stimulation. Other calculations were made using Excel.



Statistical analyses were carried out using GraphPad Prism as described in **section 2.7.** and the threshold for statistical significance was  $p < 0.05$ . Statistical significance was reported as: ns  $p > 0.05$ ; \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ ; \*\*\*\*  $p < 0.0001$ .



**Figure 4.6: Methods for ASAP5f experiments.**

**A:** The AAV9-packaged jGCaMP8m was injected in the midbrain of DAT-Cre +/- mice. On the right, example pictures of recording sites in the DLS (top) and in the NAcC (bottom).

**B:** Top: Example of field of view (x10 objective) with placement of the stimulating electrode and FOV during recording. Bottom: example of FOV (x60 objective) chosen for data acquisition with 20  $\mu$ m x 20  $\mu$ m ROI used for data collection.

**C:** Example raw trace (purple) along with linear fit (red) used to correct slope (black). Arrows indicate time of stimulation, here single pulse.

#### 4.2.6. Drugs

Drugs were diluted into aCSF (saturated with 95% O<sub>2</sub> / 5% CO<sub>2</sub>) to their final working concentration (**Table 4-1**) on the day of experiment from concentrated aliquots diluted in dH<sub>2</sub>O and kept at -20°C. The aCSF-drug solution was washed on for 10 to 20 min before probing effect of the drug on DA release.

| Drug                                                       | Action                                | Supplier                | Solvent           | Concentration                | References                                   |
|------------------------------------------------------------|---------------------------------------|-------------------------|-------------------|------------------------------|----------------------------------------------|
| 4-aminopyridine (4-AP)                                     | VGKC blocker                          | Sigma-Aldrich           | dH <sub>2</sub> O | 100 $\mu$ M                  | Condon et al., 2019                          |
| (+)-Bicuculline                                            | GABA <sub>A</sub> receptor antagonist | Cayman chemical company | DMSO              | 10 $\mu$ M<br>(DMSO: 0.05%)  | Lopes et al., 2019                           |
| CGP 55845, HCl                                             | GABA <sub>B</sub> receptor antagonist | AbCam                   | DMSO              | 4 $\mu$ M<br>(DMSO: 0.05%)   | Roberts et al., 2020                         |
| Dihydro- $\beta$ -erythroidine hydrobromide (DH $\beta$ E) | $\beta$ 2-nAChR antagonist            | Tocris Bioscience       | dH <sub>2</sub> O | 1 $\mu$ M                    | Threlfell et al., 2012                       |
| Oxotremorine (Oxo-m)                                       | mAChR agonist                         | Tocris Bioscience       | dH <sub>2</sub> O | 250 nM – 500 nM              | Threlfell et al., 2010                       |
| Veratridine                                                | Prolongs VGSC opening                 | Chem Cruz               | DMSO              | 2 $\mu$ M<br>(DMSO: 0.05%)   | (Otoom et al. 1998)                          |
| Tetrodotoxin (TTX)                                         | Broad VGSC blocker                    | Bio-Techne              | dH <sub>2</sub> O | 4 nM – 24 nM<br>1 $\mu$ M    | Brody et al., 2000<br>Threlfell et al., 2012 |
| Rufinamide                                                 | Prolongs VGSC inactivation            | Cayman chemical company | DMSO              | 100 $\mu$ M<br>(DMSO: 0.25%) | (Lin et al. 2022b)                           |

**Table 4-1: Drugs used in Chapter 4, including their mode of action and references to studies clarifying their use for our purposes and chosen concentrations.**

### 4.3. Results

#### 4.3.1. nAChR activation support prolonged depolarisation of DA axons and limit spike height

In this section, we explore the effect that nAChR activation by ACh has on the membrane voltage of a population of DA axons mainly using the novel GEVI ASAP5f (Hao et al. 2024) (**Figure 4.7 A and F**). We first describe the effect of nAChR activity on membrane voltage changes evoked by single pulse stimulation (**section 4.3.1.1**) and then focus on the corollary of these observations for train-pulse stimulation (**section 4.3.1.2**).

##### 4.3.1.1 nAChR activity provokes prolonged depolarisation of DA axonal membranes

*i. Single pulse stimulation provokes an axonal spike and prolonged depolarisation when nAChRs are activated, but only a spike when nAChRs are antagonised*

First, we explored how nAChR activity affects the membrane voltage of DA axons. We used electrical stimulations to evoke activity at the DA membrane – akin to FCV experiments – and the  $\beta 2$ -nAChR antagonist DH $\beta$ E (1  $\mu$ M). We will first present result from two striatal regions (DLS and NAcC) side-by-side and will draw out some differences later.

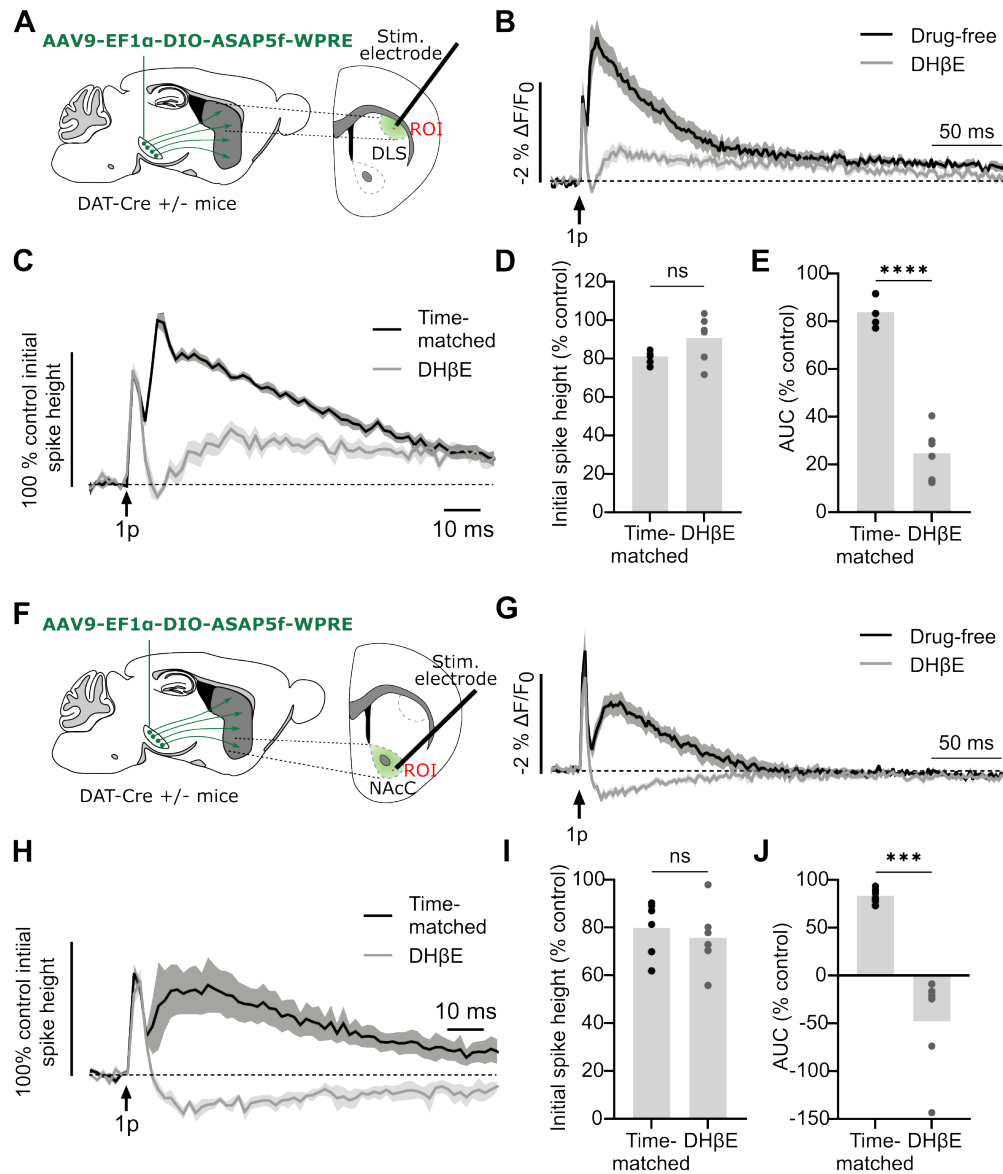
We found that, in both regions when nAChRs are active, a single pulse stimulation provokes a rapid spike (see method **section 4.2.4.5** for spike characterisation), followed by a prolonged depolarisation (**Figure 4.7**). However, when nAChRs are antagonised by DH $\beta$ E,

only the initial spike remains, and the subsequent prolonged depolarisation seems either greatly decreased in DLS (**Figure 4.7 B**) or completely abolished in NAcC (**Figure 4.7 G**).

We next quantitatively assess the differences in the signal shape caused by the antagonism of nAChRs by DH $\beta$ E. We found that initial spike height (Section 4.2.4.5.) is unchanged between the drug-free time-matched control and in the presence of DH $\beta$ E (**Figure 4.7 C, D, H and I**). Next, we compared the AUC (taken over 100 ms) between the time-matched control traces and the wash-on traces in the presence of DH $\beta$ E and found the AUC plummets in the presence of DH $\beta$ E in both regions (**Figure 4.7 C, E, H and J**).

These data highlight an important depolarisation of the membrane voltage of DA axons upon opening of nAChRs. Since nAChRs are non-selective cation channels (Decker and Dani 1990; Fieber and Adams 1991; Mülle et al. 1992; Fucile 2004), it is plausible that their activation provoked a depolarisation at the membrane as a result of the cation influx within the axons. Although these channels are non-selective, the permeability to K<sup>+</sup> is negligible in this context, and nAChRs have been demonstrated to be mostly permeable to Na<sup>+</sup>, with Ca<sup>2+</sup> conductances representing only 2 to 5% of the current flowing through open nAChRs (Vernino et al. 1994; Dani and Mayer 1995). Based on DA release data (Rice and Cragg 2004; Threlfell et al. 2012; Zhang et al. 2025) these data suggests that that the initial spike triggers a first DA release event, and that the prolonged depolarisation could also be triggering a second release event. Indeed, the latency of the maximum peak of this second depolarisation following the initial spike is in the same order of magnitude (few ms after stimulation) as the second DA release event measured by amperometry studies (Wang et al. 2014b, a). Finally, this prolonged depolarisation is unlikely to be equivalent to or exceeding the size of the spike since the sensor used here (ASAP5f) is likely to better report slower

events *ie.* with larger amplitudes (Hao et al. 2024). These points will be more thoroughly considered in the discussion (**section 4.4**).



**Figure 4.7: Single pulse stimulation evokes spike and activity at nAChRs provokes prolonged depolarisation at DA axons.**

**A, F:** Diagram illustrating the injection site (midbrain) of the virus containing ASAP5f, and the recording site in the striatum in DLS (**A**) and in NAcC (**F**). The arrow indicates the time of stimulation.

**B, G:** Mean ( $\pm$  SEM)  $-\% \Delta F/F_0$  evoked by single electrical stimulation (1p), in drug-free condition (black) or after nAChR antagonist DH $\beta$ E (1  $\mu$ M) wash on (grey), in DLS (**B**) and in NAcC (**G**). n = 6 in both regions. The arrow indicates the time of stimulation.

**C, H:** Mean ( $\pm$  SEM) normalised to respective control initial spike height evoked by single pulse stimulation (1p) in drug-free time-matched control (black) and or after nAChR antagonist DH $\beta$ E (1  $\mu$ M) wash on (grey). In DLS, n = 6 for time-matched dataset, and n = 6 for DH $\beta$ E application dataset; in NAcC, n = 5 for time-matched dataset, and n = 6 for DH $\beta$ E application dataset.

**D, I:** Spike height normalised to respective controls. Each dot represents a slice. The grey bars represent the mean. Unpaired t-test, in DLS (**D**) p = 0.0928; in NAcC (**I**) p = 0.5921.

**E, F:** AUC (100 ms post-stimulation) normalised to respective controls. Each dot represents a slice. The grey bars represent the mean. Unpaired t-test, in DLS (**E**) p < 0.0001; in NAcC (**F**) p = 0.0001.

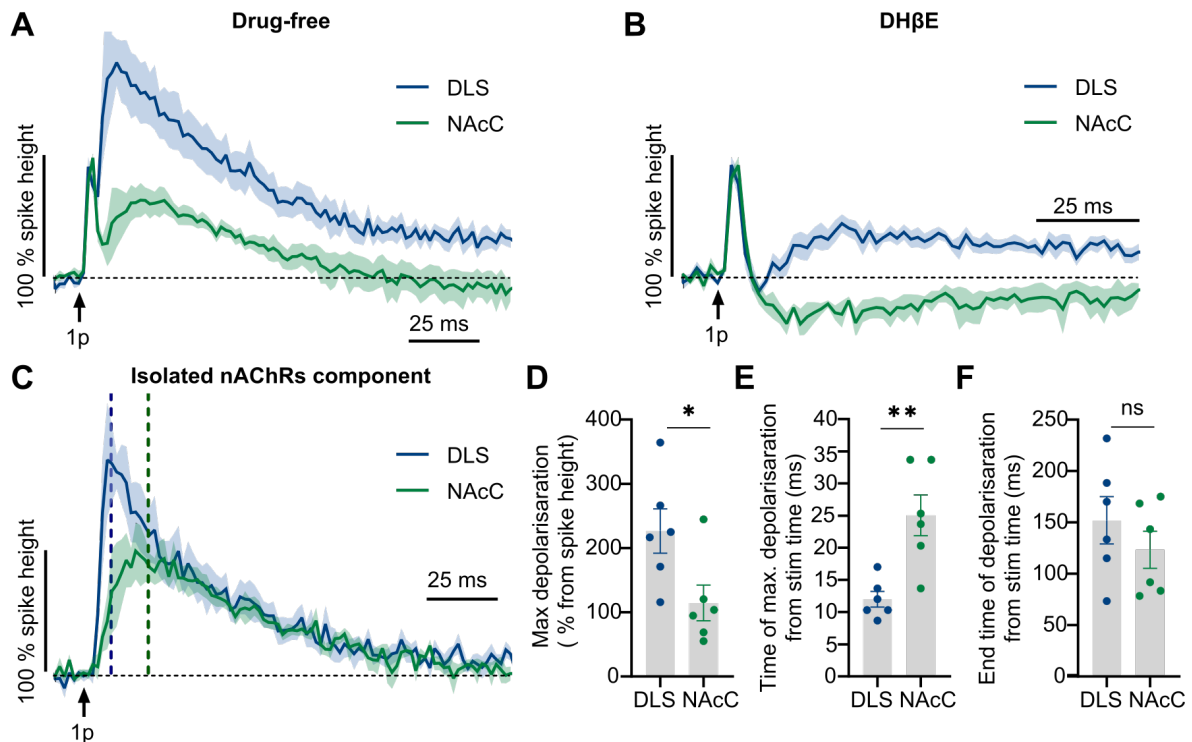
We next addressed the topographical heterogeneity (between DLS and NAcC) of the membrane voltage responses evoked by single electrical pulse when nAChRs are active (drug-free condition) and when they are antagonised (**Figure 4.8**). Although the initial component of the signal – the spike – exhibits a similar shape across regions, the second part of the signal is drastically different. In DLS, the suspected nAChR-mediated depolarisation is far greater than in NAcC (**Figure 4.8 A**). However, in the absence of nAChR activity (DH $\beta$ E condition), the membrane voltage post-initial spike seem to first hyperpolarise briefly in DLS, before repolarisation slightly (at ~9-10 ms post-stimulation) and reaching a plateau, which contrasts with NAcC where the membrane voltage hyperpolarises strongly and does not repolarise before at least 100 ms post-stimulation (**Figure 4.8 B**). Thus, we tested whether the total depolarisation potentially triggered by nAChR activation taking in account the initial state of the membrane voltage (in the absence of nAChR activation) was dissimilar between regions (**Figure 4.8 C**). We found that the kinetics at the onset of the signal are the main dissimilarity between the regions: indeed, the subtracted signal is larger and reaches its peak sooner after stimulation in DLS than it does in NAcC (**Figure 4.8 C, D and E**). Firstly, the peak of the isolated nAChRs-driven event is equivalent to more than 200% the size of the initial spike in DLS but only represents about the same size as the initial spike in NAcC (**Figure 4.8 D**). Secondly, although the isolated nAChR event seems to have the same latency to onset (~3 ms post-stimulation), their latency to peak is different. In DLS, this peak occurs ~11 ms after the stimulation onset, while it occurs ~22 ms in NAcC (**Figure 4.8 E**). Finally, we found that the total duration of the isolated nAChR event is similar between the two regions, approximately 150 ms in DLS and 135 ms in NAcC (**Figure 4.8 F**).

These regional disparities could have multiple causes:

- a. The differences in nAChR subunit composition between DLS and NAcC (Exley and Cragg 2008; Exley et al. 2008, 2011, 2012a) could be the cause for these different kinetics since subunit composition can greatly alter the properties of the channels (Alkondon et al. 1999; Albuquerque et al. 2009; Dani 2015).
- b. Varied nAChR and ChIs densities across regions could also play a key role in these differences.
- c. A differential catabolism of ACh by acetylcholinesterase (AChE), by a difference in the enzyme's efficacy or density could affect ACh level.

These regional differences could arise from different composition of the axons in various voltage-gated ions channels altering the behaviour of the membranes. For example, VGSCs density could be different in each region since the axons innervating the DLS display more branching than in the NAcC and therefore might require different VGSCs or more VGSCs to support propagation of APs.

We will explore further the potential causes for regional differences in the potential nAChRs-mediated response in the discussion of this chapter (**Section 4.4**). Because the overall effect of nAChR activity causes a prolonged depolarisation in both regions, we will present both regions simultaneously for the remainder of the chapter.



**Figure 4.8: The isolated nicotinic component of the signal presents regional heterogeneity**

**A:** Mean (± SEM) of traces evoked by single electrical stimulation (1p) normalised to their own initial spike height in drug-free. The traces were normalised to their own initial spike height in order to compare the relative size of the prolonged depolarisation succeeding the initial spike. The arrow indicates the time of stimulation. n = 6 in both regions.

**B:** Mean (± SEM) of traces evoked by single electrical stimulation (1p) normalised to their own initial spike height in presence of DHβE. The arrow indicates the time of stimulation. n = 6 in both regions.

**C:** Mean (± SEM) of isolated nAChR component calculated by subtracting traces from panel A (Drug-free) by traces from panel B (in DHβE). The vertical dotted lines label the time at which the peak is reached on averaged (panel E) in each region. The arrow indicates the time of stimulation. n = 6 in both regions.

**D:** Peak (% from initial spike height) from the subtracted traces, representing effectively the ratio of nAChRs component size:initial spike height size. Unpaired t-test, p = 0.0283.

**E:** Time at which the peak described in D occurs. Unpaired t-test, p = 0.0010.

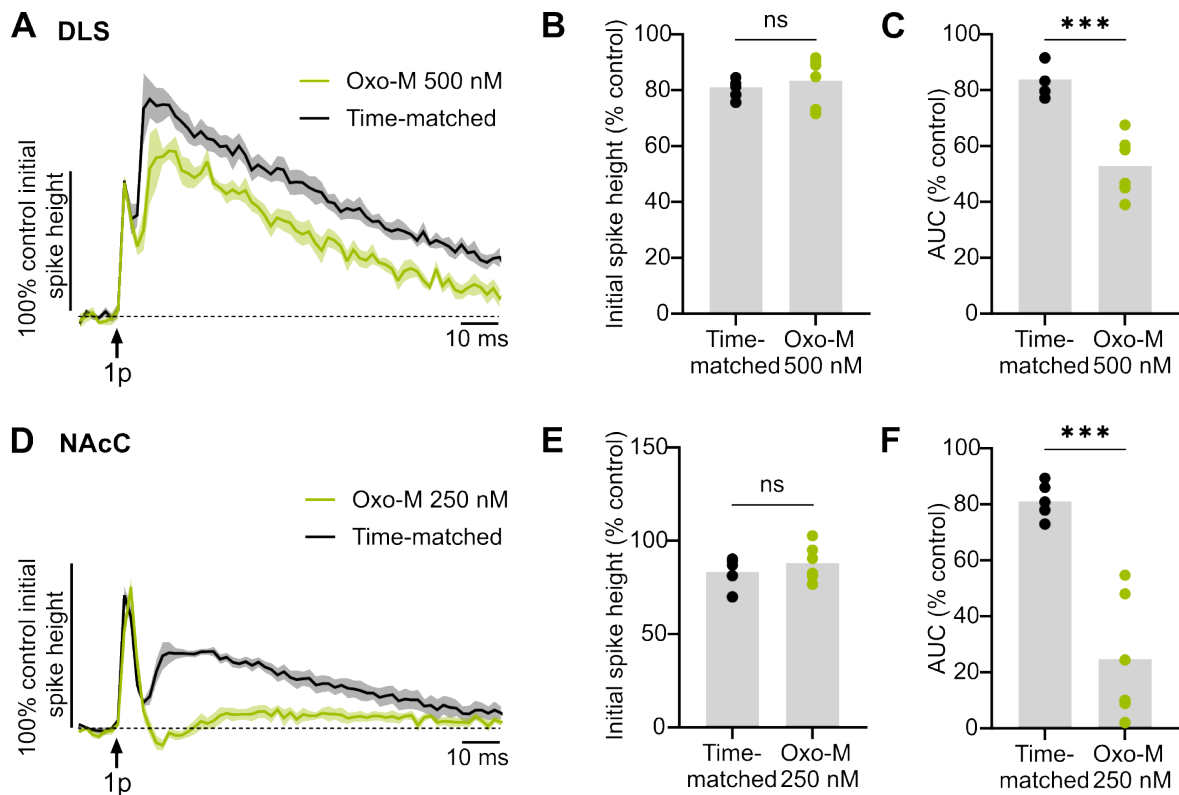
**F:** Duration of the isolated nAChR component calculated from stimulation time on traces shown in C. Unpaired t-test, p = 0.3506.

*ii. The prolonged depolarisation is attenuated when ACh levels are reduced by oxotremorine*

If the prolonged depolarisation is indeed caused by ACh binding to nAChRs at DA axons, lowering ACh release without abolishing it should diminish the amplitude of the prolonged depolarisation without affecting the size of the initial spike. Hence, we manipulated the quantity of ACh release from ChIs by partially activating mAChRs which are auto-receptors on ChIs inhibiting release. We used mAChR agonist oxotremorine (oxo-M) to manipulate ACh release levels. We used oxo-M concentrations previously reported (Vietti-



Michelina 2022) to decrease DA release evoked by light-activation of ChIs by 75% in DLS (500  $\mu$ M), and by 25% in NAcC (250 nM).



**Figure 4.9: The prolonged depolarisation is attenuated when ACh levels are reduced by oxo-M.**

**A, D:** Mean ( $\pm$  SEM) normalised to respective control initial spike height evoked by single pulse stimulation (1p) in drug-free time-matched control (black) or after mAChR agonist Oxo-M application (green), in DLS (**A**) and in NAcC (**D**). The arrow indicates the time of stimulation. In DLS, for time-matched  $n = 6$ , and for Oxo-M wash on  $n = 6$ ; in NAcC, for time-matched  $n = 5$ , and for Oxo-M wash on  $n = 6$ .

**B, E:** Initial spike height normalised to respective controls. Each dot represents a slice. The grey bars represent the mean. Unpaired t-test, in DLS (**B**)  $p = 0.5673$ ; in NAcC (**E**)  $p = 0.4274$ .

**C, F:** AUC (100 ms post-stimulation) normalised to respective controls. Each dot represents a slice. The grey bars represent the mean. Unpaired t-test, in DLS (**B**)  $p = 0.0001$ ; in NAcC (**E**)  $p = 0.0004$ .

In line with our hypotheses that only the prolonged depolarisation is triggered by nAChR activity, we found that the initial spike height is unchanged in the presence of oxo-M in both DLS and NAcC (**Figure 4.9 A, B, D and E**). Second, we found that AUC was reduced by 48 % for normalised wash-on traces in the presence of 500 nM oxo-M in DLS compared to 16% for normalised time-matched control traces (**Figure 4.9 A and C**). In NAcC, AUC was reduced by 75 % for normalised wash-on traces in the presence of 250 nM oxo-M in compared to 17% for normalised time-matched control traces (**Figure 4.9 D and F**). We

concluded that manipulating ACh release can affect the amplitude of the prolonged depolarisation occurring when nAChRs are active and that decrease ACh release also decrease the amplitude of the prolonged depolarisation.

### *iii. Probing occurrence of prolonged depolarisation*

We next performed pilot experiments to explore the time-course of this nAChRs mediate prolonged depolarisation using paired pulse ratios at different IPIs. In order to compare the signal evoked by the second pulse to the signal evoked by the first pulse normalised the signal evoked by the second pulse by either the initial spike height or AUC of the signal evoked by the first pulse (**Figure 4.10 A and G**). We use the AUC to measure whether or not the prolonged depolarisation occurred and its amplitude.

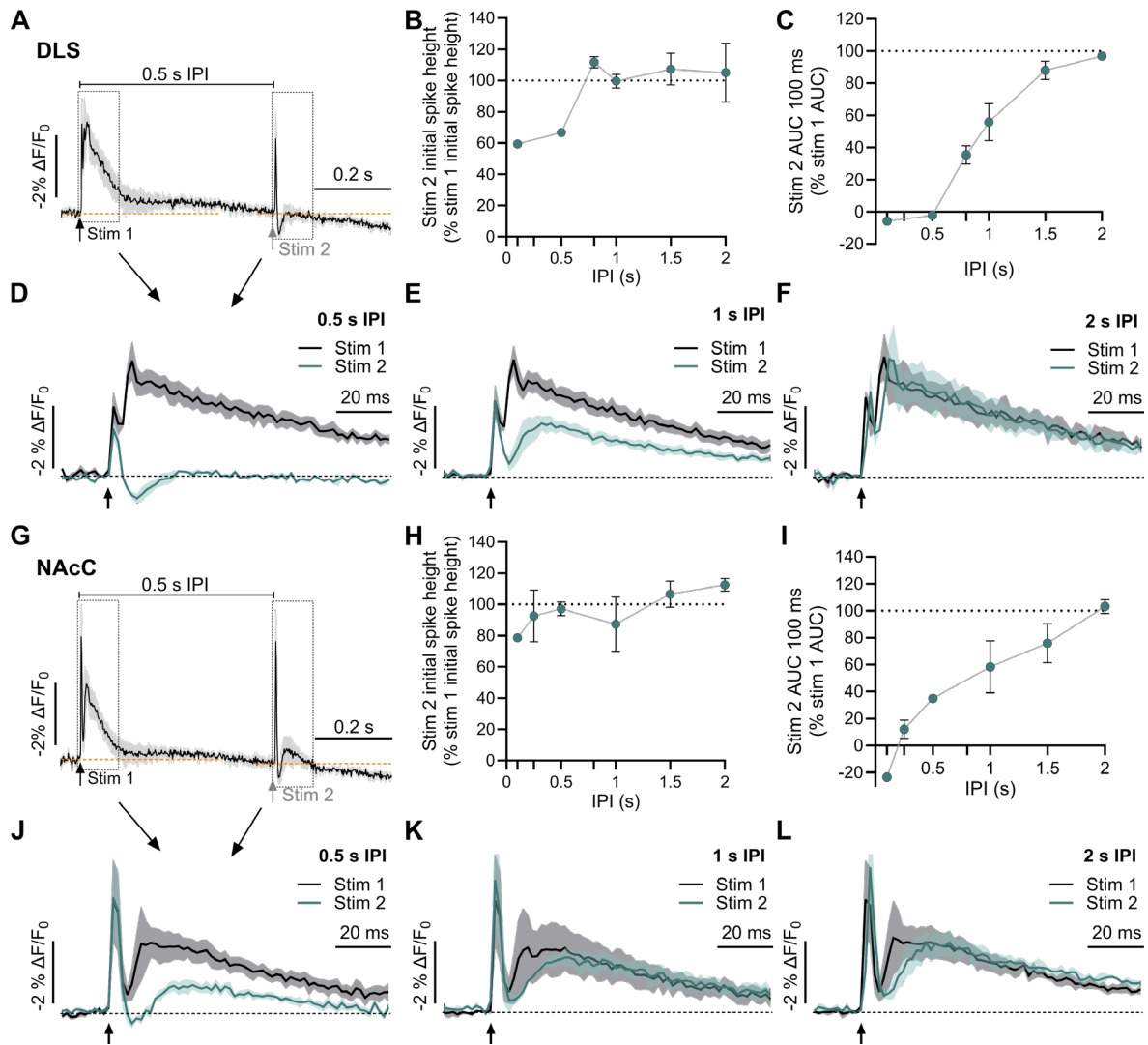
We found that at short IPIs (0.25 s and 0.5 s) the initial spike height of the second pulse is lowered by 40% in DLS, and no prolonged depolarisation is evoked (**Figure 4.10 B and D**). This suggests that in DLS, the membrane excitability is altered for longer than the duration of the prolonged depolarisation – approximately 150 ms – found in **Figure 4.8 F**. A possible explanation is that VGSCs in this region are not yet de-inactivated and therefore affect the subsequent spike formation. From IPIs equal or over 0.8 s onwards, spike height evoked by the second pulse is similar to the spike height evoked by the first pulse (**Figure 4.10 B, E and F**). The AUC of the second pulse however is abolished completely at 0.25 s and 0.5 s IPIs and gradually increase to reach AUC similar to that evoked by the first pulse at an IPI of 2 s (**Figure 4.10 C, D, E and F**).

In NAcC, the spike height evoked by the second stimulation is only smaller at an IPI of 0.25 s, but reaches the same height as the initial spike even for IPI as short as 0.5 s (**Figure 4.10**

**H** and **J**). These timings seem to echo that of the duration of the nAChR-mediated depolarisation found in **Figure 4.8 F** which is approximately 135 ms in NAcC. Similarly to DLS, in NAcC the AUC of the signal highlights that the prolonged depolarisation gradually reaches a similar AUC as evoked by the first pulse by 2 s IPI (**Figure 4.10 I, J, K and L**).

First, this data highlights that dynamics of the spike and the prolonged depolarisation are following different time-course, which supports our hypothesis that they originate from different sources: the spike reflects direct activation of the membrane voltage, while the prolonged depolarisation is due to opening of nAChRs upon ACh binding.

Secondly, we found overall that an electrical pulse does elicit a prolonged depolarisation at IPIs for which ACh release is greatly dampened (Vietti-Michelina 2022), suggesting that indeed the prolonged depolarisation needs ACh present in the environment and nAChRs to not be antagonised. However, the time needed for the AUC to fully recover seems shorter than what was found for ACh release to recover from paired pulse (Vietti-Michelina 2022). This could be due to the difference in aCSF used. This discrepancy could also reflect of the density or sensitivity of nAChRs on DA axons as perhaps just a fraction of the ACh released by electrical pulse is required to activate 100% of the nAChRs on DA axons.



**Figure 4.10: Probing occurrence of prolonged depolarisation.**

**A, G:** Mean ( $\pm$  SEM)  $-\% \Delta F/F_0$  (ASAP5f) evoked two electrical pulses (stim 1 and stim 2) with an IPI of 0.5 s in DLS (**A**) and in NAcC (**G**). Example traces of data before response to each stimulation is overlapped in zoomed in traces below. The arrow indicates the time of stimulation.  $n = 3$  in both regions.

**B, H:** Mean ( $\pm$  SEM) initial spike height evoked by stim 2 normalised to initial spike height evoked by stim 1.

**C, I:** Mean ( $\pm$  SEM) initial spike height evoked by stim 2 normalised to initial spike height evoked by stim 1.

**D, J:** Mean ( $\pm$  SEM)  $-\% \Delta F/F_0$  (ASAP5f) evoked by stim 1 aligned and overlapped with response evoked by stim 2 with an IPI of 0.5 s in DLS (**D**) and in NAcC (**J**). The arrow indicates the time of stimulation.  $n = 3$  in both regions.

**E, K:** Mean ( $\pm$  SEM)  $-\% \Delta F/F_0$  (ASAP5f) evoked by stim 1 aligned and overlapped with response evoked by stim 2 with an IPI of 1 s in DLS (**E**) and in NAcC (**K**). The arrow indicates the time of stimulation.  $n = 4$  in DLS and  $n = 3$  in NAcC.

**F, L:** Mean ( $\pm$  SEM)  $-\% \Delta F/F_0$  (ASAP5f) evoked by stim 1 aligned and overlapped with response evoked by stim 2, with an IPI of 2 s in DLS (**F**) and in NAcC (**L**). The arrow indicates the time of stimulation.  $n = 2$  in DLS and  $n = 3$  in NAcC.

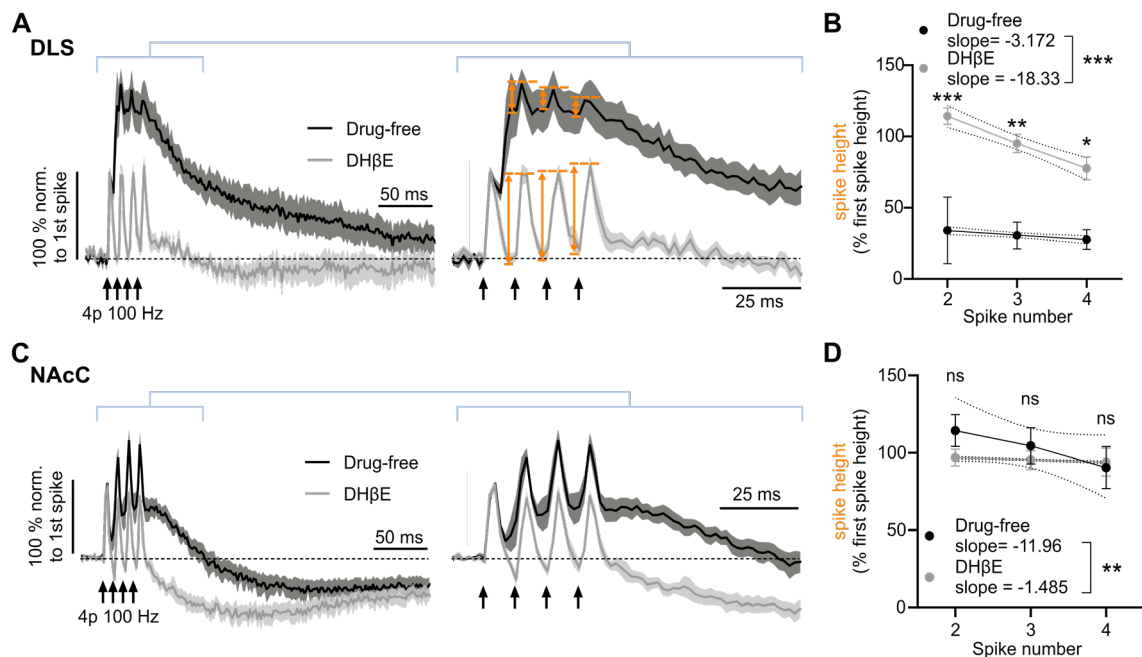
#### 4.3.1.2. nAChR activity limits successive population spike

Since we found that a single electrical pulse can provoke a prolonged depolarisation at DA axonal membrane, we tested how the membrane behaves in response to a pulse-train when nAChRs are operative or when nAChRs are antagonised by DH $\beta$ E (1  $\mu$ M) and in particular whether this prolonged depolarisation has an impact on the ability of the membrane to respond to the successive pulses.

To assess how the shape of the signal has changed because of the drug wash on, we normalised the traces to the height of the initial spike (**Figure 4.11 A**), in order to control for the general time decay due to bleaching. The effect of the drug on the initial spike has been discussed in the study of the single pulse stimulation in **section 4.3.1.1**.

In drug-free condition, we can see distinguish the prolonged depolarisation observed with the single pulse stimulation in both regions (**Figure 4.11 A and C**). In both conditions, we also noticed that during pulse-trains each electric pulse creates a fast depolarisation – population spike – and that these seem to lay on top of the prolonged depolarisation. We first measure each population spike as described on figure (**Figure 4.11 A, B and D**). We found that in DLS, all subsequent population spikes are drastically smaller when nAChRs are active in drug-free condition than they are when nAChRs are antagonized by DH $\beta$ E (**Figure 4.11 A and B**, Šídák's multiple comparisons test between Drug-free and DH $\beta$ E, 2<sup>nd</sup> spike  $p=0.0005$ , 3<sup>rd</sup> spike  $p=0.0032$ , 4<sup>th</sup> spike  $p=0.0207$ ). As opposed to DLS, NAcC population spike height is similar in both drug-free and DH $\beta$ E condition (**Figure 4.11 C and D**, Šídák's multiple

comparisons test between Drug-free and DH $\beta$ E, 2<sup>nd</sup> spike  $p=0.3575$ , 3<sup>rd</sup> spike  $p=0.8030$ , 4<sup>th</sup> spike  $p=0.9862$ ).

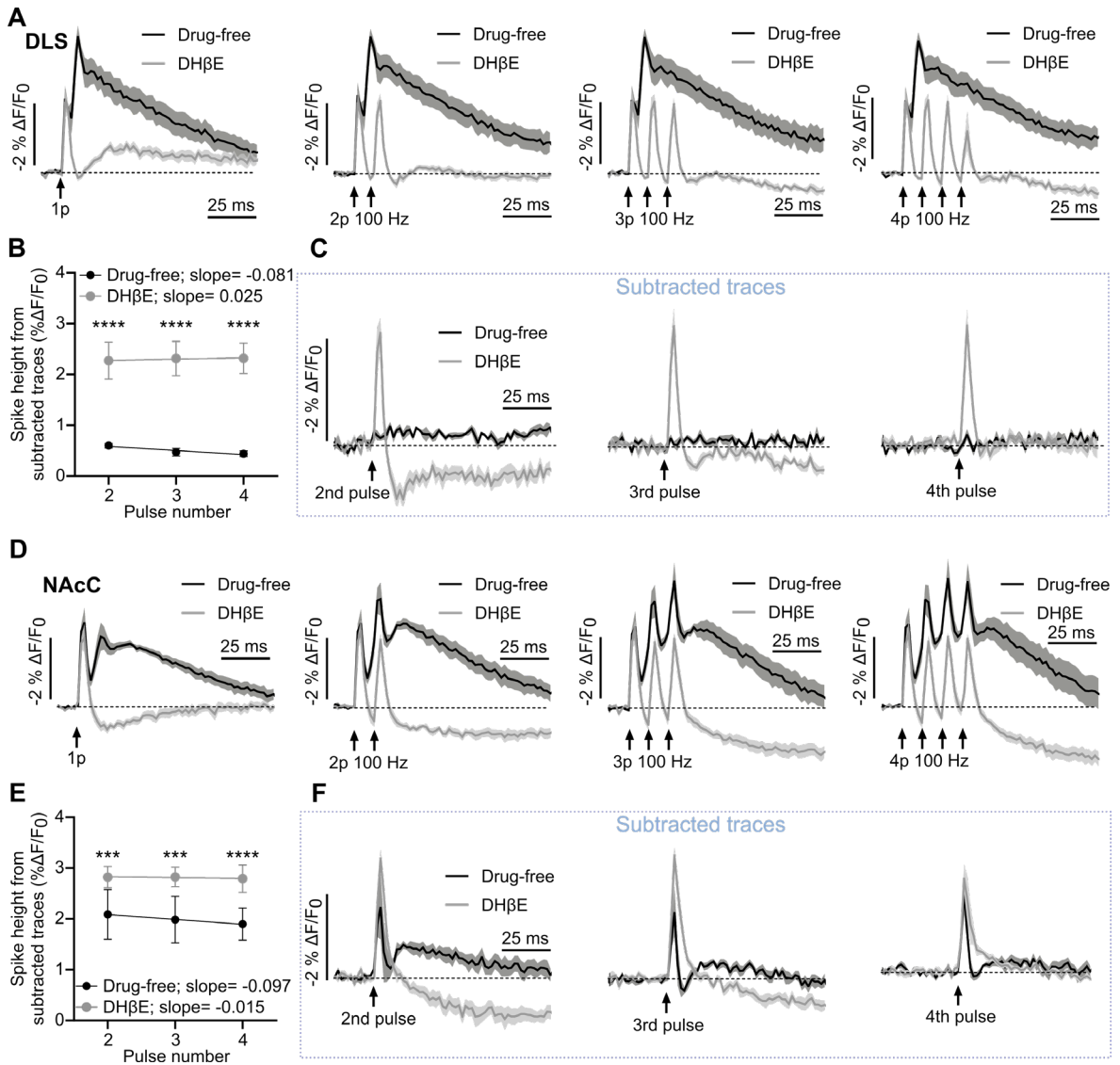


**Figure 4.11: nAChR activity limits successive population spike during pulse-train stimulation in DLS.**

**A, C:** Mean ( $\pm$  SEM) normalised to their respective initial spike height of traces evoked by four electrical train-pulses at 100 Hz (4p 100 Hz), in drug-free condition (black) or after nAChR antagonist DH $\beta$ E (1  $\mu$ M) wash on (grey), in DLS (**A**) and in NAcC (**C**). Traces on the right show a zoom of the traces on the left. The arrows indicate the time of stimulation.  $n = 6$  in both regions.

**B, D:** Mean ( $\pm$  SEM) of population spike height normalised to respective initial spike height. Two-way ANOVA (in DLS (**B**), drug effect:  $F(1, 15) = 49.52$ ,  $p < 0.0001$ ; in NAcC (**D**), drug-effect:  $F(1, 15) = 1.459$ ,  $p = 0.2458$ ) with Šídák's multiple comparisons test and simple linear regression (in DLS (**B**),  $Y_{\text{Drug-free}} = -3.172 \cdot X + 40.14$ ,  $Y_{\text{DH}\beta\text{E}} = -18.33 \cdot X + 150.6$  and slopes significantly different  $p = 0.0011$ ; in NAcC (**D**),  $Y_{\text{Drug-free}} = -11.96 \cdot X + 139.0$ ,  $Y_{\text{DH}\beta\text{E}} = -1.485 \cdot X + 99.86$  and slopes are significantly different  $p = 0.0140$ ). Dotted lines represent the 95% confidence intervals of the linear regressions.

In order to be able to isolate the depolarisations cause by each subsequent pulse rather than their summation on a dynamic background, we stimulated the slices with different pulse number (2, 3, and 4) at 100 Hz in order to obtain a background signal to subtract from each successive signal. Doing so allowed us to isolate the depolarisation evoked by the fourth electrical pulse for example, by subtracting the trace evoked by 3 pulses to the trace evoked by 4 pulses. We washed on DH $\beta$ E to antagonise nAChRs and compared signals pre- and post-drug wash on.



**Figure 4.12: nAChR activity limits successive population spike pulse-train stimulation in DLS and NAcC.** **A, D:** Mean  $(\pm \text{SEM})$   $-\% \Delta F/F_0$  evoked by one, two three or four electrical train-pulses at 100 Hz (1p, 2p, 3p or 4p 100 Hz), in drug-free condition (black) or after nAChR antagonist DH $\beta$ E (1  $\mu$ M) wash on (grey), in DLS (A) and in NAcC (D). The arrows indicate the time of stimulation.  $n = 5$  in DLS and  $n = 6$  in NAcC. **B, E:** Mean  $(\pm \text{SEM})$  of subtracted population spike height. Two-way ANOVA (in DLS (B), drug effect:  $F(1, 4) = 33.44$ ,  $p = 0.0044$ ; in NAcC (D), drug-effect:  $F(1, 5) = 3.894$ ,  $p = 0.1055$ ) with Šídák's multiple comparisons test and simple linear regression (in DLS (B),  $Y_{\text{Drug-free}} = -0.08099 \cdot X + 0.7463$ ,  $Y_{\text{DH}\beta\text{E}} = 0.02482 \cdot X + 2.227$  and slopes are statistically significantly different  $p = 0.0744$ , but their intercepts are  $p < 0.0001$ ; in NAcC (D),  $Y_{\text{Drug-free}} = -0.09590 \cdot X + 2.278$ ,  $Y_{\text{DH}\beta\text{E}} = -0.01543 \cdot X + 2.860$  and slopes different  $p = 0.0199$ ). The arrows indicate the time of stimulation. **C, F:** Mean  $(\pm \text{SEM})$   $-\% \Delta F/F_0$  subtracted traces from A and D to isolate the event triggered by the 2<sup>nd</sup> (left), the 3<sup>rd</sup> (middle) and the 4<sup>th</sup> (right) pulse of a train pulse at 100 Hz. Traces in black are from the drug-free condition or and traces in grey are from DH $\beta$ E (1  $\mu$ M) wash on, in DLS (C) and in NAcC (F).

In DLS, similarly to the previous experiment using solely 4p 100 Hz, we found that the isolated fast depolarisations caused by the second (2p - 1p), third (3p - 2p) and fourth (4p - 3p) electrical pulses were drastically attenuated in drug-free condition when nAChRs are

active compared to when nAChRs are antagonised by DH $\beta$ E (**Figure 4.12 C and B**, Šídák's multiple comparisons test between Drug-free and DH $\beta$ E, 2<sup>nd</sup>, 3<sup>rd</sup> and 4<sup>th</sup> pulses  $p < 0.0001$ ).

In NAcC, the trace subtractions revealed that the population spikes evoked by each individual second (2p - 1p), third (3p - 2p) and fourth (4p - 3p) electrical pulses were significantly smaller in drug-free condition when nAChRs are active compared to when nAChRs are antagonised by DH $\beta$ E (**Figure 4.12 F and E**, Šídák's multiple comparisons test between Drug-free and DH $\beta$ E, 2<sup>nd</sup> pulse  $p = 0.0003$ , 3<sup>rd</sup> pulse  $p = 0.0001$  and 4<sup>th</sup> pulse  $p < 0.0001$ ). Plus, subtracted traces in drug-free condition do not reveal additional prolonged depolarisations meaning that the second, third and fourth electrical stimulations activate the DA axon directly without further added impact of nAChRs (**Figure 4.12 C and F**).

Fitting a linear regression between subtracted population spike height and pulse number revealed no attenuation in the spike height over the pulse-train (all slopes were statistically not significant from zero, **Figure 4.12 B and E**). This contrasts with the un-subtracted data where all linear fit displayed negative slopes which had appeared to indicate a spike attenuation throughout the pulse-trains (**Figure 4.11**). However, the un-subtracted is still providing useful information concerning the subsequent population spike height, but might underestimate the height of these population spikes.

Together, these data suggest that the prolonged depolarisation occurring upon nAChR activation on DA axons limits subsequent spike height. One important point to note here, is that our signals are averaged the responses of a population of axons as opposed to a single axon. It is therefore possible that a smaller population spike indicates that a smaller proportion of the population is spiking, rather than that each axon is generating 'small' spikes (further discussed in **section 4.4**).



#### 4.3.1.3. Forced opening of VGSCs restores prolonged depolarisation and supports population spike attenuation

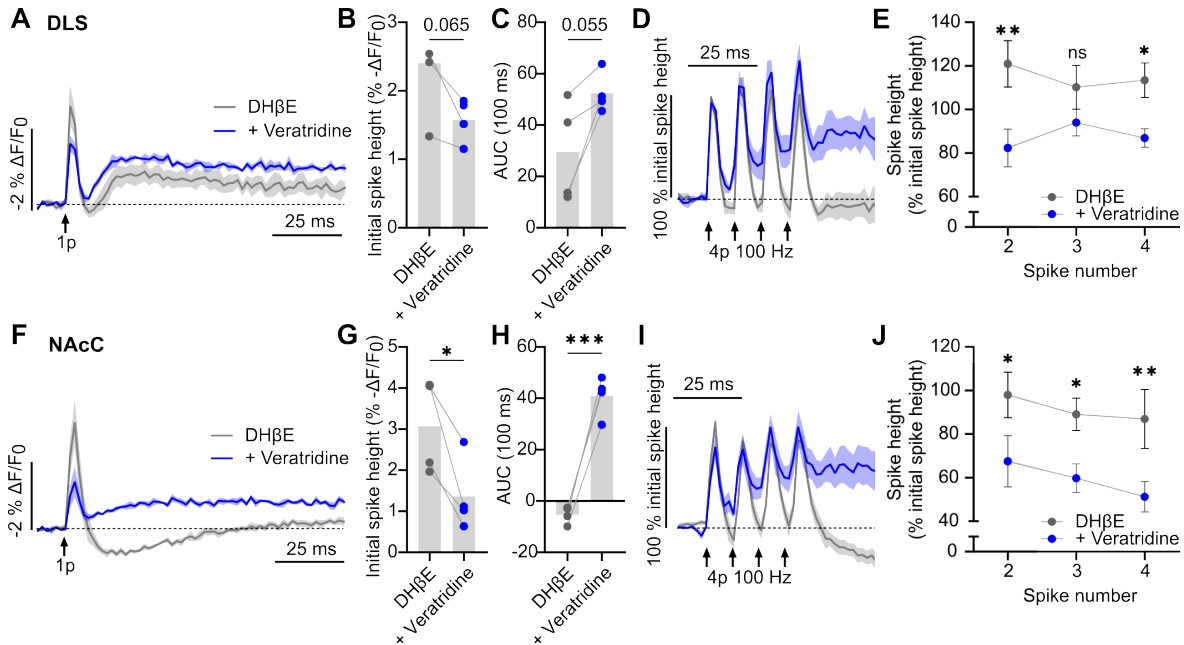
Our data suggest that activation of nAChRs creates a sustained depolarisation of DA axonal membranes, potentially through Na<sup>+</sup> entry (Decker and Dani 1990; Fieber and Adams 1991; Mülle et al. 1992; Vernino et al. 1994; Dani and Mayer 1995; Fucile 2004), that attenuates the ability of DA axons to spike again.

To corroborate this relationship, we tested the principle that the forced opening of a depolarising channel on DA axons could drive subsequent spike attenuation. We used DH $\beta$ E to prevent nAChR-mediated depolarisation and used veratridine (2  $\mu$ M) to keep VGSCs open. Veratridine (VTD) bind to VGSCs when they are in an open state, and to prolong that state (Sutro 1986; Barnes and Hille 1988; Ulbricht 1998; Zhang et al. 2018a).

Since prolonging the opening of VGSCs lowers AP amplitude (Sutro 1986; Barnes and Hille 1988; Ulbricht 1998; Zhang et al. 2018a) and ultimately limits the pool of available VGSCs for subsequent stimulation, and VGSCs are responsible for the upstroke of action potential and their propagation, we expect a decline in evoked signals. We also expect that following the initial spike, the sustained opening of VGSCs might limit population spike height when evoked by pulse trains.

As expected, application of veratridine (2  $\mu$ M) (in the presence of DH $\beta$ E) reduced initial spike height evoked by a single stimulus compared DH $\beta$ E only in NAcC (**Figure 4.13 F and G**) and shows a trend towards a reduction in DLS (**Figure 4.13 A and B**). We also found that a sustained depolarisation occurs after the initial spikes (**Figure 4.13 A, and F**) consistent with the prolonged opening of VGSCs by veratridine. Plus, despite a smaller initial spike, general AUC (taken over 100 ms) is enhanced in veratridine + DH $\beta$ E condition

compared to DH $\beta$ E only in NAcC (**Figure 4.13 F and H**), with strong trend towards an increase in DLS too (**Figure 4.13 A and C**) signifying that the post-stimulation depolarisation is larger in the presence of veratridine.



**Figure 4.13: Forced opening of VGSCs using veratridine restores prolonged depolarisation and supports population spike attenuation.**

**A, F:** Mean ( $\pm$  SEM)  $-\Delta F/F_0$  evoked by single electrical stimulation (1p), in nAChR antagonist DH $\beta$ E (1  $\mu$ M) in grey, or after addition of veratridine (2  $\mu$ M) in blue in DLS (**A**) and in NAcC (**F**). The arrow indicates the time of stimulation.  $n = 4$  in both regions.

**B, G:** Initial spike height ( $-\Delta F/F_0$ ). Each dot represents a slice. The grey bars represent the mean. Paired t-test, in DLS  $p=0.0653$ , in NAcC  $p=0.304$ .

**C, H:** AUC of traces shown in **A** and **F** respectively. Each dot represents a slice. The grey bars represent the mean. Paired t-test, in DLS  $p=0.0552$ , in NAcC  $p=0.0006$ .

**D, I:** Mean ( $\pm$  SEM) traces evoked by 4p 100 Hz stimulation normalised to their respective initial spike height. The arrows indicate the time of stimulation.  $n = 4$  in both regions.

**E, J:** Spike height normalised to respective controls. Two-way ANOVA (in DLS (**B**), drug effect:  $F(1, 3) = 4.242$ ,  $p=0.1315$ ; in NAcC (**D**), drug-effect:  $F(1, 3) = 3.397$ ,  $p=0.1625$ ) with Šídák's multiple comparisons test.

Finally, in order to assess whether subsequent population spikes are attenuated in veratridine during pulse-trains, we normalised traces evoked by pulse-trains to the initial spike height and compared the normalised height of the population spikes. We found that in both regions, although the drug effect does not come up as significant, population spike height are in general smaller upon veratridine addition compared to when only DH $\beta$ E is present (**Figure 4.13**, in DLS **D** and **E** Šídák's multiple comparisons test between DH $\beta$ E and

DH $\beta$ E + veratridine, 2<sup>nd</sup> spike  $p=0.0034$ , 3<sup>rd</sup> spike  $p=0.1411$ , 4<sup>th</sup> spike  $p=0.0210$ ; and in NAcC I and J Šídák's multiple comparisons test between DH $\beta$ E and DH $\beta$ E + veratridine, 2<sup>nd</sup> spike  $p=0.0194$ , 3<sup>rd</sup> spike  $p=0.0230$ , 4<sup>th</sup> spike  $p=0.0092$ ).

These results suggests that prolonged cation entry following an initial spike can indeed create a prolonged depolarisation at the DA axonal membrane. Furthermore, this prolonged depolarisation can also limit the subsequent population spike height during pulse trains. Hence, we mimicked nAChR activation by prolonging VGSCs opening using veratridine.

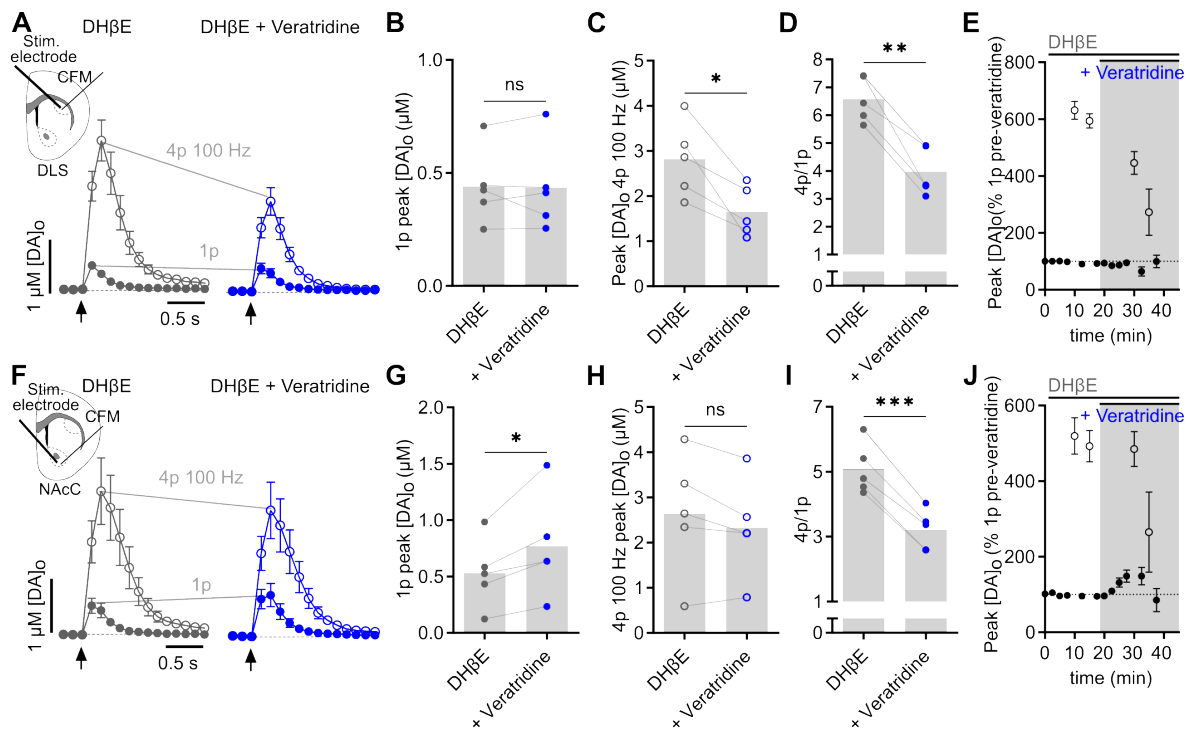
Our predictions are that population spike height govern DA release, and therefore smaller spikes trigger smaller release events.

Therefore, if our predictions hold true, we expect that veratridine would impact DA release in the following way when nAChRs are antagonised:

- (1) Release would be decreased across regions and stimulation paradigms because of the overall decrease in population spike height.
- (2) The decrease in DA release evoked by single pulse might be slightly compensated by a post-spike prolonged depolarisation.
- (3) The release evoked by train pulses will be disproportionally decreased compared to single pulse evoked release.

Altogether these predictions also converge towards mimicking the opening of nAChRs by forcing the opening VGSCs using veratridine when nAChRs are antagonised using DH $\beta$ E.

We performed pilot FCV experiments during which we antagonised nAChRs with DH $\beta$ E throughout, and wash on the 2  $\mu$ M of veratridine in order to assess whether our predictions are verified.



**Figure 4.14: Forced opening of VGSCs using veratridine reduces 4p/1p ratio of DA release.**

**A, F:** Mean ( $\pm$  SEM) [DA]<sub>o</sub> transients in response to 1 pulse electrical stimulation (1p) and 4 pulses at 100 Hz (4p 100 Hz), in DHβE (1 μM), and following by a bath application of veratridine (2 μM) in addition to the DHβE. The arrow indicates the time of stimulation.  $n = 5$  in both regions.

**B, G:** Peak [DA]<sub>o</sub> evoked by 1p stimulation in DLS (**B**) and in NAcC (**G**). Each dot corresponds to a slice. The grey bar represents the mean. Paired  $t$ -test, in DLS  $p=0.8761$ , in NAcC  $p=0.0294$ .

**C, H:** Peak [DA]<sub>o</sub> evoked by 4p 100 Hz stimulation in DLS (**C**) and in NAcC (**H**). Each dot corresponds to a slice. The grey bar represents the mean. Paired  $t$ -test, in DLS  $p=0.0157$ , in NAcC  $p=0.1246$ .

**D, I:** Ratio of peak [DA]<sub>o</sub> in response to 4p 100 Hz and peak [DA]<sub>o</sub> in response 1p in DLS (**D**) and in NAcC (**I**). The grey bar represents the mean. Paired  $t$ -test, in DLS  $p=0.0024$ , in NAcC  $p=0.0002$ .

**E, J:** Mean peak [DA]<sub>o</sub> ( $\pm$  SEM) evoked by 1p (plain circles) or by 4p 100 Hz (empty circles) over time in DLS (**E**) and NAcC (**J**) starting in DHβE and after addition of veratridine. Data is normalised to the average of the four first [DA]<sub>o</sub> values evoked by 1p shown on the graph. The mean [DA]<sub>o</sub> peak for release evoked by single pulse in **B** and **G** are average from the values flanking the peak [DA]<sub>o</sub> evoked by 4p 100 Hz.

Surprisingly, in neither region did prolonging the opening state of VGSCs using veratridine (2 μM) lessen [DA]<sub>o</sub> evoked by single pulse stimulation compared to when only the nAChRs are antagonised. Indeed, in DLS, [DA]<sub>o</sub> evoked by single pulse remained the same between veratridine + DHβE condition and DHβE condition (**Figure 4.14 A and B**). In NAcC, addition of veratridine increased [DA]<sub>o</sub> evoked by single pulse stimulation (**Figure 4.14 F and G**).

This data could suggest that the prolonged depolarisation caused by veratridine might be compensating for the lessened spike height. Our prediction (1) that release would overall be decreased is therefore not verified. Nevertheless, our prediction (2) revealed to be true, since the compensation in DLS prevent DA release decreased and in NAcC it even allowed release to exceed its initial release levels in DH $\beta$ E condition.

[DA]<sub>o</sub> evoked by pulse-train stimulation was decreased in DLS upon application of veratridine (**Figure 4.14 A, C and E**), while it left [DA]<sub>o</sub> unchanged compared to the DH $\beta$ E conditions in NAcC (**Figure 4.14 F, H and J**). This leads to the prediction (3) holding true, since the 4p/1p ratio in both regions is lower in veratridine compared to control (**Figure 4.14 D and I**).

This experiment showed that forced and maintained opening of VGSCs can drive prolonged depolarisation and accompanying spike attenuation, mimicking key effects of activation of nAChRs.

#### 4.3.1.4. Partial conclusion

We have seen that electrical stimulation evoked a compound depolarisation event in membranes of DA axons:

- First, a fast spike corresponding to the direct activation of DA axons
- Second, a few milliseconds after the initial spike, a second depolarisation occurs and is sustained over approximately 150 ms. This prolonged depolarisation is evoked when nAChRs are active (in absence of DH $\beta$ E) and can be attenuated by lowering ACh release either pharmacologically (using oxo-m) or during IPIs that are known to evoke little ACh release.

Since nAChRs are permeable to cations – especially Na<sup>+</sup> - upon binding of ACh (Decker and Dani 1990; Fieber and Adams 1991; Mülle et al. 1992; Fucile 2004), we tested whether entry of cations in the membrane could be causing the prolonged depolarisation. We found that we could mimic the depolarisation when nAChRs are antagonised (by DH $\beta$ E) by keeping VGSCs open after the initial depolarisation using veratridine (Sutro 1986; Barnes and Hille 1988; Ulbricht 1998; Zhang et al. 2018a). We could mimic opening of nAChRs both in membrane voltage and DA release levels, by keeping VGSCs open.

Our data also indicate that during pulse-trains, nAChR opening limits population spike height, by raising the membrane voltage. These shortened spike population could be due to propagation failure across the axonal population imaged. These results therefore force the following predictions on the mechanisms at play here:

- Since VGSCs are known to be responsible for the upstroke of AP, to inactivate rapidly during depolarisation and they require subsequent repolarisation or

hyperpolarisation to be de-inactivated, **we hypothesise that VGSC inactivation is the driving force behind those shortened population spike.**

- Next, because FCV data measuring DA release indicates that DA release is lower when nAChRs are active compared to when they are antagonised, we hypothesised that the **attenuated population spikes lead to attenuated release events** too. The converse would be also true, when nAChRs are inactive, the DA axon membrane will more freely repolarise and even hyperpolarise during train pulse, and therefore, each spike evokes more release than the small spikes evoked when nAChRs are active.

Finally, both predictions could provide alternative explanations for the regional differences observed. In DLS, nAChR-mediated prolonged depolarisation occurs closer to the initial spike almost without hyperpolarising at all, therefore preventing most VGSCs from de-inactivating, hence explaining why the subsequent population spike-during pulse-trains are so small/non-existent.

In NAcC, the nAChR-mediated prolonged depolarisation has a slower onset than in DLS, and we can observe a more pronounced hyperpolarisation after the initial spike. The hyperpolarisation could allow for more VGSCs to de-inactivate and therefore allow more reliable subsequent spikes despite nAChR activation in this striatal region.

### 4.3.2. nAChR activation provokes $\text{Ca}^{2+}$ transient upon initial stimulation but prevents subsequent events despite further stimulation

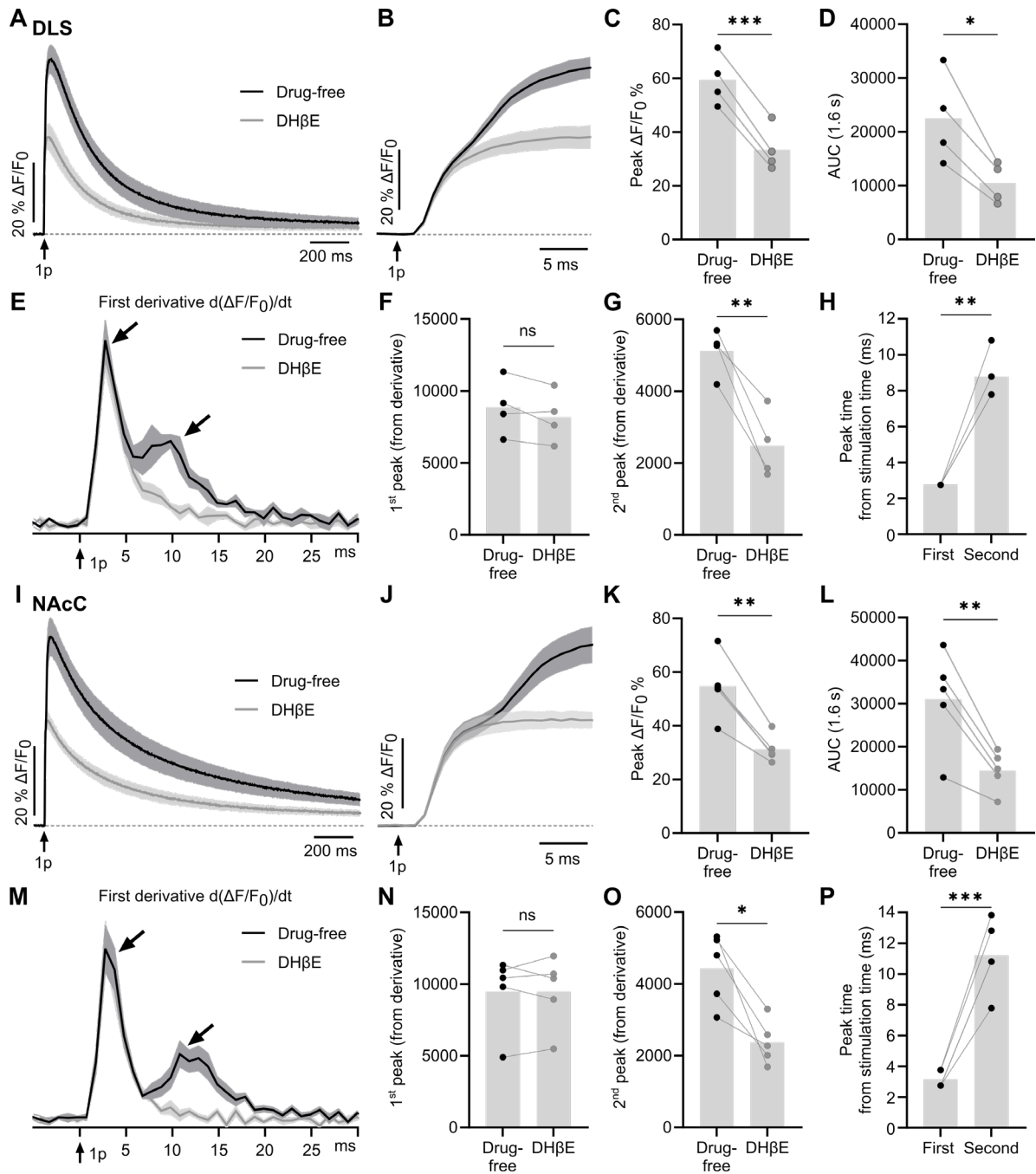
To bridge our voltage-sensor-based hypothesis and known DA release dynamics we assessed accompanying axonal  $\text{Ca}^{2+}$  dynamics that are intermediates between changes of membrane potential and release. we hypothesised that the first pulse of stimulation triggers a first event of  $\text{Ca}^{2+}$  event, quickly followed by a second event corresponding the activation of nAChRs, few milliseconds later. When nAChRs are active, we expect that any further stimulation elicit much smaller  $\text{Ca}^{2+}$  transients compared to the initial transient, suggesting that smaller spikes are indeed the caused less DA release. On the contrary, when nAChRs are blocked, only one  $\text{Ca}^{2+}$  event occurs corresponding to the initial spike. When nAChRs are blocked, each subsequent stimulation evoke  $\text{Ca}^{2+}$  events similar in magnitude to the initial event. In order to confirm that the STD in DA release evoked by pulse trains when nAChRs are active is due inability of the membrane to produce spikes and therefore trigger sufficient  $\text{Ca}^{2+}$  transient for release, we performed pilot  $\text{Ca}^{2+}$  imaging experiments using jRCaMP1.2 at a sampling rate of 1000 Hz (see Methods **section 4.2.5**) during which I bath-applied nAChR antagonist (DH $\beta$ E).

In both regions we could successfully identify the dual event occurring at DA axons following a stimulation when nAChRs are active (**Figure 4.15 A, B, I and J**). Indeed, the traces in drug-free and DH $\beta$ E overlap for the first  $\text{Ca}^{2+}$  event, but few milliseconds later a second ample  $\text{Ca}^{2+}$  event occurs in drug free condition, while the signal in DH $\beta$ E reaches a plateau (**Figure 4.15 A, B, I and J**). This resulted, in both regions, in the peak  $\Delta F/F_0$  to be significantly smaller in DH $\beta$ E compared to the drug free condition (**Figure 4.15 C and K**). Similarly, in both



regions, the AUC of the traces evoked by single pulse in DH $\beta$ E was significantly lower than the AUC evoked by single pulse in drug-free condition (**Figure 4.15 D and L**).

The first derivative of the traces – which highlight rate of change at any given moment – allowed us to confirm the existence of two separate Ca<sup>2+</sup> events in both regions since the first derivative exhibits two clear maxima with different kinetics (**Figure 4.15 E and M**). The first event shows a fast onset which reaches its maximum speed 2.75 ms after stimulation. It also exhibits the same characteristics in drug-free (nAChRs active) and DH $\beta$ E (nAChRs antagonised) conditions suggesting this first event corresponds in the Ca<sup>2+</sup> transient following direct activation of DA axons (**Figure 4.15 E, F, M and N**). The second event however, is only present in drug-free condition and not in the presence of DHBE, suggesting this second Ca<sup>2+</sup> transient corresponds to an event triggered by the opening of nAChRs (**Figure 4.15 E, G, M and O**). This second event reaches its maximum speed at 8.7 ms after the stimulation in DLS (**Figure 4.15 E and H**), and at 11.2 ms after the stimulation in NAcC (**Figure 4.15 M and P**).



**Figure 4.15: nAChRs activation provokes large  $\text{Ca}^{2+}$  transient in DA axons measured with jRCaMP8m.**

**A, I**: Mean ( $\pm$  SEM)  $\% \Delta F/F_0$  evoked by single electrical stimulation (1p), in drug-free condition (black) or after addition of nAChR antagonist DH $\beta$ E (1  $\mu$ M) in grey. The arrow indicates the time of stimulation. The arrow indicates the time of stimulation.  $n = 4$  in DLS, and  $n = 5$  in NAcC.

**B, J**: Zoom on the initial rise from traces shown in **A** and **I** respectively. Mean ( $\pm$  SEM)  $\% \Delta F/F_0$  evoked by single electrical stimulation (1p). The arrow indicates the time of stimulation.  $n = 4$  in DLS, and  $n = 5$  in NAcC.

**C, K**: Peak  $\% \Delta F/F_0$  of traces shown in **A** and **I**. Each dot represents a slice. The grey bars represent the mean. Paired t-test, in DLS  $p = 0.0003$ , in NAcC  $p = 0.0017$ .

**D, L**: AUC (taken over 1.6 s) of traces shown in **A** and **F** respectively. Each dot represents a slice. The grey bars represent the mean. Paired t-test, in DLS  $p = 0.0166$ , in NAcC  $p = 0.0054$ .

**E, M**: Mean ( $\pm$  SEM) of first derivative  $d(\Delta F/F_0)/dt$  of traces shown in **B** and **J**. The arrow indicates the time of stimulation.

**F, N**: Max value for 1<sup>st</sup> peak from the first derivative in **E** and **M** respectively. Each dot represents a slice. The grey bars represent the mean. Paired t-test, in DLS  $p = 0.1541$ , in NAcC  $p = 0.9894$ .

*Rest of legend on next page*

**G, O:** Max value for 2<sup>nd</sup> peak from the first derivative in **E** and **M** respectively. Each dot represents a slice. The grey bars represent the mean. Paired t-test, in DLS  $p=0.0100$ , in NAcC  $p=0.0112$ .

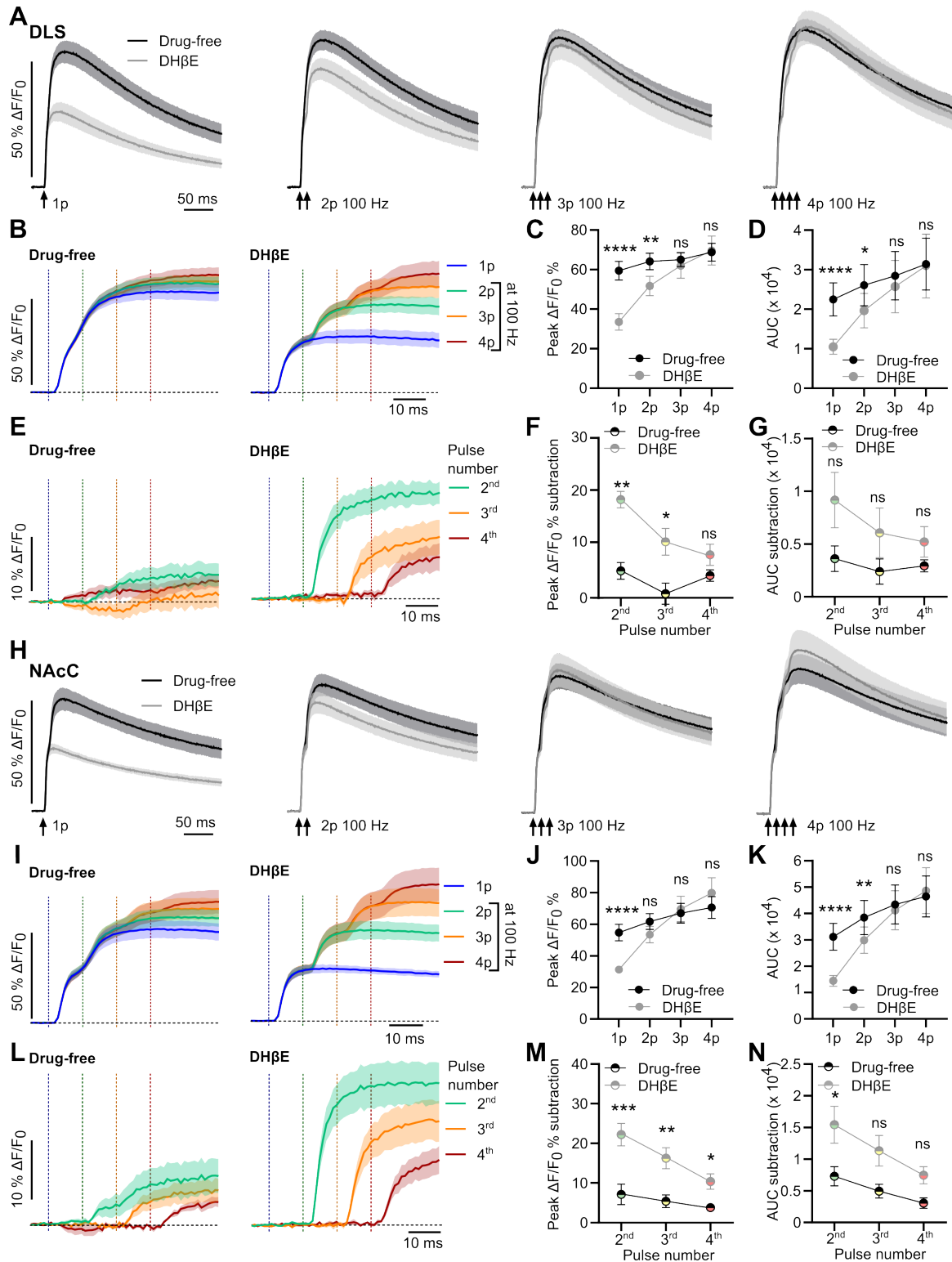
**H, P:** Time (post-stimulation) of 1<sup>st</sup> and 2<sup>nd</sup> peaks from the first derivative in **E** and **M** respectively. Each dot represents a slice. The grey bars represent the mean. Paired t-test, in DLS  $p=0.0034$ , in NAcC  $p=0.0007$ .

---

Next, we applied two, three or four electrical pulses at 100 Hz. In DLS, peak  $\Delta F/F_0$  and AUC evoked by 2p 100 Hz stimulation was higher when nAChRs are active in drug free condition compared to when nAChRs are antagonised (**Figure 4.16 A, B, C** Šídák's multiple comparisons test between Drug-free and DH $\beta$ E for 2<sup>nd</sup> pulse (2p)  $p = 0.0015$  and **D** Šídák's multiple comparisons test between Drug-free and DH $\beta$ E for 2<sup>nd</sup> pulse (2p)  $p = 0.0108$ ). We observed a similar trend in NAcC but only the AUC of the traces evoked 2p 100 Hz is statistically higher in drug-free conditions compared to when nAChRs are antagonised with DH $\beta$ E (**Figure 4.16 H, I, J** Šídák's multiple comparisons test between Drug-free and DH $\beta$ E for 2<sup>nd</sup> pulse (2p)  $p = 0.1778$  and **H** Šídák's multiple comparisons test between Drug-free and DH $\beta$ E for 2<sup>nd</sup> pulse (2p)  $p = 0.0030$ ). In both regions, peak  $\Delta F/F_0$  and AUC evoked by 3p and 4p 100 Hz was similar between drug free conditions and when nAChRs are antagonised with DH $\beta$ E (**Figure 4.16** in DLS **A, B, C** with Šídák's multiple comparisons test between Drug-free and DH $\beta$ E for 3<sup>rd</sup> pulse (3p)  $p = 0.6843$ , for 4<sup>th</sup> pulse (4p)  $p = 0.9948$  and **D** Šídák's multiple comparisons test between Drug-free and DH $\beta$ E for 3<sup>rd</sup> pulse (3p)  $p = 0.4372$ , for 4<sup>th</sup> pulse (4p)  $p = 0.9980$ ; and in NAcC **H, I, J** Šídák's multiple comparisons test between Drug-free and DH $\beta$ E for 3<sup>rd</sup> pulse (3p)  $p = 0.9358$ , for 4<sup>th</sup> pulse (4p)  $p = 0.1159$  and **H** Šídák's multiple comparisons test between Drug-free and DH $\beta$ E for 3<sup>rd</sup> pulse (3p)  $p = 0.7623$ , for 4<sup>th</sup> pulse (4p)  $p = 0.7664$ ). This suggest that total  $\text{Ca}^{2+}$  transient is the similar whether nAChRs are open or closed. Nevertheless, the event dynamics are highly different between the two conditions (**Figure 4.16** in DLS **A, B, C** and in NAcC **H, I, J**). In drug-free conditions when nAChRs are active, the total  $\text{Ca}^{2+}$  transient seems mostly unchanged by pulse number. On the contrary

when nAChRs are antagonised by DH $\beta$ E, total Ca<sup>2+</sup> transient seems to gradually increase with pulse number (**Figure 4.16** in DLS **A, B, C** and in NAcC **H, I, J**).

To be able to isolate the added Ca<sup>2+</sup> transient caused by each pulse of a pulse train, we subtracted the signal evoked by 1 pulse to the trace evoked by 2 pulses, the signal evoked by 2 pulses to the signal evoked by 3 pulses and so on (**Figure 4.16 E and L**). In both regions, the peak  $\Delta F/F_0$  of the subtracted traces is significantly higher when nAChRs are antagonised compared to when they are active for every pulse (**Figure 4.16** in DLS **F**, Šídák's multiple comparisons test between Drug-free and DH $\beta$ E for 2<sup>nd</sup> pulse (2p)  $p = 0.0044$ , for 3<sup>rd</sup> pulse (3p)  $p = 0.0258$ , for 4<sup>th</sup> pulse (4p)  $p = 0.3955$ ; in NAcC **M**, Šídák's multiple comparisons test between Drug-free and DH $\beta$ E for 2<sup>nd</sup> pulse (2p)  $p = 0.0002$ , for 3<sup>rd</sup> pulse (3p)  $p = 0.0021$ , for 4<sup>th</sup> pulse (4p)  $p = 0.0418$ ). Although the AUC in both regions shows the same trend, not all pulses evoked significantly higher AUCs (**Figure 4.16** in DLS, **G** Šídák's multiple comparisons test between Drug-free and DH $\beta$ E for 2<sup>nd</sup> pulse (2p)  $p = 0.1190$ , for 3<sup>rd</sup> pulse (3p)  $p = 0.2669$ , for 4<sup>th</sup> pulse (4p)  $p = 0.3557$ ; in NAcC, **N** Šídák's multiple comparisons test between Drug-free and DH $\beta$ E for 2<sup>nd</sup> pulse (2p)  $p = 0.0219$ , for 3<sup>rd</sup> pulse (3p)  $p = 0.0595$ , for 4<sup>th</sup> pulse (4p)  $p = 0.0914$ ).



**Figure 4.16: nAChRs activation limits  $\text{Ca}^{2+}$  transient evoked by subsequent pulses during pulse-train stimulation.**

**A, H:** Mean ( $\pm$  SEM)  $\% \Delta F/F_0$  evoked by electrical stimulation composed of 1, 2, 3 or 4 pulses given at 100 Hz, in drug-free condition (black) or after addition of nAChR antagonist DHβE (1  $\mu\text{M}$ ) in grey. Arrows indicate the time of stimulation.  $n = 4$  in DLS, and  $n = 5$  in NAcC.

**B, I:** Zoom on the initial rise from traces shown in **A** and **H** respectively and overlapped by condition drug free (left) and DHβE (right). Mean ( $\pm$  SEM)  $\% \Delta F/F_0$ . The dotted vertical lines indicate the time of stimulation.

*Rest of legend on next page*

**C, J:** Mean ( $\pm$  SEM) peak  $\% \Delta F/F_0$  of traces shown in **A** and **H**. Two-way ANOVA (in DLS (**C**), drug effect:  $F(1, 12) = 63.30$ ,  $p < 0.0001$ , pulse effect:  $F(3, 12) = 3.877$ ,  $p = 0.0377$ , interaction:  $F(3, 12) = 21.90$ ,  $p < 0.0001$ ; in NAcC (**I**), drug effect:  $F(1, 16) = 6.581$ ,  $p = 0.0207$ , pulse effect:  $F(3, 16) = 5.278$ ,  $p = 0.0101$ , interaction:  $F(3, 16) = 13.62$ ,  $p = 0.0001$ ) with Šídák's multiple comparisons test.

**D, K:** Mean ( $\pm$  SEM) AUC (taken over 1.6 s) of traces shown in **A** and **H**. Two-way ANOVA (in DLS (**D**), drug effect:  $F(1, 12) = 40.05$ ,  $p < 0.0001$ , pulse effect:  $F(3, 12) = 1.253$ ,  $p = 0.3342$ , interaction:  $F(3, 12) = 8.643$ ,  $p = 0.0025$ ; in NAcC (**K**), drug effect:  $F(1, 16) = 37.39$ ,  $p < 0.0001$ , pulse effect  $F(3, 16) = 2.787$ ,  $p = 0.0743$ , interaction:  $F(3, 16) = 15.76$ ,  $p < 0.0001$ ) with Šídák's multiple comparisons test.

**E, L:** Subtraction of traces in **B** and **I** to isolate contribution of each pulse in drug free (left) and DH $\beta$ E (right). Mean ( $\pm$  SEM)  $\% \Delta F/F_0$ . The dotted vertical lines indicate the time of stimulation.

**F, M:** Mean ( $\pm$  SEM) peak  $\% \Delta F/F_0$  of subtracted traces shown in **E** and **L**. Two-way ANOVA (in DLS (**F**), drug effect:  $F(1, 3) = 61.18$ ,  $p = 0.0044$ , pulse effect:  $F(2, 6) = 21.22$ ,  $p = 0.0019$ , interaction:  $F(2, 6) = 3.843$ ,  $p < 0.0843$ ; in NAcC (**M**), drug effect:  $F(1, 4) = 18.05$ ,  $p = 0.0132$ , pulse effect:  $F(2, 8) = 48.87$ ,  $p < 0.0001$ , interaction:  $F(2, 8) = 4.446$ ,  $p = 0.0503$ ) with Šídák's multiple comparisons test.

**G, N:** Mean ( $\pm$  SEM) AUC (taken over 1.6 s) of subtracted traces shown in **E** and **L**. Two-way ANOVA (in DLS (**G**), drug effect:  $F(1.000, 3.000) = 8.371$ ,  $p = 0.0628$ , pulse effect:  $F(1.316, 3.949) = 5.040$ ,  $p = 0.0865$ , interaction:  $F(1.133, 3.400) = 5.350$ ,  $p = 0.0928$ ; in NAcC (**N**), drug effect:  $F(1.000, 4.000) = 16.99$ ,  $p = 0.0146$ , pulse effect  $F(1.046, 4.183) = 17.36$ ,  $p = 0.0126$ , interaction:  $F(1.659, 6.636) = 19.50$ ,  $p = 0.0020$ ) with Šídák's multiple comparisons test.

---

Altogether, this data suggests that upon nAChR activation, no further  $\text{Ca}^{2+}$  event is added by subsequent pulses of a pulse train but when nAChRs are antagonised, each subsequent pulse of the train evoked a new event of  $\text{Ca}^{2+}$  event. These results mirror the data collected using the voltage sensor ASAP5f. Indeed, when nAChRs are active, nAChRs limit population spike height at DA axons, which in turns limit  $\text{Ca}^{2+}$  transients, as the opposite of when nAChRs are antagonised, and each subsequent pulse provokes tall population spikes, which leads to increased  $\text{Ca}^{2+}$  transients after each pulse. Hence, this data indicates that the STD in DA release evoked by pulse trains when nAChRs are active is due to the inability of the membrane to produce spikes ample enough to trigger further  $\text{Ca}^{2+}$  transient, and not due to a different management of intracellular  $\text{Ca}^{2+}$ .

### **4.3.3. VGSC availability is limiting subsequent release when nAChRs are active**

Next, using FCV, we tested the hypothesis that **nAChRs activity reduces DA release during pulse trains by limiting the pool of available VGSCs, therefore limiting population spike height which reduces  $\text{Ca}^{2+}$  transients necessary for release.**

#### **4.3.3.1. Partial inhibition of VGSCs limits subsequent DA release when nAChRs are active**

In this section, to investigate whether VGSC unavailability causes strong STD of DA release when nAChRs are active, we tested whether limiting their availability further using low concentrations of tetrodotoxin (TTX) would abolish re-release completely.

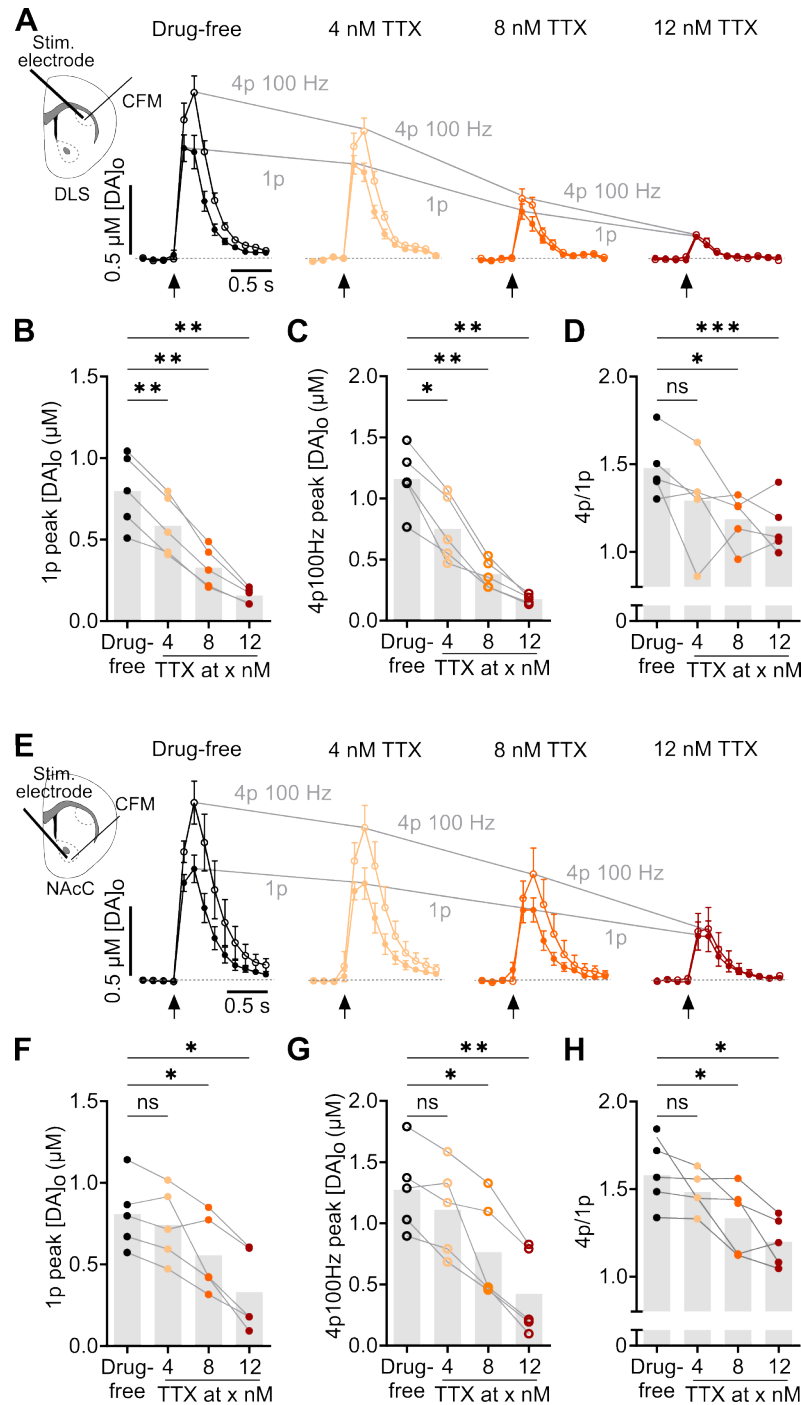
TTX is a broad-spectrum VGSC blocker that is known to abolish completely APs when used at concentrations in the range of  $\mu\text{M}$  (Marshall et al. 1996; Khaliq and Bean 2010). At lower concentrations in the nM range, TTX can be used to reveal situations where VGSCs are extremely limiting (Brody and Yue 2000; Debanne 2004), when even partial addition block of VGSCs therefore has detectable impact. We hypothesised that if nAChR activation is compromising the VGSC pool available to mediate successive depolarisation events, then (i) DA release evoked by pulse trains should be disproportionately sensitive to low concentrations of TTX compared to release evoked by single electrical pulses, and (ii) more so when nAChRs are active than inactive.

*i. Compromising the pool of VGSCs using low concentration of TTX disproportionately impairs release evoked by pulse-trains.*

First, we tested whether TTX in the nM range could affect DA release and with disproportionate impact on the 4p/1p ratio. We applied increasing concentrations of TTX 4, 8 and 12 nM to find the lowest concentration for which the 4p/1p ratio would be affected. We found that in both regions there was a progressive reduction in  $[DA]_o$  evoked by single pulse and pulse-trains with increasing TTX concentration compared to compared to the drug free condition (**Figure 4.17**, in DLS **A, B** Drug-free versus TTX at 4 nM  $p = 0.0062$ , at 8 nM = 0.0020, at 12 nM  $p = 0.0035$  and **C** Drug-free versus TTX at 4 nM  $p = 0.0136$ , at 8 nM = 0.0013, at 12 nM  $p = 0.0018$ ; in NAcC **E, F** Drug-free versus TTX at 4 nM  $p = 0.1910$ , at 8 nM = 0.0435, at 12 nM  $p = 0.0109$  and **G** Drug-free versus TTX at 4 nM  $p = 0.1354$ , at 8 nM = 0.0105, at 12 nM  $p = 0.0012$ ). At 4 nM TTX, the 4p/1p ratio was not significantly different from drug-free conditions, but by 8 nM the 4p/1p ratio was significantly reduced in both DLS and NAcC (**Figure 4.17** in DLS **A** and **D** Drug-free versus TTX at 4 nM  $p = 0.2714$ , at 8 nM = 0.0223, at 12 nM  $p = 0.0009$ ; in NAcC **E** and **H** Drug-free versus TTX at 4 nM  $p = 0.4075$ , at 8 nM = 0.0439, at 12 nM  $p = 0.0298$ ) compared to drug-free condition.

Thus, further limiting the pool of available VGSCs when nAChRs are active does disproportionately reduce release evoked by pulse-trains compared to release evoked by single electrical pulse. These results seem to verify our hypothesis that availability of VGSCs at DA membrane is reduced when nAChRs are on, and in particular that their unavailability is limiting release evoked by pulse trains.





**Figure 4.17: Partially compromising the pool of VGSCs using low concentrations of TTX disproportionately attenuates release evoked by pulse-train stimulations.**

**A, E:** Mean ( $\pm$  SEM) [DA]<sub>o</sub> transients in response to 1 pulse electrical stimulation (1p) and 4 pulses at 100 Hz (4p 100 Hz), in drug free condition first, followed by a wash on of TTX at 4 nM, then TTX at 8 nM and finally TTX at 12 nM.  $n = 5$  in DLS (**A**) and  $n = 5$  in NAcC (**E**).

**B, F:** Peak [DA]<sub>o</sub> evoked by 1p stimulation in DLS (**B**) and in NAcC (**F**). Each dot corresponds to a slice. The grey bar represents the mean. One-way ANOVA (in DLS (**B**),  $F(1.714, 6.855) = 51.02$ ,  $p$ -value  $< 0.0001$ ; in NAcC (**F**),  $F(1.174, 4.697) = 17.25$ ,  $p$ -value = 0.0092) and Dunnett's multiple comparisons test.

**C, G:** Peak [DA]<sub>o</sub> evoked by 4p 100 Hz stimulation in DLS (**C**) and in NAcC (**G**). Each dot corresponds to a slice. The grey bar represents the mean. One-way ANOVA (in DLS (**C**),  $F(1.210, 4.841) = 56.60$ ,  $p$ -value  $< 0.0001$ ; in NAcC (**G**),  $F(1.327, 5.307) = 31.55$ ,  $p$ -value = 0.0016) and Dunnett's multiple comparisons test.

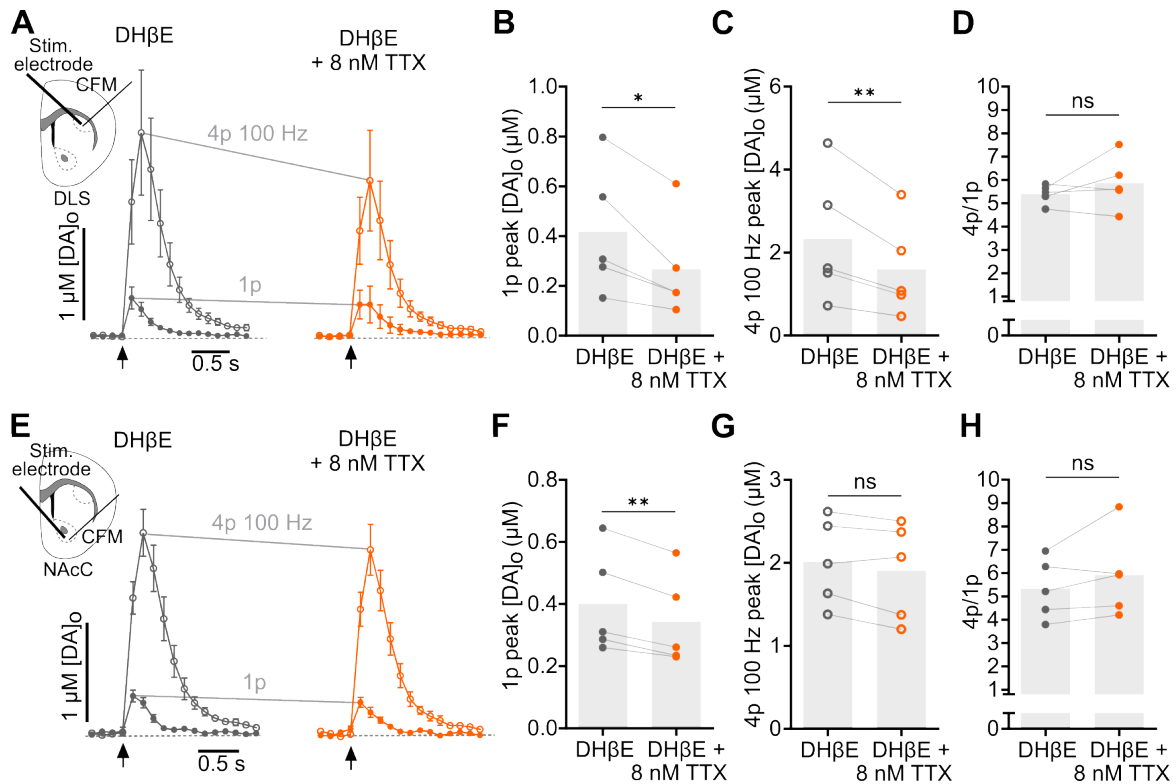
**D, H:** Ratio of peak [DA]<sub>o</sub> in response to 4p 100 Hz and peak [DA]<sub>o</sub> in response 1p in DLS (**D**) and in NAcC (**H**). The grey bar represents the mean. One-way ANOVA (in DLS (**D**),  $F(1.701, 6.803) = 5.834$ ,  $p$ -value = 0.0365; in NAcC (**H**),  $F(1.797, 7.189) = 12.70$ ,  $p$ -value = 0.0049) and Dunnett's multiple comparisons test.

*ii. When nAChRs are antagonised by DH $\beta$ E, TTX does not differently affect trains versus single pulse release.*

We antagonised nAChRs throughout the experiment using DH $\beta$ E (1  $\mu$ M) and washed on 8 nM of TTX which is the first concentration for which the 4p/1p ratio had decreased when nAChRs are active. Although we might expect a slight decrease in DA overall, we expect this decrease to be homogeneous across stimulation patterns with no differences across conditions in the 4p/1p ratio.

We found that in both region addition of 8 nM TTX when nAChRs are already antagonised using DH $\beta$ E reduces [DA]<sub>o</sub> evoked by single pulse compared to when on the nAChRs are antagonised (**Figure 4.18 A, B, E and F**). In DLS, upon bath application of 8 nM TTX when nAChRs are antagonised, [DA]<sub>o</sub> evoked by pulse-train was reduced compared to when only DH $\beta$ E was present (**Figure 4.18 A, and C**). In NAcC [DA]<sub>o</sub> evoked by pulse-train was unchanged by additional application of 8 nM TTX compared to when only DH $\beta$ E was applied (**Figure 4.18 E and G**). Finally, in neither region did the 4p/1p ratio significantly change after bath application of 8 nM TTX when nAChRs are antagonised compared to the DH $\beta$ E only condition (**Figure 4.18 D and H**).

Taken together these results highlight that low nM concentrations of TTX only disproportionally affect DA release evoked by pulse-trains when nAChRs are active but not when they are antagonised. Therefore, availability of VGSCs at DA axons seems to govern DA release evoked by pulse-trains when nAChRs active but not when they are antagonised.



**Figure 4.18: Partially compromising the pool of VGSCs using a low concentration of TTX when nAChRs are antagonised does not differently affect release evoked by single or pulse-train stimulations.**

**A, E:** Mean ( $\pm$  SEM)  $[DA]_o$  transients in response to 1 pulse electrical stimulation (1p) and 4 pulses at 100 Hz (4p 100 Hz), initially in nAChR antagonist DH $\beta$ E (1  $\mu$ M) only, following a bath application of 8 nM TTX along with DH $\beta$ E (1  $\mu$ M).  $n = 5$  in DLS (**A**) and  $n = 5$  in NAcC (**E**).

**B, F:** Peak  $[DA]_o$  evoked by 1p stimulation in DLS (**B**) and in NAcC (**F**). Each dot corresponds to a slice. The grey bar represents the mean. Paired t-test, in DLS  $p = 0.0203$ ; in NAcC  $p = 0.0038$ .

**C, G:** Peak  $[DA]_o$  evoked by 4p 100 Hz stimulation in DLS (**C**) and in NAcC (**G**). Each dot corresponds to a slice. The grey bar represents the mean. Paired t-test, in DLS  $p = 0.0170$ ; in NAcC  $p = 0.1291$ .

**D, H:** Ratio of peak  $[DA]_o$  in response to 4p 100 Hz and peak  $[DA]_o$  in response 1p in DLS (**D**) and in NAcC (**H**). Each dot corresponds to a slice. The grey bar represents the mean. Paired t-test, in DLS  $p = 0.3174$ ; in NAcC  $p = 0.1952$ .

Data acquired in this panel have been collected by Mahnoor Khurram and me.

#### 4.3.3.2. Prolonging inactivation of VGSCs limits DA release when nAChRs are active

*i. When nAChRs are active, prolonging the inactivation of VGSCs reduces DA release evoked by a train more than for a single pulse*

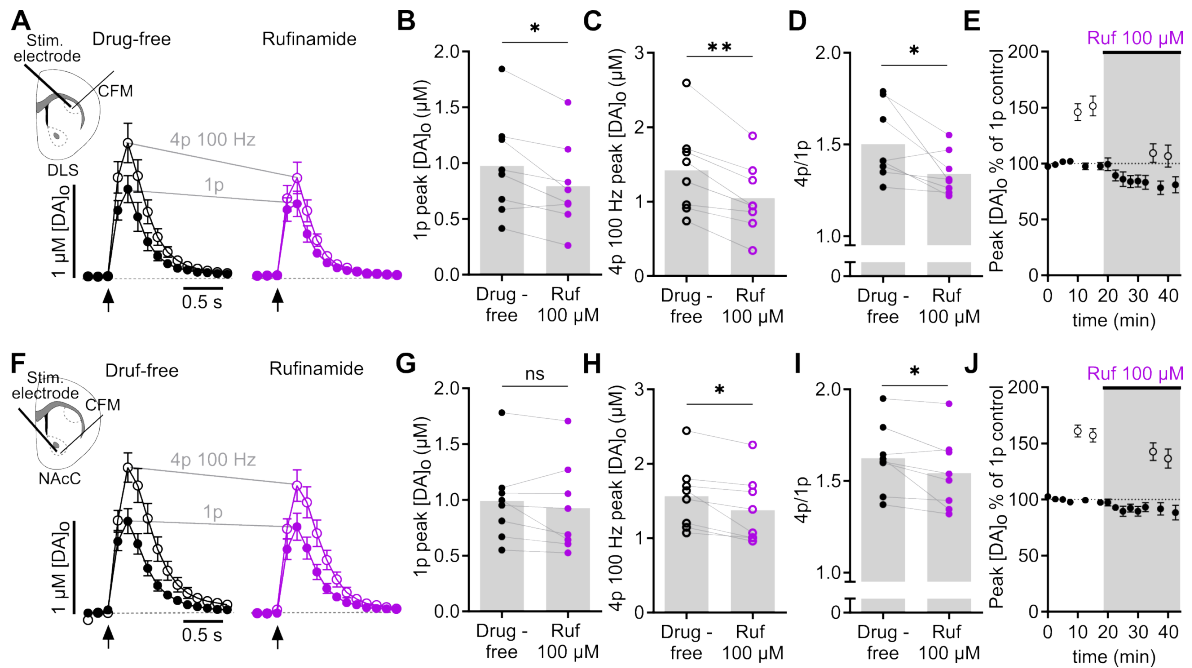
We tested whether pharmacologically prolonging inactivation of VGSCs using rufinamide would limit subsequent release even further during pulse-trains when nAChRs are active and diminish the 4p/1p ratio.

We washed on 100  $\mu$ M rufinamide which is a drug that binds to inactive VGSCs and prolong their inactive state (Hille 2001; Deeks and Scott 2006; Arroyo 2007; White et al. 2010; Lin et al. 2022c). We expect release to we overall decrease across stimulation paradigm since our hypothesis suggest that more VGSCs are inactive when nAChRs are active which therefore would allow rufinamide to bind even in the absence of a stimulation. However, we expect a more ample decay in DA release evoked by pulse-train since rufinamide would bind to the VGSCs inactivated by the first electric pulse which should further impede the membrane to produce spikes upon the subsequent pulses.

In DLS,  $[DA]_o$  evoked by single pulse (**Figure 4.19 A, B and E**) and pulse-train (**Figure 4.19 A, C and E**) was lowered upon rufinamide application compared to the drug-free condition. The 4p/1p ratio was also decreased upon rufinamide wash on compared to the drug-free condition (**Figure 4.19 D**), suggesting  $[DA]_o$  evoked by pulse-train was disproportionally decreased by rufinamide compared to the  $[DA]_o$  evoked by single pulse.

In NAcC, rufinamide did not lower  $[DA]_o$  evoked by single pulse (**Figure 4.19 F, G and J**) but slightly decreased  $[DA]_o$  evoked by pulse-train (**Figure 4.19 F, H and J**) compared to drug-free condition. In consequences, the 4p/1p ratio also decreased following rufinamide wash on (**Figure 4.19 I**).

Altogether, this data indicates that prolonging the inactivation of VGSCs disproportionally reduces DA release evoked by pulse-trains compared to release evoked by single pulse. It therefore supports our hypothesis that limited availability of VGSCs hinders release evoked pulse-train when nAChRs are active.



**Figure 4.19: Prolonging inactivation of VGSCs using rufinamide limits DA release evoked by pulse-trains when nAChRs are active.**

**A, F:** Mean ( $\pm$  SEM)  $[DA]_o$  transients in response to 1 pulse electrical stimulation (1p) and 4 pulses at 100 Hz (4p 100 Hz), in drug-free aCSF first, and followed by a bath application of rufinamide (100  $\mu$ M, Ruf).  $n = 8$  in DLS (**A**) and  $n = 8$  in NAcC (**F**).

**B, G:** Peak  $[DA]_o$  evoked by 1p stimulation in DLS (**B**) and in NAcC (**G**). Each dot corresponds to a slice. The grey bar represents the mean. In DLS paired t-test  $p = 0.0011$ ; in NAcC, Wilcoxon test  $p = 0.1484$ .

**C, H:** Peak  $[DA]_o$  evoked by 4p 100 Hz stimulation in DLS (**C**) and in NAcC (**H**). Each dot corresponds to a slice. The grey bar represents the mean. In DLS paired t-test  $p = 0.0103$ ; in NAcC, Wilcoxon test  $p = 0.0134$ .

**D, I:** Ratio of peak  $[DA]_o$  in response to 4p 100 Hz and peak  $[DA]_o$  in response 1p in DLS (**D**) and in NAcC (**I**). Each dot corresponds to a slice. The grey bar represents the mean. Paired t-test, in DLS  $p = 0.0319$ ; in NAcC  $p = 0.0436$ .

**E, J:** Mean peak  $[DA]_o$  ( $\pm$  SEM) evoked by 1p (plain circles) or by 4p 100 Hz (empty circles) over time in DLS (**E**) and NAcC (**J**) starting drug-free conditions and followed by a rufinamide (100  $\mu$ M, ruf) wash on. Data is normalised to the average of the four first  $[DA]_o$  values evoked by 1p shown on the graph. The mean peak  $[DA]_o$  evoked by single pulse in **B** and **G** are averaged from the values flanking the peak  $[DA]_o$  evoked by 4p 100 Hz.

*ii. When nAChRs are antagonised by DH $\beta$ E, prolonging inactivation of VGSCs*

*does not differently affect DA release by single versus train pulses*

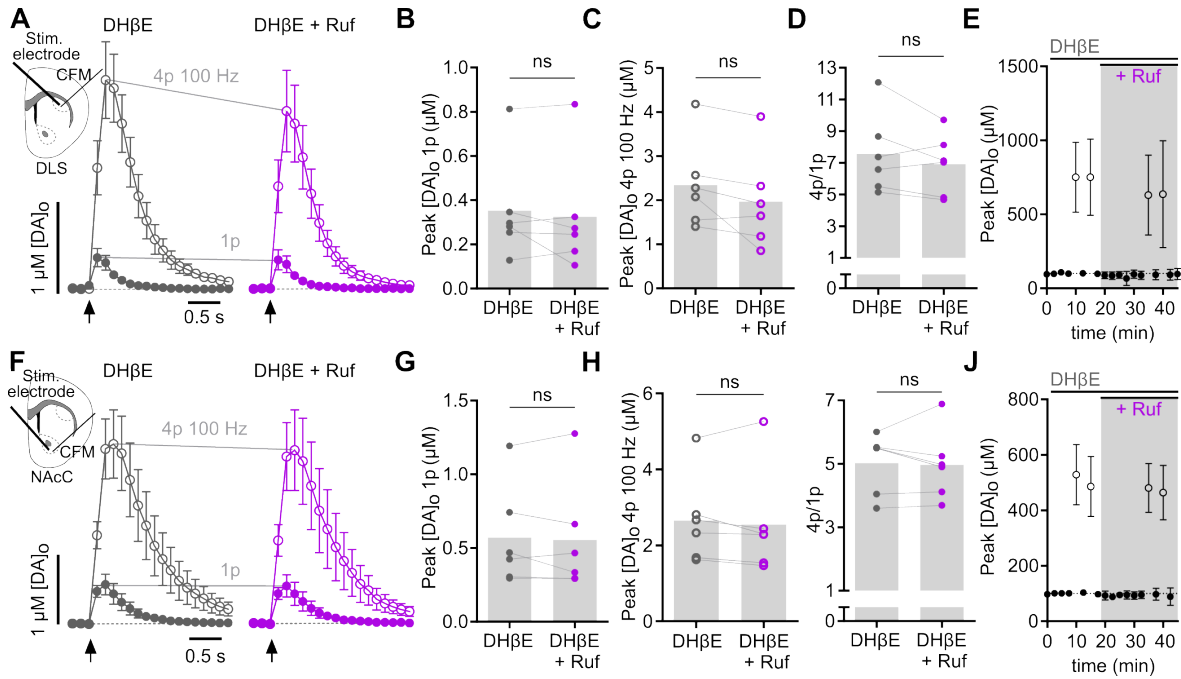
Next, we wanted to make sure the decrease in 4p/1p ratio seen upon rufinamide wash on when nAChRs are active, was not seen when nAChRs are antagonised. Indeed, when nAChRs are antagonised, the hypothesis taken from our voltage-sensor data suggest that VGSCs are available – and therefore not inactivated – for generating population spikes tall enough to trigger DA release. Hence, washing on rufinamide, which is a drug that prolongs

inactivation state of VGSCs that are already inactivated, should not affect release or 4p/1p ratio when nAChRs are antagonised.

Using FCV, we monitored  $[DA]_o$  before and after washing on rufinamide (100  $\mu$ M) while nAChRs were antagonised throughout the experiment using DH $\beta$ E (1  $\mu$ M).

When nAChRs are antagonised by DH $\beta$ E, rufinamide does not affect  $[DA]_o$  evoked by single pulse (**Figure 4.20 A, B, E, F, G and J**) nor does it change  $[DA]_o$  evoked by pulse trains (**Figure 4.20 A, C, E, F, H and J**). In both regions, the 4p/1p ratio also remain unchanged in rufinamide+ DH $\beta$ E condition compared to when only nAChRs are antagonised by DH $\beta$ E (**Figure 4.20 D and I**).

These results are in accordance with what we were expecting and therefore suggest that most of the VGSCs pool is available when nAChRs are antagonised, since prolonging inactivation of already inactivated VGSCs does not impact release. This is likely due to the fact that enough other VGSCs are available to support release despite some being effectively antagonised through prolonged inactivation during pulse trains.



**Figure 4.20: Prolonging inactivation of VGSCs using rufinamide does not limit DA release when nAChRs are antagonised by DHβE.**

**A, F:** Mean ( $\pm$  SEM) [DA]<sub>0</sub> transients in response to 1 pulse electrical stimulation (1p) and 4 pulses at 100 Hz (4p 100 Hz), initially in nAChR antagonist DHβE (1 μM) only, following a bath application of rufinamide (100 μM, Ruf) along with DHβE (1 μM).  $n = 6$  in DLS (**A**) and  $n = 6$  in NAcC (**F**).

**B, G:** Peak [DA]<sub>0</sub> evoked by 1p stimulation in DLS (**B**) and in NAcC (**G**). Each dot corresponds to a slice. The grey bar represents the mean. In DLS Wilcoxon test  $p = 0.8438$ ; in NAcC, paired  $t$ -test  $p = 0.6323$ .

**C, H:** Peak [DA]<sub>0</sub> evoked by 4p 100 Hz stimulation in DLS (**C**) and in NAcC (**H**). Each dot corresponds to a slice. The grey bar represents the mean. Paired  $t$ -test, in DLS  $p = 0.0940$ ; in NAcC  $p = 0.4365$ .

**D, I:** Ratio of peak [DA]<sub>0</sub> in response to 4p 100 Hz and peak [DA]<sub>0</sub> in response 1p in DLS (**D**) and in NAcC (**I**). Each dot corresponds to a slice. The grey bar represents the mean. Paired  $t$ -test, in DLS  $p = 0.2402$ ; in NAcC  $p = 0.8045$ .

**E, J:** Mean peak [DA]<sub>0</sub> ( $\pm$  SEM) evoked by 1p (plain circles) or by 4p 100 Hz (empty circles) over time in DLS (**E**) and NAcC (**J**) starting DHβE (1 μM) and followed by a rufinamide (100 μM, Ruf) wash on along with DHβE (1 μM). Data is normalised to the average of the four first [DA]<sub>0</sub> values evoked by 1p shown on the graph. The mean peak [DA]<sub>0</sub> evoked by single pulse in **B** and **G** are averaged from the values flanking the peak [DA]<sub>0</sub> evoked by 4p 100 Hz.

#### 4.3.3.3. DAT-Cre animals have higher DA release levels as well as different VGSCs handling compared to WT C57BL6/J animals

Next, we wanted to test our hypothesis that VGSCs are a limiting factor for release evoked by pulse trains by predicting what would happen at the DA axonal membrane when partially inhibiting VGSCs with TTX or when prolonging VGSCs inactivation using rufinamide. However, we use DAT-Cre (heterozygous DAT-Cre) for voltage sensor experiments. These animals have been demonstrated to have 15% less dopamine transporter (DAT) than their WT (C57BL/6J) counterparts (Bäckman et al.). Plus, using FCV, increased  $[DA]_o$  evoked by single electrical pulse has been reported DAT-Cre<sup>+/-</sup> compared to control DAT-Cre<sup>-/-</sup> (O'Neill et al. 2017).

Thus, this section, we first controlled for the effect of nAChR blockage on DA release in DAT-Cre animals using FCV. Next, we investigated whether 8 nM TTX and 100  $\mu$ M rufinamide would have similar effects on DA release – namely reduce the 4p/1p ratio – than these drugs have on DA release in WT animals.

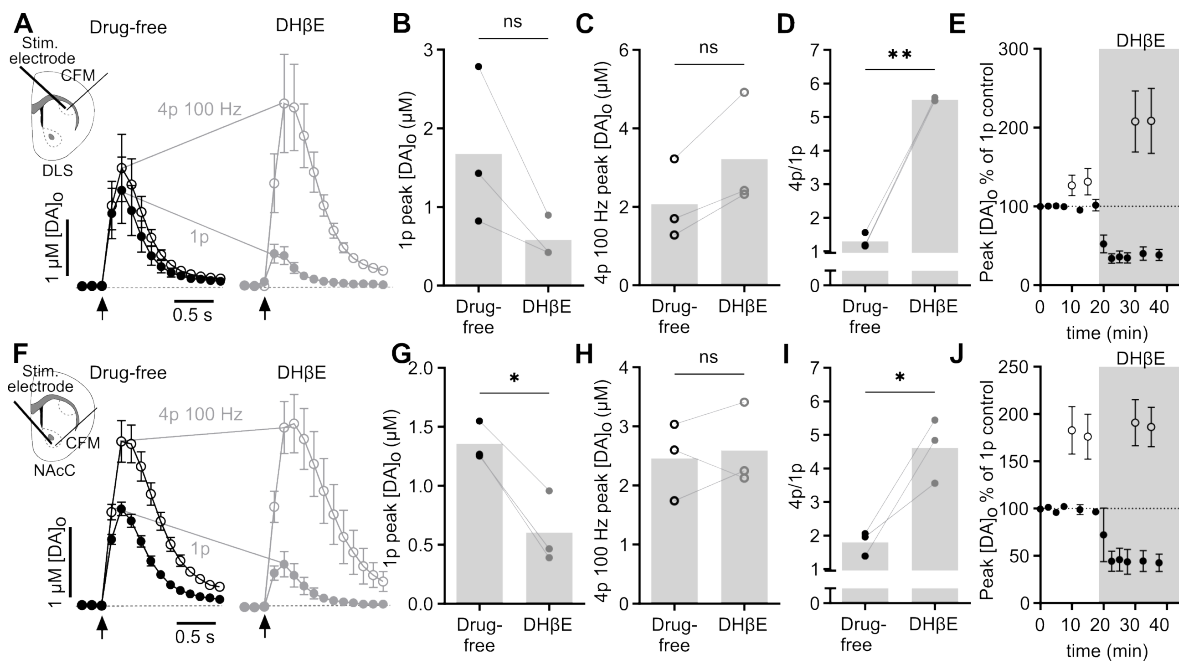
##### *i. DAT-Cre animals present higher DA release levels, but blocking nAChRs using DH $\beta$ E still produces the effects expected from WT experiments*

We first verified that blocking nAChRs through DH $\beta$ E application in DAT-Cre produces the expected effects observed in WT animals which are:

- A reduction in DA release evoked by single pulse
- An increase or trend towards an increase in DA release evoked by pulse-trains (at 100 Hz)
- And most importantly rise in the 4p/1p ratio



We performed pilot FCV experiments on mouse brain-slices of DAT-Cre animals, and bath-applied 1  $\mu$ M of DH $\beta$ E. In both regions, we noticed a strong reduction in  $[DA]_o$  evoked by 1p upon DH $\beta$ E wash on compared to the drug-free condition (**Figure 4.21 A, B, E, F, G and J**).  $[DA]_o$  evoked by 4p 100 Hz showed a slight increase after blocking nAChRs compared to the drug-free condition, but was however not statistically significant (**Figure 4.21 A, C, E, F, H and J**). Finally, the 4p/1p ratio was drastically increased by addition of DH $\beta$ E (**Figure 4.21 A, D, F and I**). Therefore, blocking nAChRs in DAT-Cre animals also provokes a drastic increase in 4p/1p ratio.



**Figure 4.21: Antagonising nAChRs with DH $\beta$ E in DAT-Cre animals increases 4p/1p.**

**A, F:** Mean ( $\pm$  SEM)  $[DA]_o$  transients in response to 1 pulse electrical stimulation (1p) and 4 pulses at 100 Hz (4p 100 Hz), in drug-free conditions initially and after a bath application of DH $\beta$ E (1  $\mu$ M).  $n = 3$  in DLS (**A**) and  $n = 3$  in NAcC (**F**).

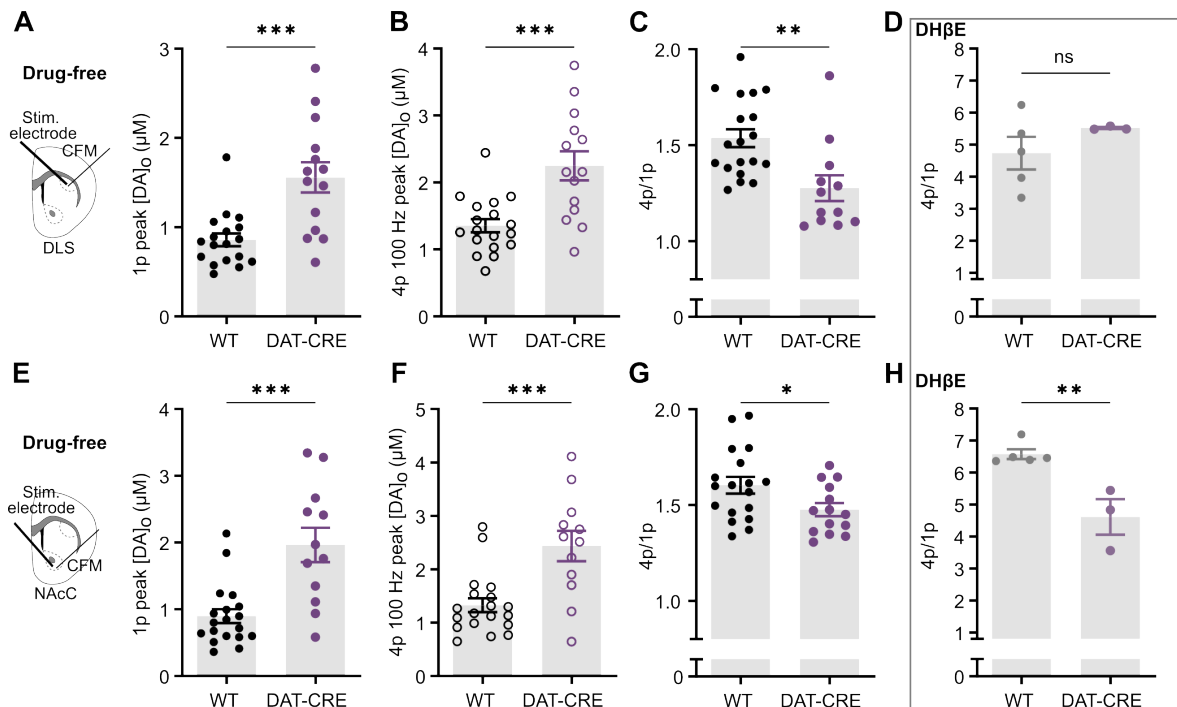
**B, G:** Peak  $[DA]_o$  evoked by 1p stimulation in DLS (**B**) and in NAcC (**G**). Each dot represents a slice. The grey bar represents the mean. Paired t-test, in DLS (**B**)  $p = 0.1273$ , in NAcC (**G**)  $p = 0.0122$ .

**C, H:** Peak  $[DA]_o$  evoked by 4p 100 Hz stimulation in DLS (**C**) and in NAcC (**H**). Each dot represents a slice. The grey bar represents the mean. Paired t-test, in DLS (**C**)  $p = 0.0567$ , in NAcC (**H**)  $p = 0.7500$ .

**D, I:** Ratio of peak  $[DA]_o$  in response to 4p 100 Hz and peak  $[DA]_o$  in response 1p in DLS (**D**) and in NAcC (**I**). Each dot represents a slice. The grey bar represents the mean. Paired t-test, in DLS (**D**)  $p = 0.0012$ , in NAcC (**I**)  $p = 0.0434$ .

**E, J:** Mean peak  $[DA]_o$  ( $\pm$  SEM) evoked by 1p (plain circles) or by 4p 100 Hz (empty circles) over time in DLS (**E**) and NAcC (**J**) starting in drug-free conditions initially and after a bath application of DH $\beta$ E (1  $\mu$ M). Data is normalised to the average of the four first  $[DA]_o$  values evoked by 1p shown on the graph. The mean peak  $[DA]_o$  evoked by single pulse in **B** and **G** are averaged from the values flanking the peak  $[DA]_o$  evoked by 4p 100 Hz.

We next compared release levels and the resulting ratios in drug-free conditions between WT and DAT-Cre animals and compared the 4p/1p ratio between genotypes in DH $\beta$ E too. In DH $\beta$ E, the 4p/1p ratio was also reduced in DAT-Cre animals compared to WT in NAcC, but was not different in DLS (**Figure 4.22 D and H**). We found that for both regions and both stimulation paradigm [DA]<sub>o</sub> was higher in DAT-Cre animals than in WT animals (**Figure 4.22 A, B, E and F**). However, the resulting 4p/1p ratio was smaller in DAT-Cre animals compared to WT animals in both region (**Figure 4.22 C and G**).



**Figure 4.22: DAT-Cre animals exhibit higher release in drug-free condition, but lower 4p/1p ratio compared to WT animals.**

**A, E:** Peak [DA]<sub>o</sub> ( $\pm$  SEM) evoked by single pulse stimulation (1p) in drug-free condition between WT and DAT-Cre animals. In DLS (**A**)  $n = 19$  in WT and  $n = 12$  in DAT-Cre, unpaired t-test  $p = 0.0001$ ; in NAcC (**E**)  $n = 18$  in WT and  $n = 14$  in DAT-Cre,  $p = 0.0003$ .

**B, F:** Peak [DA]<sub>o</sub> ( $\pm$  SEM) evoked by 4p 100 Hz stimulation in drug-free condition between WT and DAT-Cre animals. In DLS (**B**)  $n = 19$  in WT and  $n = 12$  in DAT-Cre, unpaired t-test  $p = 0.0004$ ; in NAcC (**F**)  $n = 18$  in WT and  $n = 14$  in DAT-Cre,  $p = 0.0003$ .

**C, G:** Ratio of peak [DA]<sub>o</sub> in response to 4p 100 Hz and peak [DA]<sub>o</sub> in response 1p in drug-free condition between WT and DAT-Cre animals. In DLS (**C**)  $n = 19$  in WT and  $n = 12$  in DAT-Cre, unpaired t-test  $p = 0.0027$ ; in NAcC (**G**)  $n = 18$  in WT and  $n = 14$  in DAT-Cre,  $p = 0.0364$ .

**D, H:** Ratio of peak [DA]<sub>o</sub> in response to 4p 100 Hz and peak [DA]<sub>o</sub> in response 1p in DH $\beta$ E (1  $\mu$ M) condition between WT and DAT-Cre animals. In DLS (**D**)  $n = 5$  in WT and  $n = 3$  in DAT-Cre, unpaired t-test  $p = 0.2911$ ; in NAcC (**H**)  $n = 5$  in WT and  $n = 3$  in DAT-Cre,  $p = 0.0050$ .

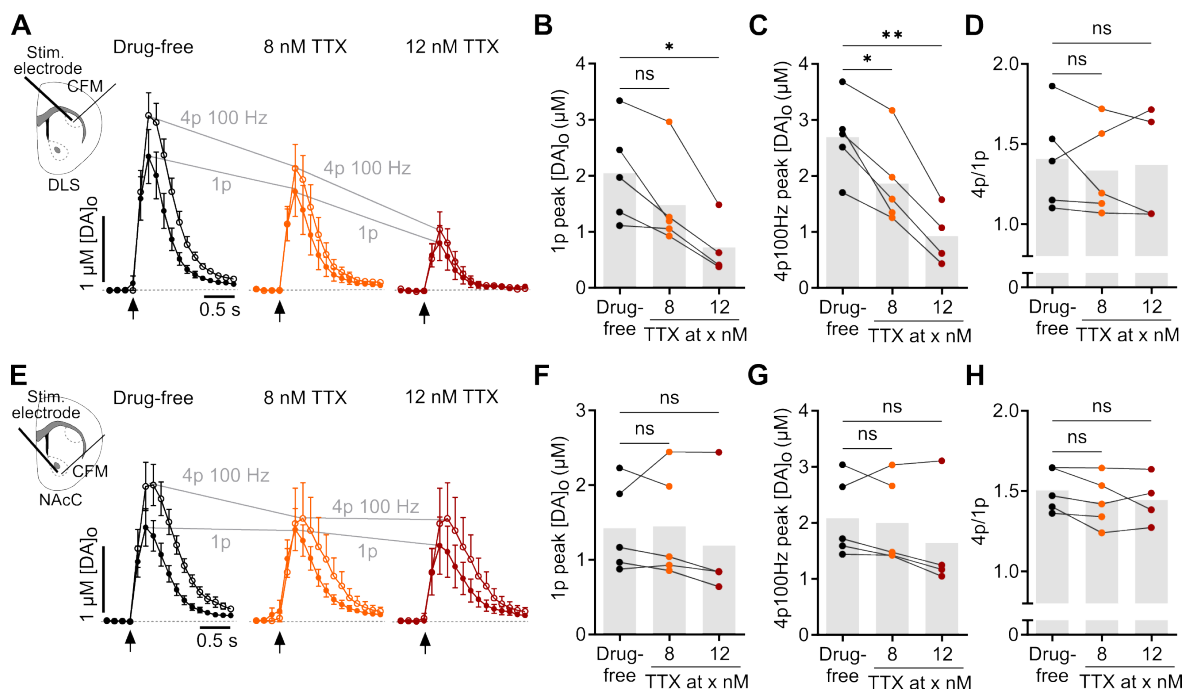
This data suggests that the axons of DAT-Cre animals are in a slightly different resting state compared to the DA axons of WT animals – potentially due to the lower DAT expression – but that the main effect of blocking nAChRs on DA axons retains its general characteristics. However, the smaller 4p/1p ratio might greatly reduce the working range of FCV experiments in which we comprise the pool of VGSCs and use 4p/1p ratio as read to highlight the disproportionate effect doing so has on pulse-trains stimulations. This flooring effect is particularly striking in DLS where the 4p/1p ratio is close to 1 in DAT-Cre animals, and the spread of the data is not following a normal distribution (D’Agostino & Pearson test for 4p/1p ratio in DLS  $p = 0.0072$ ). We therefore expect that potential effects will be more easily detectable in NAcC compared to DLS.

*ii. In DAT-Cre animals, partially impeding the pool of VGSCs available for re-release using TTX does not disproportionately affect DA release evoked by pulse-trains*

Next, we wondered whether partially compromising the pool of VGSCs using low concentrations of TTX would disproportionately affect release evoked by pulse-trains in DAT-Cre animals like it does in WT animals. We used FCV to monitor  $[DA]_o$  as we washed on increasing TTX concentrations starting at 8 nM TTX and followed by 12 nM TTX.

We found that in DLS,  $[DA]_o$  evoked by single pulse and pulse-train decreases in 8 nM and 12 nM TTX compared to drug-free, although the decrease is not significant for single pulse at 8 nM (**Figure 4.23 A, B** Šídák's multiple comparisons test between Drug-free and TTX, at 8 nM  $p = 0.0955$ , at 12 nM  $p = 0.0326$  and **C** Šídák's multiple comparisons test between Drug-free and TTX, at 8 nM  $p = 0.0223$ , at 12 nM  $p = 0.0046$ ). In NAcC,  $[DA]_o$  evoked by single pulse and 4p 100 Hz was unchanged in 8 and 12 nM TTX compared to the drug-free

condition (**Figure 4.23 E, F** Šídák's multiple comparisons test between Drug-free and TTX, at 8 nM  $p = 0.9806$ , at 12 nM  $p = 0.9873$  and **G** Šídák's multiple comparisons test between Drug-free and TTX, at 8 nM  $p = 0.8036$ , at 12 nM  $p = 0.6869$ ) which was unexpected. In both regions, the 4p/1p ratio did not decrease upon application of 8 nM or 12 nM TTX compared to the drug-free condition (**Figure 4.23 in DLS D** Šídák's multiple comparisons test between Drug-free and TTX, at 8 nM  $p = 0.6789$ , at 12 nM  $p = 0.8257$ ; in NAcC **H** Šídák's multiple comparisons test between Drug-free and TTX, at 8 nM  $p = 0.1499$ , at 12 nM  $p = 0.4077$ ).



**Figure 4.23: Partially compromising the pool of VGSCs using low concentrations of TTX does not disproportionately attenuates release evoked by pulse-train stimulations in DAT-Cre animals.**

**A, E:** Mean ( $\pm$  SEM) [DA]<sub>o</sub> transients in response to 1 pulse electrical stimulation (1p) and 4 pulses at 100 Hz (4p 100 Hz), in drug free condition first, followed by a wash on of TTX at 8 nM and TTX at 12 nM.  $n = 5$  in DLS (**A**) and  $n = 5$  in NAcC (**E**).

**B, F:** Peak [DA]<sub>o</sub> evoked by 1p stimulation in DLS (**B**) and in NAcC (**F**). Each dot represents a slice. The grey bar represents the mean. Mixed-effect analysis (in DLS (**B**) drug effect:  $F(0.6009, 2.103) = 15.59$ ,  $p = 0.3005$ ; in NAcC (**F**) drug effect:  $F(0.5193, 1.817) = 0.2340$ ,  $p = 0.2596$ ) with Šídák's multiple comparisons test.

**C, G:** Peak [DA]<sub>o</sub> evoked by 4p 100 Hz stimulation in DLS (**C**) and in NAcC (**G**). Each dot represents a slice. The grey bar represents the mean. Mixed-effect analysis (in DLS (**C**) drug effect:  $F(0.3820, 1.337) = 42.76$ ,  $p = 0.1910$ ; in NAcC (**G**) drug effect:  $F(0.6409, 2.243) = 1.214$ ,  $p = 0.3205$ ) with Šídák's multiple comparisons test.

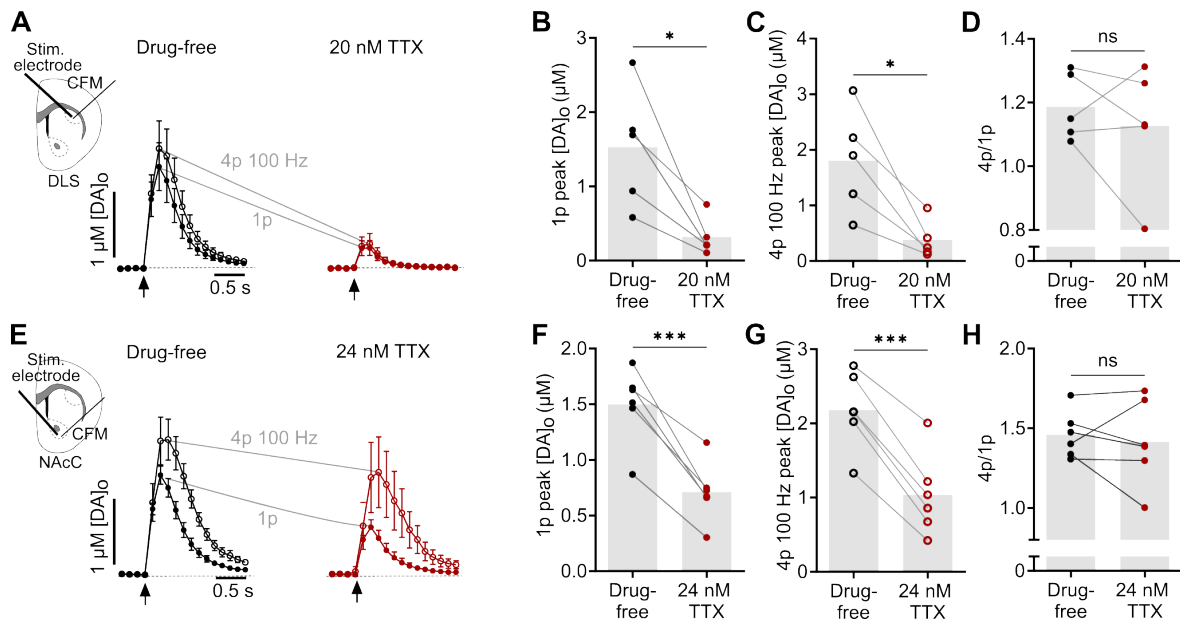
**D, H:** Ratio of peak [DA]<sub>o</sub> in response to 4p 100 Hz and peak [DA]<sub>o</sub> in response 1p in DLS (**D**) and in NAcC (**H**). Each dot represents a slice. The grey bar represents the mean. Mixed-effect analysis (in DLS (**D**) drug effect:  $F(0.7893, 2.763) = 0.4114$ ,  $p = 0.3947$ ; in NAcC (**H**) drug effect:  $F(0.9680, 3.388) = 2.151$ ,  $p = 0.4840$ ) with Šídák's multiple comparisons test.

Therefore, in DAT-Cre animals, partially blocking VGSCs using low concentrations of TTX does not affect the 4p/1p ratio the same way it does for WT animals. This could be due to the fact that their membrane is in a slightly different resting state as shown by comparing  $[DA]_o$  between DAT-Cre and WT animals in drug-free condition (**Figure 4.22**). Thus, these TTX concentrations (8 and 12 nM) might not be enough to compromise the pool of VGSCs in DAT-Cre animals. The lower 4p/1p ratio observed in DAT-Cre animals compared to WT animals could suggest that the pool of VGSCs is already more compromised than it is in WT, however it does not seem to be the case here especially in NAcC where 12 nM TTX barely reduced release at all.

Thus, we hypothesised that DA axons of DAT-Cre animals may be less prone to be affected very low TTX concentrations because of the potentially different state of their membrane, and that they might therefore require higher TTX concentrations. We tested sequentially higher concentrations of TTX to assess if we could compromise VGSC availability enough to limit release evoked by pulse-trains more than release evoked by single pulse. The data we present next was obtained from the highest TTX concentrations we used and for which the evoked DA was still reliably detectable.

In DLS, although we found that upon 20 nM TTX application,  $[DA]_o$  for both single pulse and train pulses plummeted compared to drug-free levels (**Figure 4.24 A, B and C**), the 4p/1p ratio remained unchanged (**Figure 4.24 D**).

In NAcC, application of 24 nM of TTX reduced  $[DA]_o$  for both single pulse and train pulses compared to drug-free levels (**Figure 4.24 E, F and G**), contrary to application of 8 nM or 12 nM TTX (**Figure 4.23 D, E and G**). However, it did not affect the 4p/1p ratio (**Figure 4.24 H**).



**Figure 4.24: Partially compromising the pool of VGSCs using 20 nM and 24 nM does not differently attenuate release evoked by single or pulse-train stimulations in DAT-Cre animals.**

**A, E:** Mean ( $\pm$  SEM) [DA]<sub>o</sub> transients in response to 1 pulse electrical stimulation (1p) and 4 pulses at 100 Hz (4p 100 Hz), in drug free condition first, followed by a wash on of TTX at 20 nM in DLS (**A**) or TTX at 24 nM in NAcC (**E**).  $n = 5$  in DLS (**A**) and  $n = 6$  in NAcC (**E**).

**B, F:** Peak [DA]<sub>o</sub> evoked by 1p stimulation in DLS (**B**) and in NAcC (**F**). The grey bar represents the mean. Paired t-test, in DLS  $p = 0.0230$ ; in NAcC  $p = 0.0006$ .

**C, G:** Peak [DA]<sub>o</sub> evoked by 4p 100 Hz stimulation in DLS (**C**) and in NAcC (**G**). The grey bar represents the mean. Paired t-test, in DLS  $p = 0.0171$ ; in NAcC  $p = 0.0001$ .

**D, H:** Ratio of peak [DA]<sub>o</sub> in response to 4p 100 Hz and peak [DA]<sub>o</sub> in response 1p in DLS (**D**) and in NAcC (**H**). The grey bar represents the mean. Paired t-test, in DLS  $p = 0.4666$ ; in NAcC  $p = 0.6127$ .

Thus, the use of low concentrations of TTX (8 – 24 nM) does not appear to affect the 4p/1p ratio in DAT-Cre animals. Using of low concentration of TTX partially to compromise the pool of available VGSCs when nAChRs are active does not achieve the same effect as in WT animals, *ie.* does not reduced release evoked by pulse-trains more than release evoked by single pulse. This could potentially be due to a larger pool of VGSCs available when nAChRs in DAT-Cre than in WT. In consequence, the concentration of TTX needed to reach the tipping point where the pool is compromised enough to affect release at pulse-trains more than single pulse would be higher. However, at higher TTX concentrations release reaches undetectable levels with FCV. Thus, this experiment might require a more targeted

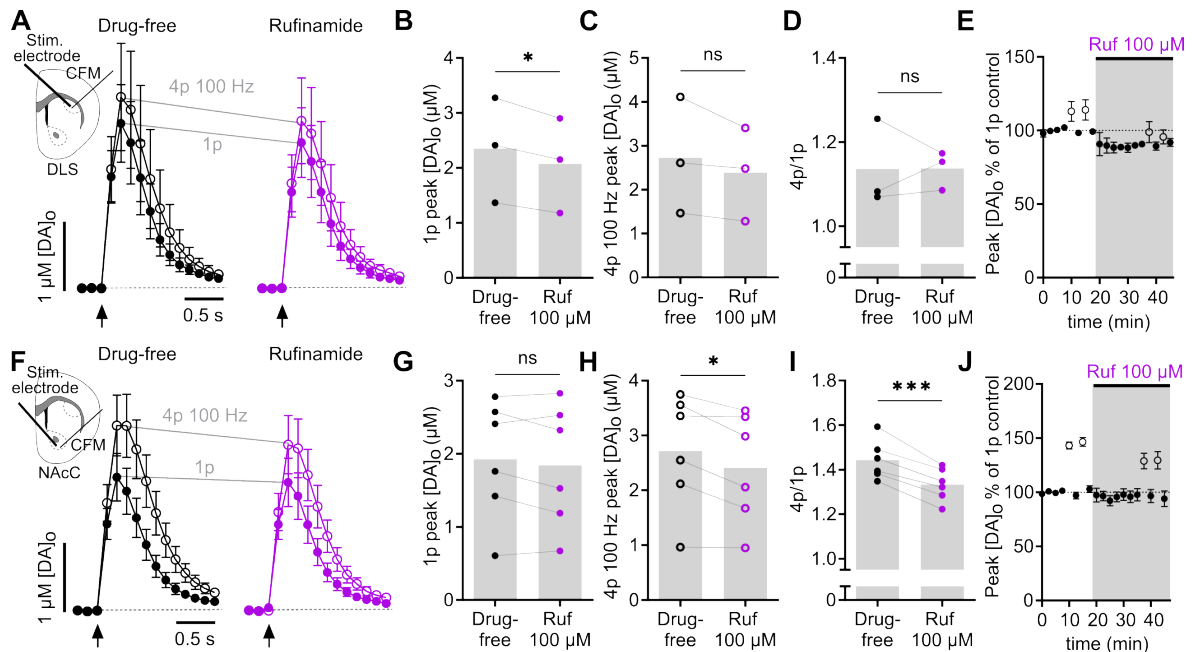
drug – such as rufinamide – that focuses on compromising the pool VGSCs after a pulse-train rather than lowering release overall like TTX being a broad VGSC blocker.

*iii. In DAT-Cre animals, prolonging inactivation of VGSCs only affects the 4p/1p ratio in NAcC*

We tested whether rufinamide (100  $\mu$ M) would be more suited to reduce the pool of VGSCs available during pulse-trains. Since rufinamide binds to the inactivated state of the VGSCs, and prolonged their inactive state (Hille 2001; Deeks and Scott 2006; Arroyo 2007; Humayun and Wadhwa 2022), this drug is likely to have a more specific and targeted effect on DA release than TTX has.

We piloted experiments in DLS, where rufinamide reduces  $[DA]_o$  evoked by single pulse (**Figure 4.25 A, B and E**) compared to drug-free condition. Although  $[DA]_o$  evoked by pulse-trains tends to decrease with rufinamide wash on compared to drug-free condition, it is not a statistically significant reduction (**Figure 4.25 A, C and E**). The 4p/1p ratio was unchanged by the bath application of rufinamide in DLS (**Figure 4.25 D**).

On the contrary in NAcC,  $[DA]_o$  evoked by single pulse was unchanged compared to drug-free condition (**Figure 4.25 F, G and J**) but  $[DA]_o$  evoked by pulse-trains was statistically significantly reduced by bath application of rufinamide (**Figure 4.25 F, H and J**). As a result, the 4p/1p ratio was also reduced in NAcC (**Figure 4.25 I**).



**Figure 4.25: Prolonging inactivation of VGSCs using rufinamide limits DA release evoked by pulse-trains when nAChRs are active only in NAcC in DAT-Cre animals.**

**A, F:** Mean ( $\pm$  SEM) [DA]<sub>o</sub> transients in response to 1 pulse electrical stimulation (1p) and 4 pulses at 100 Hz (4p 100 Hz), in drug-free aCSF first, and after bath application of rufinamide (100  $\mu\text{M}$ , Ruf).  $n = 3$  in DLS (**A**) and  $n = 6$  in NAcC (**F**).

**B, G:** Peak [DA]<sub>o</sub> evoked by 1p stimulation in DLS (**B**) and in NAcC (**G**). The grey bar represents the mean. Paired t-test, in DLS  $p = 0.0370$ ; in NAcC  $p = 0.3017$ .

**C, H:** Peak [DA]<sub>o</sub> evoked by 4p 100 Hz stimulation in DLS (**C**) and in NAcC (**H**). The grey bar represents the mean. In DLS Wilcoxon test  $p = 0.2500$ ; in NAcC paired t-test  $p = 0.0270$ .

**D, I:** Ratio of peak [DA]<sub>o</sub> in response to 4p 100 Hz and peak [DA]<sub>o</sub> in response 1p in DLS (**D**) and in NAcC (**I**). The grey bar represents the mean. Paired t-test, in DLS  $p = 0.9785$ ; in NAcC  $p = 0.0006$ .

**E, J:** Mean peak [DA]<sub>o</sub> ( $\pm$  SEM) evoked by 1p (plain circles) or by 4p 100 Hz (empty circles) over time in DLS (**E**) and NAcC (**J**) starting drug-free conditions and followed by a rufinamide (100  $\mu\text{M}$ , ruf) wash on. Data is normalised to the average of the four first [DA]<sub>o</sub> values evoked by 1p shown on the graph. The mean peak [DA]<sub>o</sub> evoked by single pulse in **B** and **G** are averaged from the values flanking the peak [DA]<sub>o</sub> evoked by 4p 100 Hz.

Thus, in DLS prolonging inactivation of VGSCs does not affect release evoked by single pulse or pulse-trains differently as the 4p/1p ratio remained constant upon rufinamide bath application. This could be explained the low starting point for 4p/1p ratio in DAT-Cre in drug-free, and the potential flooring effect revealed in **Figure 4.22**, which makes it difficult to decrease the 4p/1p ratio further. Nevertheless, the use of a higher rufinamide concentration could potentially reveal an effect in DLS.



In NAcC, prolonging inactivation of VGSCs did limit release evoked by pulse-trains more strongly than release evoked by single-pulse. This is consistent with our initial hypothesis that VGSC availability is a limiting factor for DA release evoked by pulse-trains.

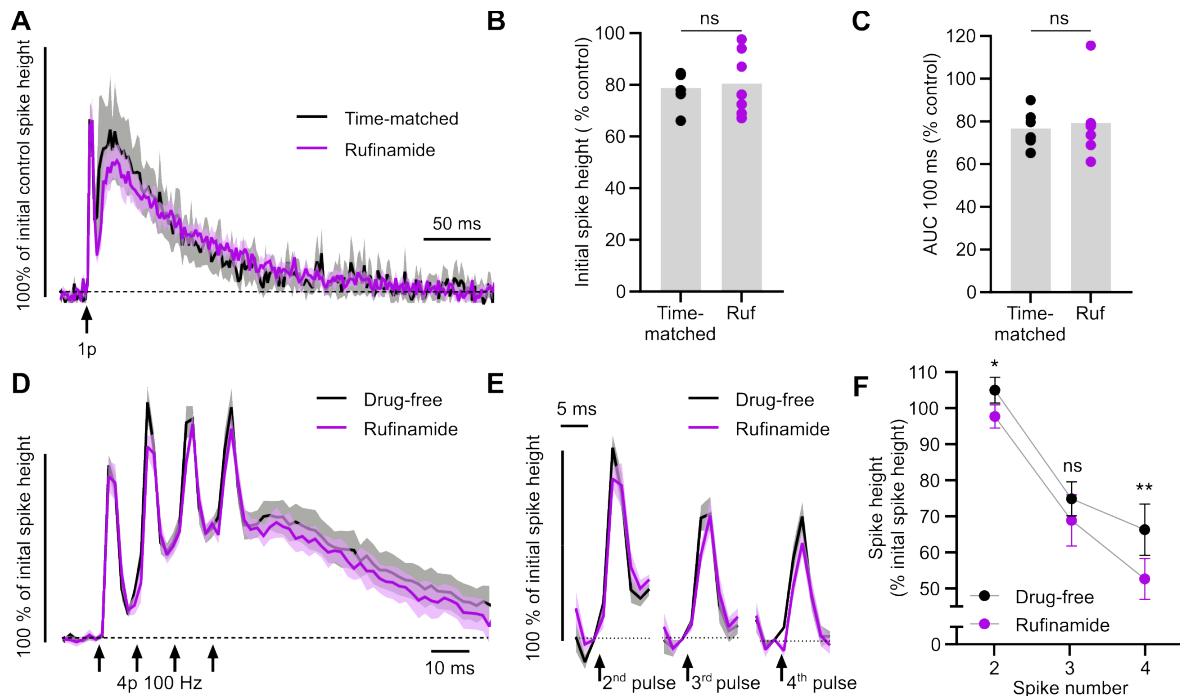
*iv. In NAcC of DAT-Cre animals, prolonging inactivation of VGSCs reduces height of fourth spike during pulse-train*

Next, we assessed if our hypothesis that the population spike height seen with the voltage sensor can dictate DA release. Thus, we hypothesised that the reduction 4p/1p ratio cause by rufinamide (which prolongs inactivation of VGSCs) is due to limited height of population spike. We performed voltage sensor experiments in DAT-Cre animals where we washed on rufinamide (100 $\mu$ M) and focused our recordings in NAcC since rufinamide only reduced DA release evoked by 4p and 4p/1p in this region (**Figure 4.25**).

First, we determined the general effect rufinamide has the signal. We found no differences in the initial spike height in rufinamide compared to the initial spike height of the drug-free time-matched dataset (**Figure 4.26 A and B**). The AUC in the presence of rufinamide was also similar to the AUC of the drug-free time-matched signals evoked by 1p (**Figure 4.26 A and C**). Therefore, in NAcC, rufinamide does not affect the signal evoked by 1p stimulations, consistent with its absence of effect on DA release evoked by 1p in DAT-Cre (**Figure 4.25 F and G**) in DAT-Cre, and in WT animals (**Figure 4.19 F and G**).

Next, assessed whether rufinamide affects the population spikes evoked by subsequent pulses of a pulse-train differently than in drug-free (pre-rufinamide) condition. We found that the population spike height evoked by the second pulse is lower in rufinamide compared to the drug-free condition (**Figure 4.26 D, E and F** Šídák's multiple comparisons test between drug-free and rufinamide, for 2<sup>nd</sup> pulse,  $p = 0.380$ ). The height of the third

population spike has not been affected by the addition of rufinamide (**Figure 4.26 D, E and F** Šídák's multiple comparisons test between drug-free and rufinamide, for 3<sup>rd</sup> pulse,  $p = 0.1037$ ). The fourth population spike height is significantly decreased compared to the drug-free condition (**Figure 4.26 D, E and F** Šídák's multiple comparisons test between drug-free and rufinamide, for 4<sup>th</sup> pulse,  $p = 0.0005$ ).



**Figure 4.26: Prolonging inactivation of VGSCs reduces subsequent population spikes during pulse-train measured with ASAP5f (in NAcC).**

**A:** Mean ( $\pm$  SEM) traces evoked by single-pulse, normalised to their respective control initial spike height in drug-free time-matched condition (black) or in rufinamide 100  $\mu$ M wash on (fuschia). The arrows indicate the time of stimulation.  $n = 6$  for time-matched dataset, and  $n = 7$  for rufinamide wash on.

**B:** Initial spike height normalised to respective control initial spike height. Each dot represents a slice. Grey bars represent the mean. Unpaired t-test,  $p = 0.7734$ .

**C:** AUC taken over 100 ms from normalised to respective AUC control. Each dot represents a slice. Grey bars represent the mean. Unpaired t-test,  $p = 0.7453$ .

**D:** Mean ( $\pm$  SEM) traces evoked by 4p 100 Hz electrical stimulation, normalised to their respective initial spike in drug-free (pre-rufinamide) condition (black) and after rufinamide 100  $\mu$ M wash on (fuschia). The arrows indicate the time of stimulation.  $n = 7$ .

**E:** Traces of spikes evoked by 2<sup>nd</sup>, 3<sup>rd</sup> and 4<sup>th</sup> electrical pulse from the pulse-train. Mean ( $\pm$  SEM) normalised to their respective initial spike in drug-free (pre-rufinamide) condition (black) and after rufinamide 100  $\mu$ M wash on (fuschia). The arrows indicate the time of stimulation.  $n = 7$ .

**F:** Mean ( $\pm$  SEM) of population spike height normalised to respective initial spike height. Two-way ANOVA drug effect  $F(1, 6) = 7.803$ ,  $p = 0.0314$ ; pulse effect  $F(2, 12) = 81.54$ ,  $p < 0.0001$ ; interaction  $F(2, 12) = 2.649$ ,  $p = 0.1114$  with Šídák's multiple comparisons test.

Prolonging the inactivation of VGSCs seem to lower spike-height during pulse trains, and especially the spike height of the last spike. This could be due to the fact that opening of nAChRs likely inactivates some VGSCs by raising the membrane voltage and each electric pulse recruits some VGSCs which then become inactive, therefore, by the fourth pulse, rufinamide has had the more VGSCs to bind to (since more are now inactivated). Altogether, this data suggests supports our hypothesis that population spike height is limited by the pool of available VGSCs and govern DA release. Indeed, the smaller spike height in rufinamide compared to control condition suggest that the pool of available VGSCs is indeed very limited. Plus, this lower population spike height is consistent with the decrease in DA release evoked by pulse-trains (seen in both DAT-Cre and WT animals) upon bath application of rufinamide. However, for more precision on the spike height, using the subtraction technique shown in **Figure 4.12** might be more adapted.

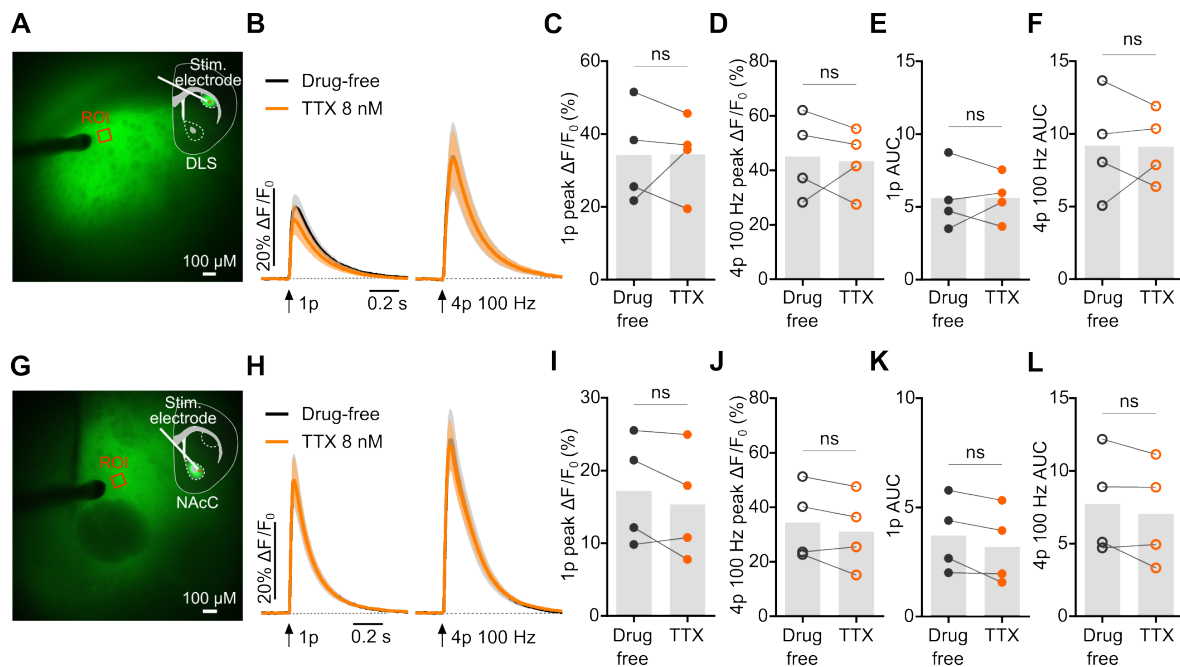
Overall, these data support our hypothesis that at a population of DA axons, nAChRs activation causes a prolonged depolarisation, which inactivates VGSCs or renders VGSCs unavailable to respond to further stimulation. In consequences, smaller population spikes are elicited following subsequent stimulation during pulse-trains, plus, they mirror the lower DA release evoked by subsequent stimulation during pulse-trains

#### 4.3.4. VGSC blockers are not acting through differential impact on ACh release

Electrical stimulations activate all neurons and brain cells surrounding the stimulating electrode. In addition, drug application affects all circuits within the striatum. Since our interest lies in investigating the mechanisms downstream of activation of nAChRs at DA axons, varying ACh release levels could modify their occupancy levels and therefore their effect on DA release.

Thus, we tested whether the manipulation done in section 4.3.3. using VGSC blockers can affect ACh release, using the GRAB<sub>ACh3.0</sub> sensor (Jing et al. 2020) to detect extracellular levels of ACh (**Figure 4.27 A and G**). The virus containing the sensor (AAV5-hSyn-ACh3.0) was injected directly in the striatum for WT (C57JBL6) for expression in all neurons.

We first tested the effect of low concentration of TTX on ACh release. We used the lowest TTX concentration (8 nM) that affect the 4p/1p ratio of DA release when nAChRs are active. If the reduction of the 4p/1p DA ratio seen with FCV in TTX was due to variations in ACh release, we might expect it to signify an increase in ACh release, since the 4p/1p ratio is lower where [ACh] is higher. However, this effect seems unlikely given that blocking VGSCs is normally expected to decrease neurotransmitter release. In order to understand whether the effects of low concentrations of TTX involve confounding effects on ACh release, it was important to establish assess if and how 8 nM TTX could affect ACh release.



**Figure 4.27: Partially blocking VGSCs using 8 nM TTX does not affect ACh release.**

**A, G:** Example slices with expression of GRAB<sub>ACh3.0</sub> in DLS (**A**) and in NAcC (**G**) with positioning of the stimulation electrode (in block from the left) and where ROI would be positioned during analysis. Pictures taken under a 10x objective.

**B, H:** Mean ( $\pm$  SEM) % $\Delta F/F_0$  transients in response to 1 pulse electrical stimulation (1p) and 4 pulses at 100 Hz (4p 100 Hz) in DLS (**B**) and NAcC (**H**), in drug-free condition (black) and after bath application of 8 nM TTX (orange). The arrow indicates the time of stimulation.  $n = 4$  in DLS and  $n = 4$  in NAcC.

**C, I:** Peak % $\Delta F/F_0$  evoked by 1p stimulation in DLS (**C**) and NAcC (**I**). Grey bars indicate the mean. Paired t-test, in DLS  $p = 0.9726$ ; in NAcC  $p = 0.2302$ .

**D, J:** Peak % $\Delta F/F_0$  evoked by 4p 100 Hz stimulation in DLS (**D**) and NAcC (**J**). Grey bars indicate the mean. Paired t-test, in DLS  $p = 0.7699$ ; in NAcC  $p = 0.1877$ .

**E, K:** AUC evoked by 1p stimulation in DLS (**E**) and NAcC (**K**). Grey bars indicate the mean. Paired t-test, in DLS  $p = 0.9861$ ; in NAcC  $p = 0.0955$ .

**F, L:** AUC evoked by 4p 100 Hz stimulation in DLS (**F**) and NAcC (**L**). Grey bars indicate the mean. Paired t-test, in DLS  $p = 0.9548$ ; in NAcC  $p = 0.2525$ .

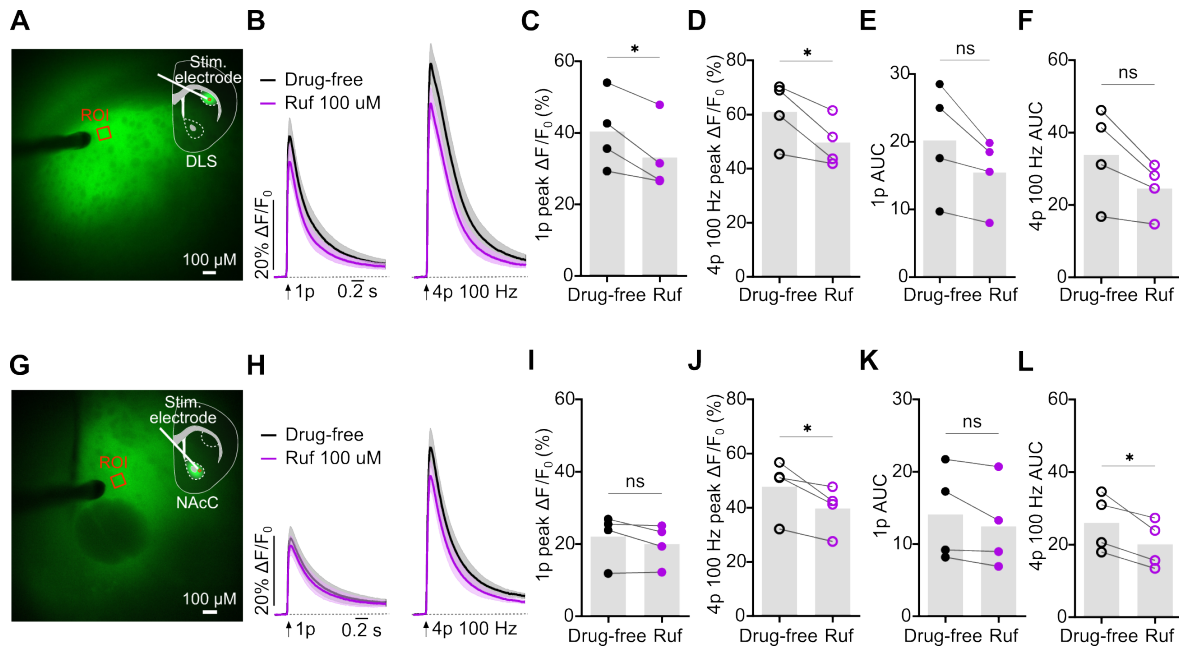
In both regions, blocking few VGSCs using 8 nM TTX did not change the peak  $\Delta F/F_0$  evoked by 1p in both regions (**Figure 4.27 B, C, H and I**) from the drug-free condition. 8 nM TTX wash on did not affect the peak  $\Delta F/F_0$  evoked by 4p 100 Hz in either region (**Figure 4.27 B, D, H and J**), compared to the drug-free condition. However, peak  $\Delta F/F_0$  might not always reflect smaller changes in ACh release levels; studies from the lab (Vietti-Michelina 2022) have found that AUC might be more reflective of finer changes in external ACh concentrations. Hence, we also used the AUC of the transients as an approximation for changes in extracellular ACh concentration. In both regions, blocking few VGSCs using 8 nM

TTX did not affect the AUC of ACh transients evoked by 1p stimulation (**Figure 4.27 B, E, H and K**), and by 4p 100 Hz stimulation (**Figure 4.27 B, F, H and L**).

Therefore, variation in [ACh] was not a confounding effect in our FCV experiments using low concentrations of TTX, since 8 nM TTX did not affect ACh release.

Next, we assessed whether ACh release was affected by rufinamide wash on. Similarly to the rationale for low TTX concentration, if the reduction of the 4p/1p DA ratio seen with FCV in rufinamide was due to variations in ACh release, we might expect it to signify an increase in ACh release since a decrease in ACh would increase 4p/1p ratio. However, similarly to TTX, ACh release is more likely to decrease upon rufinamide application, therefore suggesting that the drop in 4p/1p ratio of DA release could *not* be caused by variations in ACh release.

Prolonging the inactivation of VGSCs using rufinamide decreased the peak  $\Delta F/F_0$  evoked by 1p from the drug-free condition in DLS (**Figure 4.28 B and C**) but not in NAcC (**Figure 4.28 H and I**). Rufinamide wash on decreased the peak  $\Delta F/F_0$  evoked by 4p 100 Hz in both region (**Figure 4.28 B, D, H and J**) compared to the drug-free condition. Additionally, in DLS, prolonging VGSCs inactivation shows a trend towards a decrease the AUC of ACh transients evoked by 1p stimulation, but did not change AUC in NAcC (**Figure 4.28 B, E, H and K**). For AUC of transients evoked by 4p 100 Hz stimulation although there is a trend towards decrease in DLS (**Figure 4.28 B and F**), the decrease is only statistically significant in NAcC (**Figure 4.28 H and L**).



**Figure 4.28: Prolonging inactivation state of VGSCs using rufinamide reduces ACh release mainly evoked by pulse-train stimulations.**

**A, G:** Example slices with expression of GRAB<sub>ACh3.0</sub> in DLS (**A**) and in NAcC (**G**) with positioning of the stimulation electrode (in block from the left) and where ROI would be positioned during analysis. Pictures taken under a 10x objective.

**B, H:** Mean ( $\pm$  SEM)  $\Delta F/F_0$  transients in response to 1 pulse electrical stimulation (1p) and 4 pulses at 100 Hz (4p 100 Hz) in DLS (**B**) and NAcC (**H**), in drug-free condition (black) and after bath application of 100  $\mu$ M rufinamide (Ruf, in fuchsia). The arrow indicates the time of stimulation.  $n = 4$  in DLS and  $n = 4$  in NAcC.

**C, I:** Peak  $\Delta F/F_0$  evoked by 1p stimulation in DLS (**C**) and NAcC (**I**). Grey bars indicate the mean. Paired t-test, in DLS  $p = 0.0274$ ; in NAcC  $p = 0.1778$ .

**D, J:** Peak  $\Delta F/F_0$  evoked by 4p 100 Hz stimulation in DLS (**D**) and NAcC (**J**). Grey bars indicate the mean. Paired t-test, in DLS  $p = 0.0387$ ; in NAcC  $p = 0.0498$ .

**E, K:** AUC evoked by 1p stimulation in DLS (**E**) and NAcC (**K**). Grey bars indicate the mean. Paired t-test, in DLS  $p = 0.0712$ ; in NAcC  $p = 0.1426$ .

**F, L:** AUC evoked by 4p 100 Hz stimulation in DLS (**F**) and NAcC (**L**). Grey bars indicate the mean. Paired t-test, in DLS  $p = 0.0541$ ; in NAcC  $p = 0.0344$ .

Since [ACh] was reduced by rufinamide wash on, the expected effect on DA release would be an increase in 4p/1p ratio, however our FCV experiments revealed a *decrease* in 4p/1p ratio upon rufinamide application. Therefore, the changes in ACh release related here may reduce the amplitude effect seen in FCV, but they do not underlie the reduction in 4p/1p ratio of DA release.

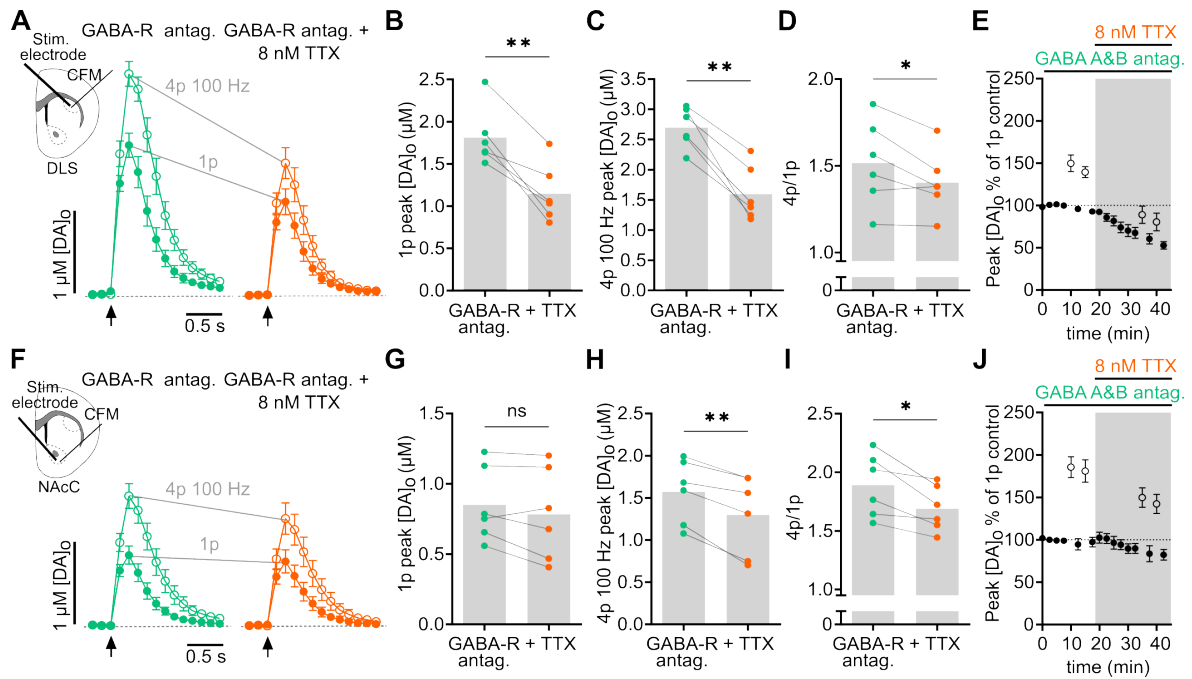
Altogether, these data indicate that changes in ACh release cause by blocking or prolonging inactivation of VGSCs do not underlie the reduction in 4p/1p of DA release since a reduction in ACh release would have opposite effect on 4p/1p of DA release.

#### 4.3.5. VGSC blockers affect DA release independently of GABA receptor control

Confounding effects from electrical stimulation of brain slices include activation of all neurones surrounding the stimulating electrode, including the GABAergic MSNs. GABA, through GABA<sub>A</sub> receptors and GABA<sub>B</sub> receptors located on DA axons, tonically inhibits DA in slices (Kramer et al. 2020). Using TTX or rufinamide (in **section 4.3.3**) to modify the excitability of DA axons may have also changed the excitability of MSNs and GABA release. Therefore, we tested (in WT animals) whether the reduction in 4p/1p ratio seen in **section 4.3.3.1**, when VGSC availability is compromised and nAChRs are active, are indirect effects of GABA on DA release. If the reduction in 4p/1p is due to an indirect effect of GABA through GABA receptors on DA release, therefore blocking the GABA receptors would negate the effects of TTX or rufinamide on DA release. To control for this, we first pharmacology antagonised GABA<sub>A</sub> receptors and GABA<sub>B</sub> receptors and washed on 8 nM TTX to partially inhibit VGSCs.

In DLS, we found that additionally blocking 8 nM TTX wash on when GABA receptors already are antagonised lessen  $[DA]_o$  evoked by both single stimulation (**Figure 4.29 A, B and E**) and pulse train (**Figure 4.29 A, C and E**) compared to when only the GABA receptors are blocked. In NAcC, only release evoked by train pulses has been reduced by additional wash on of TTX (**Figure 4.29 F, H and J**) compared to when only GABA receptors are blocked, but release evoked by single pulse stimulation remains unchanged (**Figure 4.29 F, G and J**).





**Figure 4.29: Partially compromising the pool of VGSCs using 8 nM TTX while GABA<sub>A</sub>-R and GABA<sub>B</sub>-R are antagonised still disproportionately affect release evoked pulse-train stimulations.**

**A, F:** Mean ( $\pm$  SEM) [DA]<sub>o</sub> transients in response to 1 pulse electrical stimulation (1p) and 4 pulses at 100 Hz (4p 100 Hz), initially in GABA-R antagonists (GABA<sub>A</sub>-R antagonist = (+)-bicuculline (10  $\mu$ M), GABA<sub>B</sub>-R antagonist = CGP 55845, HCl (4  $\mu$ M)), followed by a wash on of TTX at 8 nM still in the presence of GABA-R antagonists. Arrows indicate time of stimulation.  $n = 6$  in DLS (**A**) and  $n = 6$  in NAcC (**F**).

**B, G:** Peak [DA]<sub>o</sub> evoked by 1p stimulation in DLS (**B**) and in NAcC (**G**). Each dot corresponds to a slice. The grey bar represents the mean. Paired t-test, in DLS (**B**)  $p = 0.0017$ ; in NAcC (**G**),  $p = 0.1503$ .

**C, H:** Peak [DA]<sub>o</sub> evoked by 4p 100 Hz stimulation in DLS (**C**) and in NAcC (**H**). Each dot corresponds to a slice. The grey bar represents the mean. Paired t-test, in DLS (**C**)  $p = 0.0011$ ; in NAcC (**H**),  $p = 0.0026$ .

**D, I:** Ratio of peak [DA]<sub>o</sub> in response to 4p 100 Hz and peak [DA]<sub>o</sub> in response to 1p in DLS (**D**) and in NAcC (**I**). Each dot corresponds to a slice. The grey bar represents the mean. Paired t-test, in DLS (**D**)  $p = 0.0429$ ; in NAcC (**I**),  $p = 0.0178$ .

**E, J:** Mean peak [DA]<sub>o</sub> ( $\pm$  SEM) evoked by 1p (filled circles) or by 4p 100 Hz (empty circles) over time in DLS (**E**) and NAcC (**J**) starting in GABA-R antagonists (GABA<sub>A</sub>-R antagonist = (+)-bicuculline (10  $\mu$ M), GABA<sub>B</sub>-R antagonist = CGP 55845, HCl (4  $\mu$ M)), followed by a wash on of TTX at 8 nM still in the presence of GABA-R antagonists. Data is normalised to the average of the four first [DA]<sub>o</sub> values evoked by 1p shown on the graph. The mean peak [DA]<sub>o</sub> evoked by single pulse in **B** and **G** are averaged from the values flanking the peak [DA]<sub>o</sub> evoked by 4p 100 Hz.

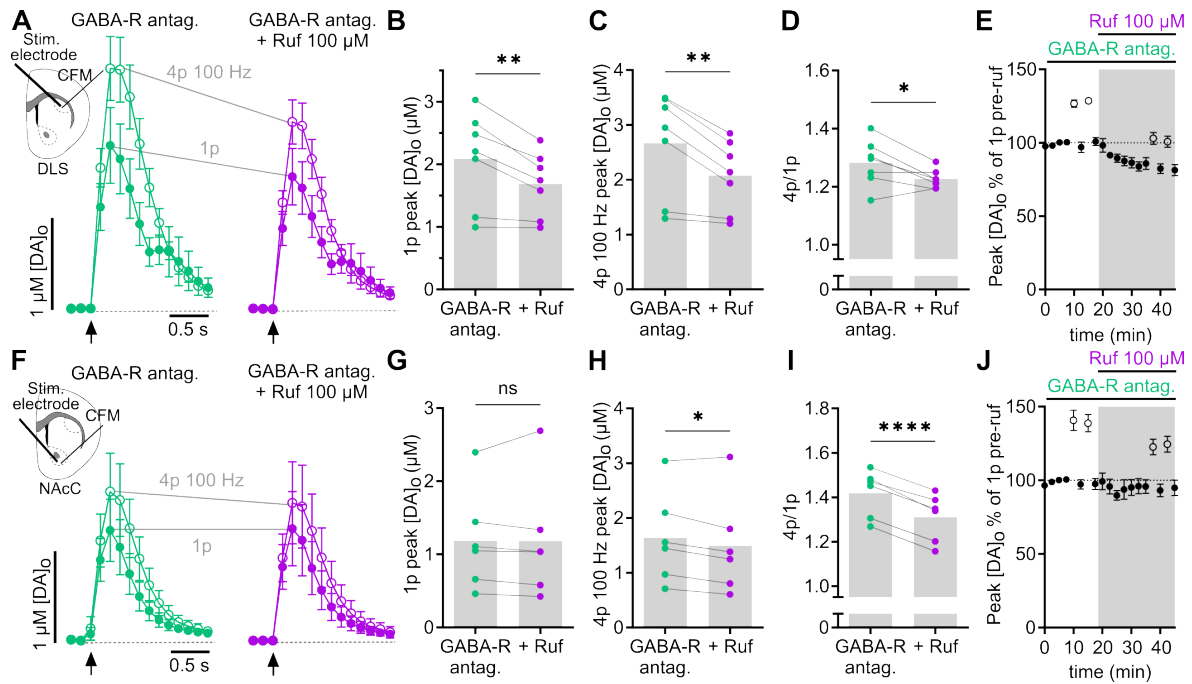
However, in both regions TTX seems to have impaired disproportionately release evoked by pulse trains compared to single pulse even in presence of GABA receptors antagonists, lowering the 4p/1p ratio compared to when only GABA receptors are blocked (**Figure 4.29 D and I**).

Next, we controlled for the potential indirect GABA effect onto DA release when rufinamide is bath-applied on striatal slices. We pharmacologically blocked GABA<sub>A</sub>

receptors and GABA<sub>B</sub> receptors and washed on rufinamide to prolong the inactivation state of VGSCs.

In DLS, additional bath application of rufinamide when GABA receptors already are blocked diminished [DA]<sub>o</sub> evoked by both single stimulation (**Figure 4.30 A, B and E**) and pulse train (**Figure 4.30 A, C and E**) compared to when only the GABA receptors are blocked. In NAcC, release evoked by train pulses was reduced by additional application of rufinamide (**Figure 4.30 F, H and J**) compared to when only GABA receptors are blocked. However, prolonging VGSC inactivation using rufinamide when GABA receptors are blocked did not lower [DA]<sub>o</sub> evoked by single pulse stimulation (**Figure 4.30 F, G and J**) compared to when only GABA receptors are blocked. Finally, akin to TTX bath application, prolonging inactivation of VGSCs when GABA receptors are already blocked lessened [DA]<sub>o</sub> evoked by pulse trains more than [DA]<sub>o</sub> evoked single pulse even in presence of GABA receptors antagonists, lowering the 4p/1p ratio compared to when only GABA receptors are blocked (**Figure 4.30 D and I**).

This data suggests that the drop in 4p/1p ratio caused by TTX or rufinamide in **section 4.3.3.2** are not due to confounding effects of potential changes in GABA release and GABA receptors are indirectly affecting DA release.



**Figure 4.30: Prolonging the inactivation state of VGSCs using rufinamide while GABA<sub>A</sub>-R and GABA<sub>B</sub>-R are antagonised still disproportionally affect release evoked pulse-train stimulations.**

**A, E:** Mean ( $\pm$  SEM) [DA]<sub>o</sub> transients in response to 1 pulse electrical stimulation (1p) and 4 pulses at 100 Hz (4p 100 Hz), initially in GABA-R antagonists (GABA<sub>A</sub>-R antagonist = (+)-bicuculline (10  $\mu$ M), GABA<sub>B</sub>-R antagonist = CGP 55845, HCl (4  $\mu$ M)), followed by a wash on of rufinamide (Ruf, 100  $\mu$ M) still in the presence of GABA-R antagonists. Arrows indicate time of stimulation.  $n = 7$  in DLS (**A**) and  $n = 6$  in NAcC (**E**).

**B, G:** Peak [DA]<sub>o</sub> evoked by 1p stimulation in DLS (**B**) and in NAcC (**G**). Each dot corresponds to a slice. The grey bar represents the mean. Paired t-test, in DLS (**B**)  $p = 0.0079$ ; in NAcC (**G**),  $p = 0.9602$ .

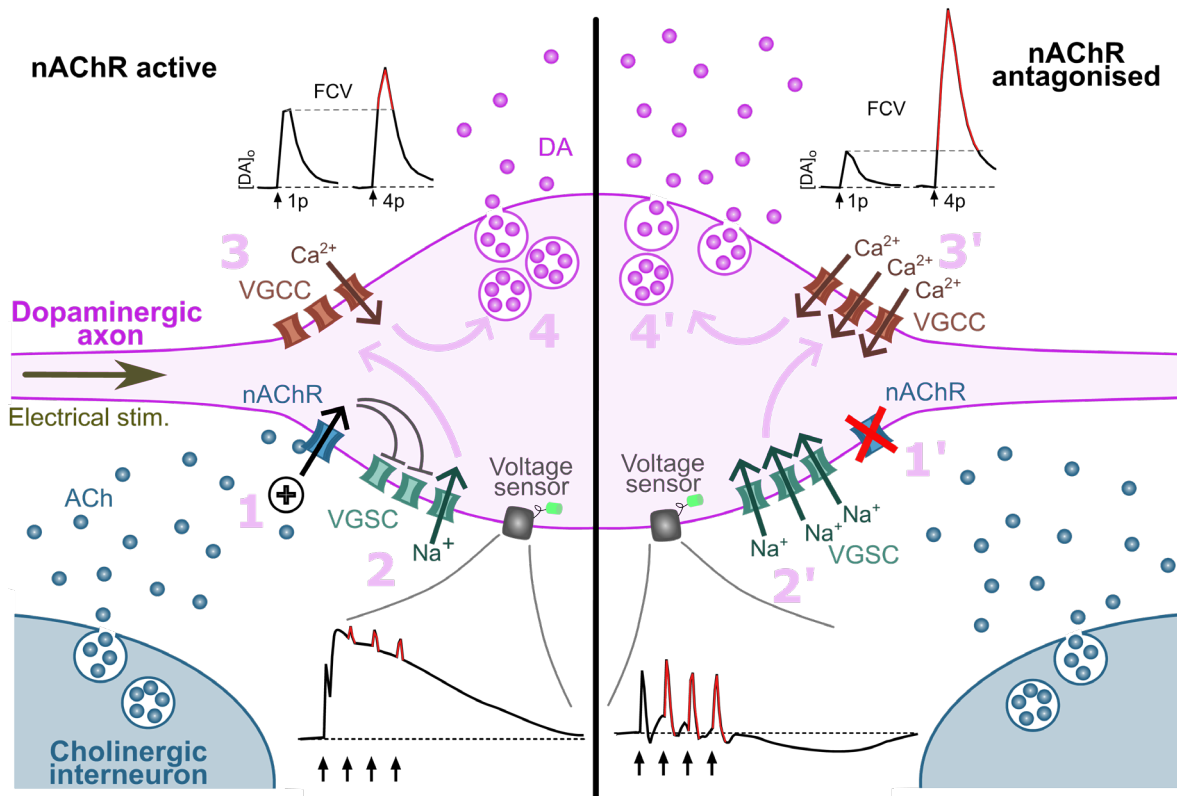
**C, H:** Peak [DA]<sub>o</sub> evoked by 4p 100 Hz stimulation in DLS (**C**) and in NAcC (**H**). Each dot corresponds to a slice. The grey bar represents the mean. Paired t-test, in DLS (**C**)  $p = 0.0034$ ; in NAcC (**H**),  $p = 0.0359$ .

**D, I:** Ratio of peak [DA]<sub>o</sub> in response to 4p 100 Hz and peak [DA]<sub>o</sub> in response 1p in DLS (**D**) and in NAcC (**I**). Each dot corresponds to a slice. The grey bar represents the mean. Paired t-test, in DLS (**D**)  $p = 0.0470$ ; in NAcC (**I**),  $p < 0.0001$ .

**E, J:** Mean peak [DA]<sub>o</sub> ( $\pm$  SEM) evoked by 1p (plain circles) or by 4p 100 Hz (empty circles) over time in DLS (**E**) and NAcC (**J**) starting in GABA-R antagonists (GABA<sub>A</sub>-R antagonist = (+)-bicuculline (10  $\mu$ M), GABA<sub>B</sub>-R antagonist = CGP 55845, HCl (4  $\mu$ M)), followed by a wash on of rufinamide (Ruf, 100  $\mu$ M) still in the presence of GABA-R antagonists. Data is normalised to the average of the four first [DA]<sub>o</sub> values evoked by 1p shown on the graph. The mean peak [DA]<sub>o</sub> evoked by single pulse in **B** and **G** are averaged from the values flanking the peak [DA]<sub>o</sub> evoked by 4p 100 Hz.

## 4.4. Conclusion and discussion

In this chapter, we investigated the role of VGSCs in the downstream mechanisms of the nAChR modulation of DA release. Using the new GEVI ASAP5f (Duquenoy and O'Connor 2024; Hao et al. 2024) to record membrane voltage variations in a population of DA axons, we found that nAChR activation provokes a **prolonged depolarisation at DA axons**, likely to arise from the cation conductances carried by the nAChRs in their open state (**Figure 4.31**). We also found when nAChRs are active, **the amplitude of subsequent population spikes is limited** during pulse-trains. Next, ultra-fast jRCaMP8m imaging allowed us to show that nAChR activation elicits a strong  $\text{Ca}^{2+}$  transient, but that any subsequent electrical pulses only elicit very limited additional  $\text{Ca}^{2+}$  signals. Finally, using FCV, we found that the **limited VGSC availability following nAChR activation provoked STD of DA release** during pulse trains. Finally, using a combination of GRAB<sub>ACh</sub> and FCV we ruled out the involvement of GABA in mediating these nAChR-gated mechanisms.



**Figure 4.31: Schematic of sequence of events after initial pulse during pulse trains when nAChRs are active (left) and when they are antagonised (right).**

This schematic represents what our data suggests happens at a *population* of DA axons (here represented as a single axon for clarity) after the initial electrical pulse of a pulse train in *ex vivo* striatal mouse brain slices. In this context, electrical stimulation provokes highly synchronous action potential firing in neurons in the vicinity of the stimulating electrode and in particular co-activates dopamine neuron axons and cholinergic interneurons.

On the **left**, when nAChRs are active: **(1)** ACh binds to nAChRs on DA axons, which triggers a cation entry in the axon and the prolonged depolarisation. **(2)** most VGSCs are unavailable due to the depolarisation, and only few VGSCs are able to support further depolarisation triggered by a subsequent pulse, and subsequent population spike height is impaired. **(3)** The limited added depolarisation hinders subsequent  $\text{Ca}^{2+}$  entry. **(4)** The limited  $\text{Ca}^{2+}$  entry only evokes little added release.

On the **right**, when nAChRs are antagonised (or when no ACh is release): **(1')** ACh does not bind to nAChRs on DA axons. **(2')** most VGSCs are available to support further depolarisation triggered by a subsequent pulse, and subsequent population spike height is similar to initial spike height. VGSCs can potentially de-inactivate during hyperpolarisation phases **(3')** The added depolarisation triggers consequent  $\text{Ca}^{2+}$  entry. **(4')** The  $\text{Ca}^{2+}$  entry evokes consequent DA release.

#### 4.4.1. Synchronous population spikes evoked by electrical stimulation

ASAP5f, when imaged from single cell bodies, can reliably report single APs, subthreshold events and even be calibrated to correlate mV and fluorescence changes. In the present study, ASAP5f is imaged from a *population* of striatal dopaminergic axons, and

although signals provide novel valuable insight into the behaviour of the membrane potential of these axons, they do not allow for direct determination of absolute mV.

We found that the sensor reliably report an apparently synchronous, short-lived population spike evoked by electrical stimulation resembling action potential spikes recorded in individual distal axons (Kramer et al. 2022) in time course. The relatively slow acquisition rate of image capture (600 Hz) compared to the near continuous (kHz) membrane potential recordings with electrophysiological methods precludes accurate quantification of millisecond order events such as AP rise and decay times, or  $dV/dt$  versus  $V$  that can be achieved with patch-clamp recordings. Our temporal resolution for imaging has however been maximised by us and precludes faster sampling without compromising signal amplitude.

#### **4.4.2. The nAChR-mediated prolonged depolarisation at a population of DA axons: occurrence at each axon, or asynchronous population signal?**

Our findings suggest that activity at nAChRs provokes a prolonged depolarisation at a population of DA axons. This prolonged depolarisation could arise from an actual depolarisation at each axon, asynchronous spiking events within the ROI, or both.

Most axons within our ROI could be experiencing a prolonged membrane depolarisation in response to activation of nAChRs. nAChRs are pentameric ligand gated ion-channels which are cation permeable (Alkondon et al. 1999; Corringer et al. 2000; Albuquerque et al. 2009; Dani 2015). Their permeability is non-selective to cations however only 2 to 5 % of their conductance seems supported by  $Ca^{2+}$ , while the rest is vastly attributed to  $Na^{+}$  entry (Vernino et al. 1994; Dani and Mayer 1995). Upon activation nAChRs have been

demonstrated to elicit prolonged depolarisation at neuromuscular junction (Hille 2001), but also in the CNS, for instance at DA and GABAergic neurons present in the VTA (Klink et al. 2001). Pressure application of ACh for 30 ms evoked large inward currents lasting up to 600 ms in DA VTA and SNc neurons (Klink et al. 2001). Plus, in this study, the  $\beta 2$  subunit seemed critical for the initiation of these currents, while the  $\alpha 4$  subunit seem to mediate the length of duration of these large inwards currents. Both of these subunits are present in nAChRs expressed at striatal DA axons in DLS and in NAcC (Exley and Cragg 2008; Exley et al. 2008, 2011, 2012a, 2013). It is therefore plausible that nAChRs on striatal DA axons can elicit a prolonged depolarisation following ACh binding, and that this depolarisation lasts approximately 150 ms on mouse brain slices *ex vivo*. Furthermore activity nAChRs can lead to further membrane depolarization through recruitment of voltage-gated sodium channels (VGSCs) leading activation of voltage-gated  $\text{Ca}^{2+}$  channels and neurotransmitter release ((Albuquerque et al. 2009)). Plus, patch-clamp recordings from dopaminergic axons reveal a rapid and sustained membrane depolarisation following spontaneous ACh release only when nAChRs are not antagonised, suggesting that single axons can indeed be depolarised by nAChR activity (Kramer et al. 2022).

The prolonged depolarisation may also represent asynchronous spiking occurring across the axonal population within our ROI. This hypothesis relies on nAChR reported ability to induce ectopic APs at DA axon (Liu et al. 2022; Kramer et al. 2022). This hypothesis also implies that DA axons receive or summate ACh input throughout the approximately 150 ms during which nAChRs mediate depolarisation, which may not be realistic due to the high AChE efficacy (Nosaka and Wickens 2022).

The prolonged depolarisation observed could also be the result of a compound effect of both of the scenario aforementioned as they are not mutually exclusive. Indeed, nAChRs could evoke prolonged depolarisation at most axons within our ROI and trigger asynchronous APs at axons for which the depolarisation exceeds the spiking threshold.

#### **4.4.3. Amplitude of nAChR-mediated prolonged depolarisation**

We next observed that the amplitude of nAChR-mediated prolonged depolarisation can surpass that of initial AP-like population spike, particularly in DLS. However, it is quite unlikely that the membrane voltage of each axon is indeed experiencing a depolarisation that exceeds the amplitude of an action potential spike. Rather, the larger, prolonged depolarisation could be a compound of multiple factors:

- (1) As discussed before, the prolonged depolarisation observed might at least partly reflect asynchronous spike within the axonal population. Asynchronous spiking could be occurring at a greater level than synchronous initial spiking across the axonal population.
- (2) The sensor might be biased towards exaggerating subthreshold event or longer-duration events integrated over a longer timescale compared to more short-lived events (Hao et al. 2024). Thus, a prolonged depolarisation by just a few mV might be reported as a larger increase in fluorescence than a fast depolarisation.

The larger amplitude of the prolonged depolarisation in DLS could be a compound of both factors mentioned here. Although its absolute amplitude cannot be directly correlated to membrane voltage, its time course and kinetics can be compared within striatal regions to infer potential regional differences.



#### 4.4.4. Regional differences in response to nAChR activation

We found that, although the onset of the nAChR-mediated depolarisation seems to occur at similar time (~3 ms post-stimulation), the onset dynamics of the nAChR-mediated response are different between striatal regions.

The DLS and NAcC are enriched in different nAChR subunits (Exley and Cragg 2008; Exley et al. 2008, 2011, 2012a). Indeed, work on mouse brain slices have shown that in DLS,  $\alpha 4\alpha 5\beta 2$ -nAChRs and  $\alpha 6\beta 2\beta 3$ -nAChRs are the main modulators of DA release. In NAcC,  $\alpha 4\alpha 6\beta 2\beta 3$ -nAChRs seems to be responsible for effects on DA release. Varying subunit composition can greatly influence the kinetics of the nAChRs (Alkondon et al. 1999; Albuquerque et al. 2009; Harpsøe et al. 2013; Dani 2015; Nosaka and Wickens 2022). Varied nAChR densities across regions could also play a key role in these differences. Different localisation of nAChRs at DA axons in relation to cholinergic release sites might also be a source of disparities.

Any regional differences concerning the ChIs and their transmission could be responsible for these disparities at DA axons. The gradient of decreased ChI density along the anterior-posterior axis (Matamales et al. 2016) could impact the amount of ACh release upon electrical stimulation. Regional variations in catabolism of ACh by acetylcholinesterase (AChE) through a difference in the enzyme's efficacy or density could also affect ACh level and in turns affect the activation of nAChRs.

However, these regional differences may as well arise from different composition of the axons themselves, therefore changing their response to nAChR activation. For example, VGSC density might be different in each region. The axons innervating the DLS display more branching than in the NAcC and might therefore be more prone to propagation failure and/or require different types or density of VGSCs to support reliable propagation of APs. Another

potential master regulator of short-term plasticity and DA axonal excitability is the DAT (Ingram et al. 2002; Cragg and Rice 2004; Condon et al. 2019), which is known to be differentially expressed between striatal regions (Block et al. 2015). Indeed, DAT exhibits electrogenic properties, such as generation of a depolarising inward current upon DA uptake (Carvelli et al. 2004). Thus, the DAT could also partially contribute for the post-initial spike depolarisation or to the regionally different after-spike behaviours of the membrane when nAChRs are blocked.

#### **4.4.5. Subsequent population spike during pulse-trains: deciphering actual membrane voltage changes from sensor dynamics**

Our data also revealed that when nAChRs are active, the amplitude of subsequent population spikes is limited during pulse-trains. The limited amplitude of the population spikes is unlikely to reflect such small depolarisation on each individual axon within our ROI. Although spike at those axons could be of slightly smaller amplitude (such as demonstrated upon GABA<sub>A</sub>R agonist application by Kramer et al., 2022) the all-or-none fundamental property of APs prevents its amplitude to be so strongly reduced. It is therefore possible that the non-linearity of the ASAP5f sensor in relation to voltage distort the shape of our signal, and depicts AP which ride on top of a prolonged depolarisation as smaller depolarisation than they are in mV. Indeed, the sensor is mostly linear very hyperpolarised potentials until -20 to -10 mV after which the relation between change in fluorescence and membrane voltage becomes much less steep (Hao et al. 2024).

These smaller population spike could also reflect that only a small proportion of axons is able to create a spike, and that most axons are unable to be further depolarised. This explanation is not mutually exclusive with the previous one, and whenever axons are able to

spike despite the prolonged depolarisation, these spikes might be smaller in amplitude too (Kramer et al. 2020).

Our accompanying GCaMP8m data also revealed limited  $\text{Ca}^{2+}$  transient during pulse-trains when nAChRs are active. This data matches out hypothesis that these smaller population spikes are causing smaller  $\text{Ca}^{2+}$  event resulting in less DA release.

#### **4.4.6. VGSC unavailability is downstream of nAChR-mediated STD of DA release**

This study highlights the role of VGSC downstream of nAChR activation at DA axons. In first instance, our findings show that reduced population spike height occurring when nAChRs are active are likely to be a result of unavailability of VGSCs at DA axons. VGSCs are responsible for the upstroke of action potential (Hodgkin and Katz 1949). They activate and inactivate rapidly, from membrane potential as low as  $-60$  mV (Hodgkin and Huxley 1952c, a, d; Hodgkin 1958) and are de-inactivated by hyperpolarisation (Dodge 1963; Hille 2001). Therefore, during pulse-trains and following nAChR-mediated depolarisation, the pool of available VGSCs is likely to be compromised, as little or no repolarisation has occurred between initial pulse and the subsequent ones.

These effects of nAChRs can be mimicked by partial inhibition/activation of VGSCs using three different ligands – including very low TTX concentrations, rufinamide which prolongs inactivation of VGSCs, and veratridine which prolongs the open state of VGSCs) giving support to the notion that nAChR activation does indeed limits VGSC availability. The prolonged depolarisation is however also conditional on the DA axonal membrane not being able to repolarise rapidly. It therefore suggests that after the initial nAChR-mediated depolarisation VGSCs are unable to efficiently and rapidly repolarise the membranes.

Modelling conductances through DA axonal membrane might enable to disentangle the time course and role that each different channel type has to play in this process.

Finally, the exact type of VGSCs expressed at DA axons is not known. In DA neurons from the midbrain, TRAP data from SpatialBrain (Kilfeather et al. 2024) revealed a relative enrichment in the SCN2B and SNC3B genes coding for  $\beta$ -regulatory subunits of VGSCs, and in SNC3a coding for the intrinsic  $\alpha 3$  subunit (Na<sub>v</sub>1.3). Mutations in the former ( $\beta$ -subunits) are known to be involved cardiac atrial fibrillation (Lin et al. 2022a), and the latter ( $\alpha$ -subunit) is involved in epilepsy (Liao et al. 2022). However, no information on whether these are located at the axonal level. Plus, since the structures of VGSCs is largely conserved within this family of channels, pharmacological agents allowing discrimination between channel types and subunits is extremely limited. Finer dissection of the mechanisms downstream of nAChRs activation would therefore require better access to genes for instance.

In order to circumvent these issues in the mouse brain, we took advantage of the highly conserved and more easily manipulable genes from *Drosophila melanogaster* to investigate the mechanisms downstream of nAChR activation in the next Chapter (**Chapter 5**). Indeed, the fly brain also provides a simpler and well understood circuits with unrivalled mechanistic insight of behavioural relevance. Plus, the *Drosophila* mushroom body offer pertinent ACh-DA interaction within its a centre for olfactory learning, the mushroom body.

Our inter-phylum approach could uncover conserved mechanisms and common function of cholinergic modulation of DA release by linking molecular mechanisms to the behaviourally relevant signals.

# 5

Inter-specie approach:  
Exploring ACh-DA interaction  
in *Drosophila melanogaster*

## 5.1. Introduction

### 5.1.1. Function and anatomy of the fly's centre for associative learning and memory: the mushroom body (MB)

The fly mushroom body (MB) appears to support functions homologous to the striatum. Indeed, similarly to the striatum which has been associated with reward learning (Schultz 1986; Schultz et al. 1997; McFarland et al. 2003; Sellings and Clarke 2003; Ikemoto 2007) motor functions and goal-directed behaviour (Graybiel et al. 1994b; Knowlton et al. 1996; Yin and Knowlton 2006), the MB has been demonstrated to be the seat of associative learning (Tempel et al. 1983; Tully and Quinn 1985; Waddell 2013), memory (Pascual and Pr  at 2001; Folkers et al. 2006; Aso et al. 2010; Waddell 2010), motivation (Krashes et al. 2009; Perisse et al. 2016; Burnett et al. 2016), as well as directing approach or avoidance behaviours. Akin to the striatum, these functions rely heavily on the activity of DA neurons (DANs) which densely innervate the mushroom body.

The MB is comprised of three main cell types (Aso et al. 2014a, b): the presynaptic fields of cholinergic Kenyon Cells (KCs) and dopaminergic neurons (DANs), and the post-synaptic fields of mushroom body output neurons (MBONs).

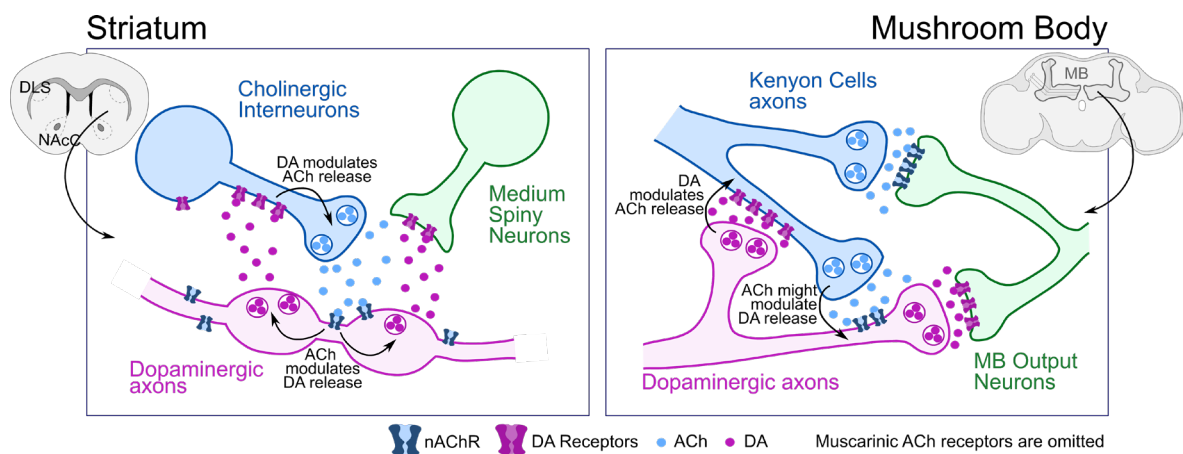
The MB is formed by the parallel axonal projections of 2 000 Kenyon-cells (KCs) that are arranged in L-shaped structures and separated into lobes, the  $\alpha\beta$ ,  $\alpha'\beta'$  and  $\gamma$  lobes. These mainly cholinergic neurons (Aso et al. 2014a) provide the MB with multisensory information (Ito et al. 1998; Vogt et al. 2016; Okray et al. 2023), although they mostly receive olfactory inputs. They are third-order olfactory neurons and relay olfactory information through sparse coding (Turner et al. 2008; Ito et al. 2008; Honegger et al. 2011). A study looking at calcium

dynamics at the KCs cell bodies (Honegger et al. 2011) has suggested that single compound odour activated around 5% of the 2 000 intrinsic KCs, and that upon first encounter with an odour, the activation pattern amongst KCs is random, and that the same neurons will fire again upon second application of the same odour. Associative memories integrating the animal's internal state and context are believed to reside at synapses between KCs and MBONs (Cognigni et al. 2018).

Next, the MBON dendrites tile the MB lobes into 15 distinct compartment (Aso et al. 2014b). These cells are mainly glutamatergic, GABAergic or cholinergic neurons (Aso et al. 2014b). Activity across the MBON network ultimately skews behaviour towards approach or avoidance of learned cues. Artificial activation of a single MBON using optogenetic tools can skewed behaviour towards approach or avoidance of an odour (Aso et al. 2014b). Studies have also highlighted that MBONs can project back to the MB, contra- or ipsi-laterally and in different compartments (Aso et al. 2014a).

Finally, axons of specific and discrete groups of DANs overlap with the specific MBON dendrites and KC axons within each of the 15 MB compartments (Aso et al. 2014b). Some compartments are innervated by as little as the axon of a single DAN (Tanaka et al. 2008; Krashes et al. 2009; Aso et al. 2014a). DANs have extremely dense and branched axons (Aso et al. 2014a; Otto et al. 2020) reminiscent of the axonal arbours of striatal DA neurons (Matsuda et al. 2009). They are crucial in the functioning of the MB as they provide teaching signal, internal state and motivational control to the MB (Mao and Davis 2009; Waddell 2010; Watabe-Uchida and Uchida 2018). During learning, DAN activity is thought to depress the strength of synapses between the cholinergic odour activated KCs and the MBONs dendrite located in the same compartment (Aso et al. 2014a; Perisse et al. 2016).

The architecture of the MB, although overall more anatomically and functionally more segregated, can be compared to that of the striatum in the way that they both rely on DA release to modulate the signal of their output neurons – the MBONs in the MB and the MSNs in the striatum – which ultimately drive behaviour. While the main function of cholinergic KCs in the striatum – which ultimately drive behaviour. While the main function of cholinergic KCs is generally defined as providing sensory information to the MB, the information ChIs encode is still debated (Morris et al. 2004; Schulz and Reynolds 2013; Aoki et al. 2015; Fleming et al. 2022; Bouabid et al. 2025), but could also carry sensory information (Schulz and Reynolds 2013).



**Figure 5.1: Schematic of simplified circuits within the mouse striatum (left) and the *Drosophila* mushroom body (right).**

### 5.1.2. Role of DANs in the MB

In the fly MB, DANs are functionally heterogeneous. Indeed, some DANs – such as those from the Protocerebral Anterior Medial (PAM) cluster code for reward while others, such as those from Protocerebral Anterior Lateral 1 (PPL1) cluster seem to be mainly responsive to aversive stimulus. Within each of these clusters discrete subsets innervating anatomically segregated compartments also code for behaviourally relevant rewarding or aversive signals. For example, sweet taste, nutrient value or water reward are encoded by different identifiable PAM DANs subsets which can differentially trigger the formation of



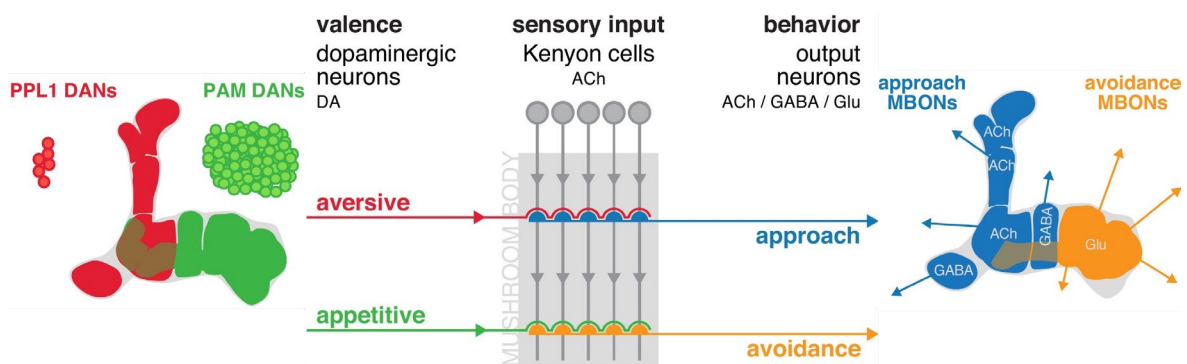
short (STM) or long-term memory (LTM) (Krashes et al. 2009; Burke et al. 2012; Lin et al. 2014; Huetteroth et al. 2015; Felsenberg et al. 2017, 2018; Senapati et al. 2019). Conversely, aversive reinforcement is encoded by PPL1 DANs innervating mostly the vertical compartments and peduncle of the mushroom body (Claridge-Chang et al. 2009; Aso et al. 2010, 2012; Galili et al. 2014; Perisse et al. 2016; Jacob and Waddell 2020; Meschi et al. 2024). These neurons are responsive to signals such as bitter taste, foot shocks and have recently been shown to be involved in memory linked to repetitive-aggression in males aggression (Kim et al. 2018). DANs innervating the mushroom body can also integrate the internal state of the animals, which modulate their activity in order to exhibit the appropriate state-dependent memory. Activity in DANs innervating specific MB compartments have also been shown to correlate with movement (Berry et al. 2015; Cohn et al. 2015; Siju et al. 2020). Altogether DANs integrate a multitude of signals and enable skews the MB network towards expression of the appropriate behaviour.

In brief, axons of DANs providing positive value to a stimulus overlap with the dendrites of MBONs driving aversion, and axons of negatively reinforcing DANs overlap with dendrites of approach-driving MBONs (Aso et al. 2014a) (**Figure 5.2**). Therefore, for the appropriate behaviour to arise, the MB network balances activity such that rewarding DANs depress synapses onto avoidance directing MBONs – ultimately creating ‘less avoidance’ and therefore more approach – and aversive DANs synapses depress odour activation of approach directing MBONs – creating ‘less approach’ and ultimately leading to avoidance (Felsenberg et al. 2017, 2018).

Although these DA mediated-processes are thought to be regulated by phasic or burst-like activity in the appropriate subset of DANs, there have been report of tonic activity in

DANs innervating the MB (Plaçais and Preat 2013; Cohn et al. 2015; Aimon et al. 2019; Siju et al. 2021). A recent suggests that the tonic level of activity in the DAN innervating the  $\gamma$ 1-pedc compartment of the MB signals codes for the modulation in internal state in particular related to hunger (Meschi et al. 2024).

Finally, DANs can modulate the strength of KCs-MBONs synapses through four types of DA receptors known in *Drosophila*. Akin to mammals, these receptors have different kinetics and sensitivity to DA, the two D1-like receptors (Dop1R1 (Dumb) and Dop1R2 (Damb)) have low affinity for DA and respond better to changes in phasic levels, while the D2-like receptor (Dop2R) has a higher affinity and is thought respond better to changes in tonic levels of DA (Siju et al. 2021). In addition, DopEcR (dopamine/ecdyteroid receptor) a third type of DA receptor is also expressed in *Drosophila*. Co-expression of several of these receptors on target cells is common. Single-cell sequencing studies (Croset et al. 2018; Kondo et al. 2020) have demonstrated that two to four DA receptors can be expressed on axons of KCs, each type being involved in different aspect of memory formation and updating (Pavlovsky et al. 2018; Handler et al. 2019)



**Figure 5.2: Schematic of MB connectivity modified from Cognigni et al, 2018.**

### 5.1.3. ACh-DA interactions within the MB

As explained above, DAN-to-KCs synapses have been extensively studied. However, little is known about the reciprocal axo-axonal KCs-to-DANs synapses.

A study revealing the MB connectome in adult *Drosophila* (Takemura et al. 2017) highlights anatomical connections between the KC and DANs at the level of their axons often occurring within tripartite or rosette synapses. Unfortunately, these tripartite synapses are often not accounted for clearly in the downstream/upstream count of synaptic partners (Otto et al. 2020). Plus, the study does not explore the pre-synaptic KC to post-synaptic DAN connections. Another study presenting the connectome data of the larval brain (Eichler et al. 2017) also accounts for some KC-to-DAN connections, however no description of such connections is discussed. Considering both that both of DA and ACh are released by en-passant synapses (Takemura et al. 2017), localisation of physical connections between KCs and DANs might not be possible.

ACh inputs from KCs onto DA axons have been shown to be involved in aversive learning (Cervantes-Sandoval et al. 2017). Cervantes-Sandoval et al., 2017 suggested that nAChRs expressed by DANs play an essential role in aversive learning. Indeed, when preventing the *Drosophila*  $\alpha 1$ ,  $\alpha 4$  and  $\alpha 5$  nAChR subunits translation in specific aversive DANs, flies would show a decreased learning score when trained to pair an odour with an aversive cue. They also showed that silencing KCs dampened the odour response of the  $\alpha 2\alpha'2$  PPL1 (aversive) DANs measured with GCaMP6s, as well as drastically reduced the response to foot shocks provided along with an odour in the same  $\alpha 2\alpha'2$  PPL1 DANs. Surprisingly, response to shocks alone was also dampened by silencing KCs, suggesting that KCs would also be necessary for shock response even when no odour is presented.

Other evidence of cholinergic modulation of DA release in flies was found in the ventral nerve chord (VNC) of *Drosophila* larvae (Pyakurel et al. 2018). This study found that puffing an external solution containing ACh elicits DA release in the VNC, and nAChRs are necessary to elicit release (Pyakurel et al. 2018). This study therefore supports that, in the fly nervous system, nAChRs on DA axons can also promote DA release upon application of ACh or nicotine similarly to what happens at mammalian striatal DA axons (Rice and Cragg 2004; Wang et al. 2014a, a).

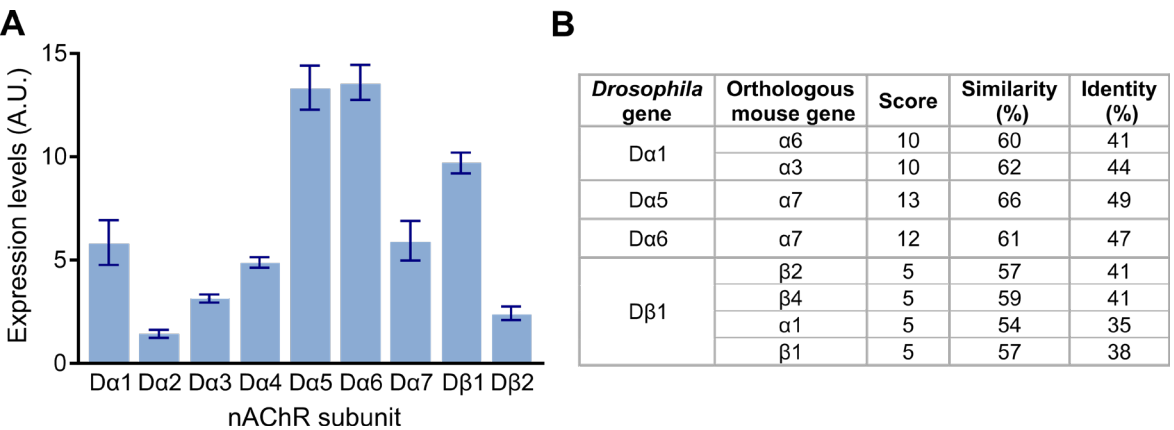
Although few pieces of evidence suggest axo-axonal cholinergic modulation occurs in the fly CNS and in particular in the MB, clear molecular and behavioural link has yet to be established. Plus, the precise release dynamics of each neurotransmitter and their potential interaction remain to be established as release patterns from neither KCs nor DANs have been studied directly in the context of associative learning.

#### **5.1.4. Comparison of nAChR subunit enrichment in DANs within the MB in flies and striatum in mice**

In *Drosophila*, 10 nAChRs subunits have been identified (Dα1-Dα7 and Dβ1-Dβ3) (Schloss et al. 1991, 1992; Schulz et al. 1998, 2000; Chamaon et al. 2000; Jones et al. 2007). This compact family of nAChR genes is however mainly composed by nAChR genes homologous to that of human and mouse nAChRs (Rosenthal and Yuan 2021) with the exception of one Dβ3 subunit (Lansdell and Millar 2002).

To confirm the presence of nAChRs on DANs, as well as explore their subunit composition, I took advantage of an existing unpublished single-cell dataset of the fly brain available in the Waddell lab. I used a pre-annotated dataset (by Dr. C. Treiber) containing

identifiable subsets of DANs innervating the MB. I focussed on extracting the information of nAChR subunits expression from subsets of DANs that are known to code for aversive reinforcement, since literature suggest these are where the reciprocal ACh-DA interaction occur (Cervantes-Sandoval et al. 2017). Interrogating the single cell sequencing dataset revealed that the Da5, Da6 and Dβ1 are the most expressed subunits in these fly DANs, along with Da1, Da7 (**Figure 5.3 A**).



**Figure 5.3 : Identification of nAChR subunits present in aversive DANs innervating the fly MB and their orthologs in the mouse.**

**A:** nAChR subunit expression levels in aversive DANs innervating the *Drosophila* MB. Expression levels for each subunit was averaged over 32 replicates. Average  $\pm$  SEM.

**B:** Table showing the correspondance between *Drosophila* nAChR subunit and the top orthologous genes in mouse (*Mus musculus*) revealed with DIOPT v8.5 using protein alignment. DIOPT combines fifteen different alignment methods, and the score reflects how many of these predicted two genes to be orthologs. The similarity column indicates how similar the amino acid sequences are, taking in account the substitution of amino acids by others with similar properties. The identity reveals how many amino acids are exactly matching between the two aligned sequences

Since *Drosophila* and mouse genes are homologous but their nomenclature is different, I used DIOPT v8.0, an ortholog prediction tool (Hu et al. 2011) to identify what mouse nAChR subunit are the closest the ones enriched in PPL1 aversive DANs in *Drosophila* (**Figure 5.3 B**). I have selected the highest ortholog matches between the two species for each *Drosophila* gene tested (Figure 3 B). The Dβ1 gene shows 57% similarities with both β1 and β2 mouse genes. The Da5 and Da6 seem to both mostly resemble most the murine α7 gene. Finally, the Da1 gene shares 60% similarities with the mouse α6 gene. In the mouse striatum, α4/β2-containing nAChRs prevail (Exley and Cragg 2008; Exley et al. 2008, 2011, 2012b).

Although the subunit composition of nAChRs receptors seems different from the striatum, it is likely that, like in the striatum, nAChRs in *Drosophila* aversive DANs are heteromeric, i.e. comprising both alpha and beta subunits. Nevertheless, little is known about the kinetics of these channels in the context of DA axons, and they could potentially possess similar properties and kinetics across species.

### 5.1.5. Overarching aims of the inter-specie approach

The use of a model with identifiable and behaviourally relevant clusters of dopaminergic neurons (DANs) as well as easy access to gene, such a *Drosophila melanogaster*, could help finding potentially conserved candidate mechanisms for nAChR-mediated modulation of DA release as well as a potential common function for this modulation by linking molecular mechanisms to the behaviourally relevant signals. Furthermore, crosstalk between models allows us to formulate clear hypothesis to be tested in flies based on what has been found in mice while circumventing the more complex striatal mouse circuitry.

In this chapter, I first present the data obtained during the optimisation GRAB sensor imaging in live flies by revealing DA and ACh release patterns in response to odours and foot shock in live *Drosophila* using GRAB<sub>DA2m</sub> and GRAB<sub>ACh4.4</sub> under a 2-photon microscope.

Next, I investigated the impact of ACh release on DA release in live flies, with the aim of uncovering the potentially conserved mechanisms of DA release modulation downstream of nAChR activation on DA axons.

## 5.2. Methods

### 5.2.1. Fly strains

All fly stocks were reared on standard cornmeal and agar food with active yeast at 25°C, 40-60% relative humidity and under a 12:12 h light cycle.

Fly strains were all acquired from the Bloomington *Drosophila* Stock Centre (BDSC), apart from the UAS-GRAB<sub>ACh4.4</sub> who had been given to the institute directly by the Li lab. We used the OK107-GAL4 (BDSC: 44404)(Aso et al. 2009) strain to target all KCs, and the MB504B-GAL4 (BDSC: 68329)(Galili et al. 2014; Aso et al. 2014a) strain to target DANs innervating the  $\gamma$ 1-peduncle compartment of the MB that has been shown to be responsive to negative reinforcement and involved in aversive learning (Aso et al. 2010). We used newer versions of the GPCR-activation-based protein (GRAB) sensors for DA (Sun et al. 2018, 2020) and ACh (Jing et al. 2018, 2020) called UAS-GRAB<sub>DA2m</sub> (BDSC : 90878) and UAS-GRAB<sub>ACh4.4</sub>.

The GAL4/UAS (Brand and Perrimon 1993) system which allow cell-specific gene expression in *Drosophila*. GAL4 is a transcription factor needed enhance expression of the gene downstream of the UAS-sequence. Expression of GAL4 can be targeted to specific group of cells defined by a particular marker. A protein of interest can be placed after the UAS and will only be expressed in cells which also express the GAL4 gene. Therefore, crossing a fly line in which GAL4 are under the promoter of specific marker genes with a fly line containing a gene of interest (such as the gene encoding for GRAB sensors), will result in an offspring line in which the gene of interest is only expressed in certain, chosen cells.

### 5.2.2. Live functional imaging in *Drosophila*

Virgin females from UAS-GRAB<sub>DA2m</sub> and UAS-GRAB<sub>ACh4.4</sub> were crossed to male flies from either OK107-GAL4 or MB504B-GAL4 lines to obtain the following lines (**Figure 5.4 C**):

- OK107-GAL4 x UAS-GRAB<sub>ACh4.4</sub> where GRAB<sub>ACh4.4</sub> is expressed in all KCs
- OK107-GAL4 x UAS-GRAB<sub>DA2m</sub> where GRAB<sub>DA2m</sub> is expressed in all KCs
- MB504B-GAL4 x UAS-GRAB<sub>DA2m</sub> where GRAB<sub>DA2m</sub> is expressed in in the DAN innervating the  $\gamma$ 1-peduncle compartment of the MB.

Mixed sex flies aged between 10 to 20 days were used for functional imaging experiments. For experiments comparing internal state of hunger, the starved condition refers to flies which were food-deprived on 1% agar for 15 to 19 h before recording.

Flies were first briefly (> 1 min) anaesthetised on ice, before being mounted in a custom-made stage and positioned so that the top of their head lies in the window – small hole – of the stage. Their wings were waxed the back of the stage, as well as the tip of the abdomen. The head was secured to the back of the stage by placing a thin wire – stuck to the platform using wax – across the neck with enough pressure to maintain the flies without damaging the neck. Similarly, the proboscis was secured in an extended position using a second wire, so head and brain movement are minimized by preventing proboscis extension reflex. Finally, the head was secured to the platform by light waxing of the top of the eyes to the back of the stage, while avoiding wax to block antennae and damaged brain by touching the cuticle at the top of the head between the eyes. The legs and antennae were left freely moving (**Figure 5.4 A**).

Next, the head capsule was opened by hand using thin forceps under a binocular microscope and under oxygenated buffer solution (95% O<sub>2</sub>, 5% CO<sub>2</sub>) (103 mM NaCl, 3 mM



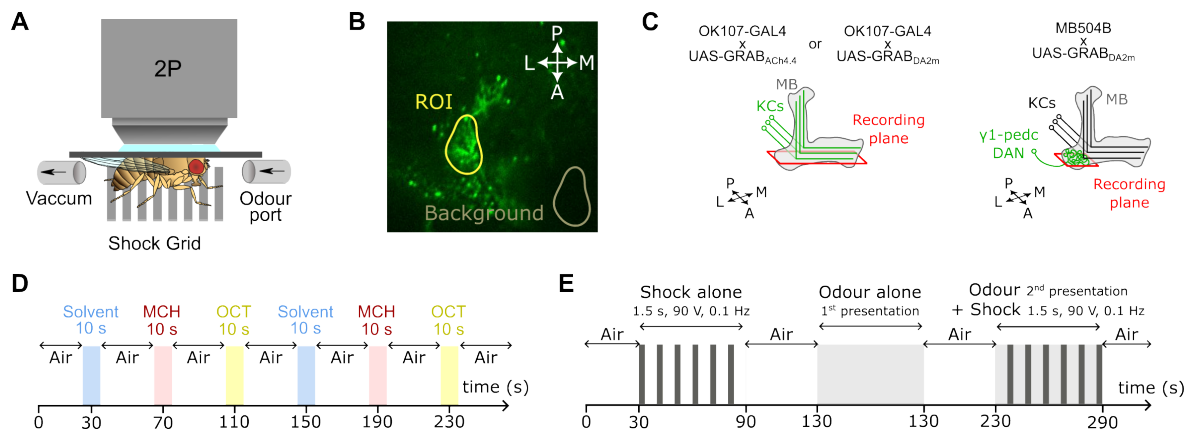
KCl, 5 mM N-Tris, 10 mM trehalose, 10 mM glucose, 7 mM sucrose, 26 mM NaHCO<sub>3</sub>, 1 mM NaH<sub>2</sub>PO<sub>4</sub>, 1.5 mM CaCl<sub>2</sub>, 4 mM MgCl<sub>2</sub>, osmolarity 275 mOsm, pH 7.3). To avoid fluorescence contamination during recordings and allow for visual better access to structure deep in the brain, fat and tracheas were removed from the surface of the brain. During this step, special care was accorded to preserve the integrity of the brain and the neural connections between the brain and the antennae. Next, flies were left to recover in the dark for a minimum of 5 to 15 min before being placed in the recording chamber.

Individual flies in the recording chamber were placed under a two-photon microscope (2P, Scientifica), in front of the odour port, and above an electric shock grid which was positioned in contact with the fly's legs. However, the legs were still allowed move which avoids potential burns or the flies and short circuits. The electric shock grid is built such that the circuit is completed only when the fly's leg touches the positive and negative bands at the same time. This system however has the caveat of not providing shocks are each stimulation since the flies have to position their legs in a particular way to receive the current. Finally, this also does not allow us to control for the length nor the intensity of the shock received despite fixing them to six 1.5 s long 90V electric shocks provided over for 1 min (0.1Hz). In order to monitor when a shock was actually received by the fly, we created a custom-made device that visually signalled when the circuit was completed, and current was going through. The visual information was recorded and synced to the functional recording.

A 40x, 0.8 NA water immersion objective and wide field blue laser ( $\lambda = 470$  nm) were used to locate the region of interest (ROI). The ROI was visually assessed from anatomical landmarks and the hemisphere it was selected in was randomly assigned. Next, fluorescence was excited using  $\sim 140$  fs pulses, 80 MHz repetition rate, centred on 910 nm

generated by a Ti-Sapphire laser (Chameleon Ultra II, Coherent) and the ROI region was adjusted and zoomed in x 4 when recording from the  $\gamma$ 1-peduncle DAN (MB504B-GAL4 line). The start of the recording (controlled by ScanImage 3.8 software) was synced to the start of the protocol controlled custom-made in conjunction with the odour delivery system (Shang et al. 2007). Images (256x256 pixels) were acquired at 5.92 Hz using a with a 40x, 0.8 NA water immersion objective.

Each n represents a fly, and each fly was presented with a single protocol in order to avoid potential confound of learning or plasticity induced by the protocol presented.



**Figure 5.4 : Functional GRAB sensor imaging in live flies under a two-photon microscope**

**A:** Schematic of the set up for functional imaging in live flies. Legs and antennae are left intact and freely moving. The fly is attached to a custom-made platform by the wings and abdomen. The head capsule is open, submerged by external solution and exposed to the microscope (two-photon, 2P) through a small whole in the platform. The flies are placed onto a shock grid, and in between an odour port (in front of them) and a vacuum port (behind them).

**B:** Example of a field of view during recording from the  $\gamma$ 1-peduncle compartment. The raw fluorescence was extract from the yellow ROI. The background fluorescence was extracted from zone delimited by the brown line.

**C:** Schematic of the different genotypes and where the recordings were taken from.

**D:** Schematic of the protocol with successive 10 s presentation of solvent and odour.

**E:** Schematic of the protocol which allow for testing the ACh>DA interaction.

### 5.2.3. Odour delivery and foot shock protocols used for functional imaging in *Drosophila*

The odour delivery system used in this body of work is a custom-made system providing a constant airflow in which either odorants or solvents can be mixed through (Shang et al. 2007). Odours (3-Octanol (OCT) and 4-methylcyclohexanol (MCH)) were diluted in mineral oil (solvent) to  $1 \times 10^{-3}$  M. These odours and concentration are the commonly used in learning paradigms especially for T-mazes (Krashes and Waddell 2011a, b), and therefore were good starting points for this study, and left the opportunity to potentially do behaviour using odours matching the functional imaging.

We used a first protocol (**Figure 5.4 D**) to confirm that ACh is indeed release from KCs in response to odour presentation as expected from the literature, and presented in sequence: 10 s of odour or solvent separated by 30 s of airflow to leave enough time for the previous odour to be fully dissipated before the next one is presented. The flies are facing the odour port, and a vacuum port to help extract odours is place at the behind the flies and is active throughout. The first 10 s presentation was a presentation of the solvent (mineral oil), then MCH and finally OCT both diluted in mineral were presented, all presentation interspaced by 30 s of air. This sequency was repeated twice, so each odour was presented twice to the fly. At the beginning of the protocol, 25 s of air in order to allow the fly to habituate to the air flow, and to allow for the initial short exponential decay in fluorescence seen in the recordings to reach a plateau.

Next, we used the following protocol to assess whether prolonged odour presentation (1 min long) would evoke sustained neurotransmitter release. Similarly to the first protocol,

airflow was first presented for 30 s, followed by 1 min of either MCH or OCT (both diluted in mineral oil).

Finally, we wanted to assess whether DA release from the aversive stimuli responsive  $\gamma$ 1-pedc compartment of the MB during foot shock was altered by the presence of ACh in the environment. We therefore designed a protocol (**Figure 5.4 E**) such as: we could record responses to foot shock only, when only DA is released, then, we could control for the integrity of the olfactory system by monitoring DA release to odour onset, which also infers ACh is released upon odour presentation too, and finally, we would present both the shocks and the odour at the same time to compare DA release in the shock + odour condition compared to the shock only condition.

First, 30 s of air were presented in order to allow the fly to habituate to the air flow, and to allow for the initial short exponential decay in fluorescence seen in the recordings to reach a plateau. Next, 1.5 s-long 90 V electric shocks were presented 6 times over 1 min at a rate of 0.1 Hz. Electric shock of 1.5 s at 90 V spread over 1 min are traditionally used for aversive learning in T-mazes (Tully and Quinn 1985; Krashes and Waddell 2011b) although the rate used is 0.2 Hz. We chose a lower rate of 0.1 Hz to allow signal to go back to baseline after the rise in DA evoked by electric pulses. An airflow was also presented during shock presentation. After the end of the shocks, there was a 45 s break during which only air flow was provided.

Next, we presented an odour for 1 min, as a control for the integrity of the olfactory pathways. This step was placed after the shock to avoid any associative learning to arise. If there no response to the odour onset was detected, the signal from that fly was discarded altogether, because we could not exclude the possibility that either antennae or neuronal

connection had been damaged and therefore could not infer that ACh would be release in response to the odour. After the odour presentation, 45 s of clean air was presented.

Finally, the shock + odour condition was delivered as follow: the odour was delivered first for 1 min and the shock sequence (6 six 90 V shock, 1.5 s long, at 0.1 Hz rate over 1 min) was started 10 s later. The delay in the start of the shock sequence was put in place to avoid confounding effects of the increase in DA release due to the second odour presentation.

For this protocol, odours presented were either OCT throughout or MCH throughout (both diluted in mineral oil), and the use of one odorant or the other was chosen at random and aimed to have an equal split in the final data sets.

#### 5.2.4. Analysis of functional imaging in live flies

Files were recorded were first manually segmented on FIJI ImageJ. The ROI was then visually drawn around the compartment(s) of interest base on morphological landmarks and detectable fluorescence. For recordings in the  $\gamma$ 1-peduncle compartment, an aera of the same size and shape as the ROI was placed in a zone where no fluorescent changes other than those potentially caused by small brain movements occur to account for those slight movements and is referred to as the background fluorescence (**Figure 5.4 B**). In general, fly movements were small enough such that images did not require registration. Average fluorescence over time within the ROI and the background-ROI were from ImageJ and pasted into excel, along with the information determining the time of odour onset and offset, as well as the time at which shocks are being received by the flies.

Next, analysis was made using custom-made MatLab codes adapted to the different protocol used. The fluorescence  $F$  was calculated by subtracting the background fluorescence to the fluorescence extracted from the ROI. The baseline fluorescence  $F_0$  was

then defined before each stimulation. For odour presentations, the  $F_0$  was calculated as the mean fluorescence  $F$  from 11.5 s to 1.5 s before the stim onset. For shock presentations, the  $F_0$  was calculated as the mean fluorescence  $F$  between 1.5 s and 0.5 s before shock onset. Variations in the fluorescence were then calculated as  $\Delta F / F_0 = (F - F_0) / F_0$ . The AUC were calculated on the  $\Delta F / F_0$  traces using the Trapz function in MatLab. The peak  $\Delta F / F_0$  was defined as the maximum value over the 5 s after shock onset, or 10 s after odour onset on excel. Responses to shock were averaged per fly, so that each  $n$  corresponds to a fly in graphs related to shock responses – for peak  $\Delta F / F_0$ , AUC or  $F_0$ . Therefore, on all graphs, a  $n$  corresponds to a fly.

Statistical analyses were carried out using GraphPad Prism as described in **section 2.7.** and the threshold for statistical significance was  $p < 0.05$ . Statistical significance was reported as: ns  $p > 0.05$ ; \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ ; \*\*\*\*  $p < 0.0001$ .

### 5.2.5. Single cell data extraction and analysis

Single-cell sequencing was performed by Dr. C. Treiber as described in (Croset et al. 2018). Data was sorted, analysed and annotated by Dr. C. Treiber (Treiber et al., unpublished). From the analysed dataset, we extracted expression levels of nAChR subunits exclusively from clusters of cells that have been identified as being dopaminergic cells innervating the mushroom body using custom-made code on R.

## 5.3. Results

### 5.3.1. Establishing ACh and DA release patterns from odour responsive

#### Kenyon cells in behaving flies

##### 5.3.1.1. Kenyon cells ACh release dynamics upon short odour presentation

First, we aimed to assess the release dynamics of ACh from KCs in response to odour stimulations. We expressed GRAB<sub>ACh4.4</sub> (UAS-GRAB<sub>ACh4.4</sub>) in all KCs (OK107-Gal4). We recorded from the MB where KC axons are overlapped tightly with DAN axons and MBON dendrites. In particular, we recorded from the horizontal lobe of the MB (**Figure 5.5 A**), since we know DANs carrying shock information project in some of these compartments.

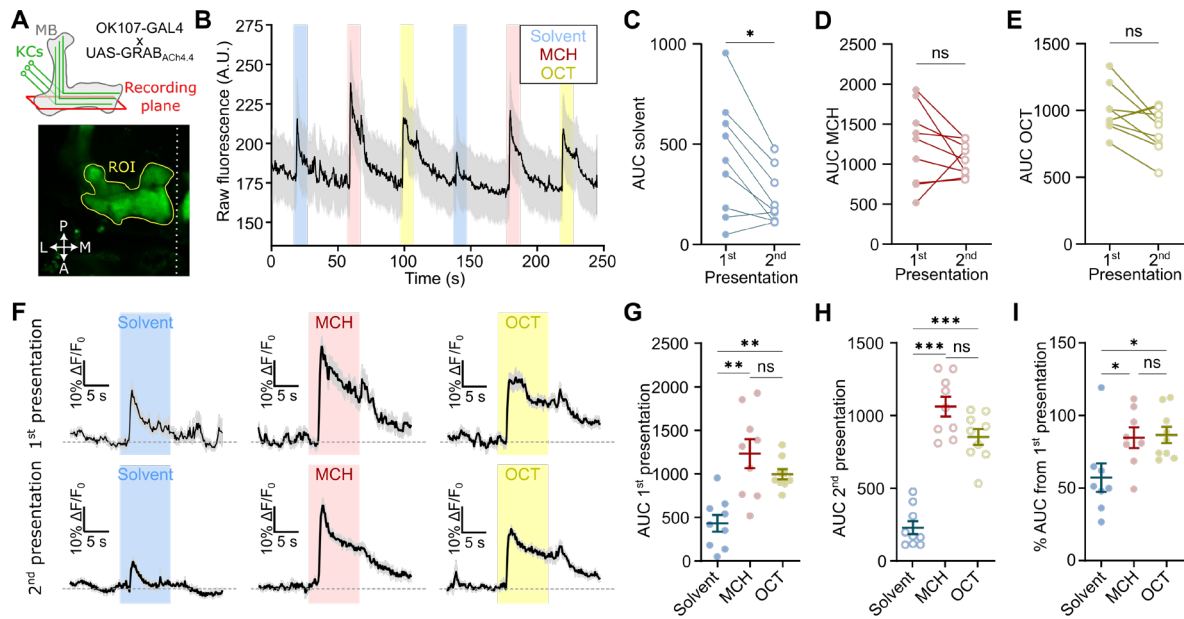
Flies were exposed to two odours – Octanol (OCT) and MCH – that are commonly used to train flies in learning paradigms (Krashes and Waddell 2011a, b), and to the solvent – mineral oil – in which those odours are diluted into. We presented each solution for 10 s with 30 s intervals in which air was puffed. For the order of presentations, we used the following sequence mineral oil, MCH and OCT seen on **Figure 5.5 B**. We expect to find increase in ACh release upon odour delivery, and a lower or no response to the solvent mineral oil.

We found that all three solutions tested evoke an increase in  $\Delta F/F_0$  (**Figure 5.5 B** and **F**) which suggest an increase in ACh release to both odours and their solvent. Nevertheless, OCT and MCH elicit transients which have significantly higher AUCs than mineral oil does during the first presentation (**Figure 5.5 B, F** and **G**, solvent versus MCH p-value = 0.0035, solvent versus OCT p-value = 0.0018) and the second presentation (**Figure 5.5 B, F** and **H**,

solvent versus MCH p-value < 0.0001, solvent versus OCT p-value < 0.0001). Therefore, although KCs are responsive to the solvent (mineral oil), ACh release is mostly due to the odour mixed in the solvent. Plus, both odours elicit ACh transients with similar AUC for both presentations (**Figure 5.5 B, F, G** MCH versus OCT p-value = 0.4512, and H MCH versus OCT p-value = 0.1122).

Next, we compared responses between the first and the second presentation for each solution. The response to mineral oil was significantly reduced (**Figure 5.5 F and C**), however it was not completely abolished. For presentation of OCT and MCH, no decline in AUC was found to be significant for either odours (**Figure 5.5 F, D and E**). Finally, the AUC upon second odour presentation is more reduced after mineral oil exposure compared to MCH and OCT exposure (**Figure 5.5 B, F and I**, solvent versus MCH p-value = 0.020, solvent versus OCT p-value = 0.043). These results suggest that although ACh release may decrease slightly upon second presentation of the solvent, but that KCs seem to release similar ACh amount in response the first and second odour presentations.





**Figure 5.5 : ACh is released in response to first and second odour presentation.**

**A:** Top, schematic of the genotype used for the experiment.  $GRAB_{ACh4.4}$  is expressed in all KCs labelled by OK107-GAL4. The recording plane is shown in red and is located on the horizontal lobe of the mushroom body. Bottom, example of field of view during recording and ROI used for analysis. The dotted line represents the midline.

**B:** Mean ( $\pm$  SEM) of raw fluorescence throughout the experiment over time. Shaded areas represent odour delivery times. In the whole panel,  $n = 9$  flies undergoing the protocol once each.

**C, D and E:** comparison of AUC of the transients in response first and second presentation of the solvent (**C**, Wilcoxon test,  $p$ -value = 0.02), MCH (**D**, Paired  $t$ -test,  $p$ -value = 0.287) or OCT (**E**, Wilcoxon test,  $p$ -value = 0.055).

**F:** Mean ( $\pm$  SEM)  $\Delta F/F_0$  in response to solvent (left), MCH (middle) or OCT (right) delivery during first (top row) or second (bottom row) odour presentation. The shaded area represents the time on odour delivery.

**G, H:** Mean AUC of each odour during first odour presentation (**G**, RM One-Way ANOVA  $F(1.606, 12.85) = 16.01$ ,  $p$ -value = 0.0005, with Tukey's multiple comparison tests), and during second odour presentation (**H**, RM One-Way ANOVA  $F(1.832, 14.66) = 68.13$ ,  $p$ -value < 0.001, with Tukey's multiple comparison tests).

**I:** Mean of AUC of second odour presentation plotted as a percentage of the AUC from first odour presentation. RM One-Way ANOVA  $F(1.981, 11.89) = 8.348$ ,  $p$ -value = 0.006, with Tukey's multiple comparison tests).

### 5.3.1.2. Kenyon cells ACh release dynamics upon prolonged odour

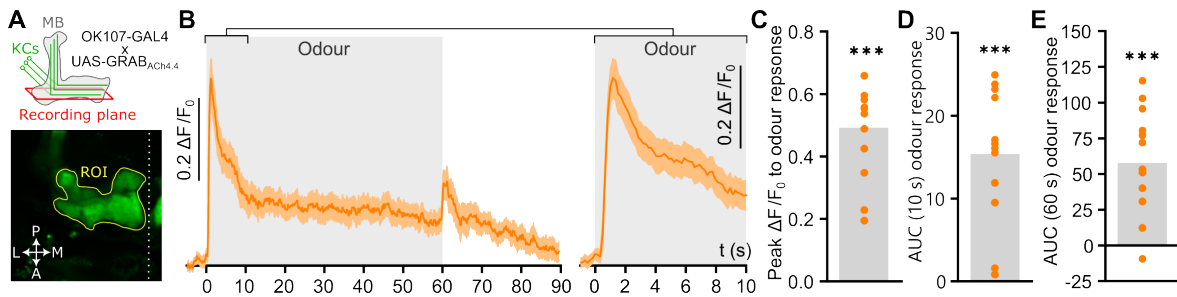
#### presentation

In the protocol we use to interrogate the impact of coincident ACh release on DA release evoked by foot shock, we exposed the flies to an odour for 60 s, to mimic what flies usually go through during aversive training (Krashes and Waddell 2011b), and to allow sufficient time for the flies to receive at least one electric foot shock during the session.

Therefore, we wanted to assess whether ACh release was sustained throughout a prolonged odour presentation.

Similarly to **section 5.3.1.**, we imaged from the KC axons innervating the horizontal lobe of the MB from OK107-Gal4 x UAS-GRAB<sub>ACh4.4</sub> flies (**Figure 5.6 A**). We exposed them to either OCT or MCH. We found that, like with 10 s stimulation from **section 5.3.1.**, there is strong on response with a fast peak in ACh release when the odour first reaches the fly (**Figure 5.6 B, C and D**). Although release quickly decreases, it reaches a plateau. This plateau indicates sustained release throughout the odour presentation as the AUC of the trace taken over the 60 s of odour presentation is significantly different from zero (**Figure 5.6 B and E**).

We noticed what seems to be an off response to the odour being turned off. The off response is likely to be due to transient changes in airflow speed. The signal takes approximately 30 s to return to pre-odour stimulus baseline. The slow decay could be due to a few factors. Although quite unlikely due to the presence of a vacuum port behind the fly, the tail of the signal could be due to the odour still surrounding the flies for few seconds after being switched off. The slow return to baseline is also unlikely to be due to the intrinsic properties of the sensor since the off kinetics are faster than the slow decay observed here (Jing et al. 2020). Finally, it is more likely to be caused by biologically relevant processes whereby a prolonged exposure to a high odour concentration changes plasticity for release and flies habituate to the odour (Semelidou et al. 2018).



**Figure 5.6 : ACh released is sustained throughout long odour presentation.**

**A:** Top, schematic of the genotype used for the experiment. GRAB<sub>ACh4.4</sub> is expressed in all KCs labelled by OK107-GAL4. The recording plane is shown in red and is located on the horizontal lobe of the mushroom body. Bottom, example of field of view during recording and ROI used for analysis. The dotted line represents the midline.

**B:** Left, mean ( $\pm$  SEM)  $\Delta F/F_0$  during 60 s of odour presentation. Right, zoom on the first 10 s of odour presentation, when the peak occurs. In the whole panel,  $n = 13$  flies undergoing the protocol once each with either MCH or OCT as an odour.

**C:** Peak  $\Delta F/F_0$  of odour response within the first 10 s of odour exposure. Grey bars represent the mean. One-sample Wilcoxon, theoretical median = 0,  $p$ -value = 0.0002.

**D:** AUC of odour response taken over the first 10 s of odour exposure. Grey bars represent the mean. One-sample Wilcoxon, theoretical median = 0,  $p$ -value = 0.0002

**E:** AUC of odour response taken over the whole 60 s of odour exposure. Grey bars represent the mean. One-sample t-test, theoretical mean = 0,  $p$ -value = 0.0001.

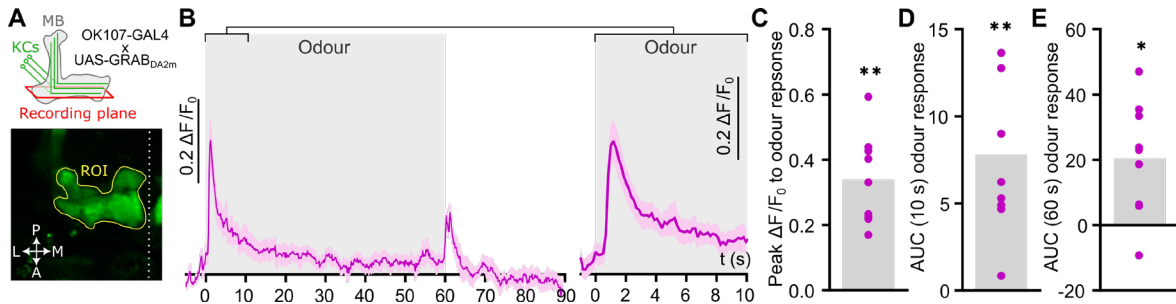
### 5.3.1.3. Odour presentation triggers DA release from DANs

Finally, we tested whether odour presentation was also eliciting DA release. Although previous work used indirect methods for assessing neurotransmitter release such as GCaMP, previous studies (Dylla et al. 2017) suggest DA release should also be elicited by odour presentation in several compartment of the horizontal lobe of the MB.

We expressed GRAB<sub>DA2m</sub> (UAS- GRAB<sub>DA2m</sub>) in all KCs (OK107-Gal4). We recorded from the whole horizontal lobe of the MB (**Figure 5.7 A**). Although KCs are not known to release DA, they are in close contact with DA axons within the MB, which allows us to measure DA release from different compartments all at once to control for a general response to odours.

We found that odour onset triggers a sharp increase in  $\Delta F/F_0$  (**Figure 5.7 B, C and D**). However, although the AUC over 60 s of odour exposure is statistically different from zero (**Figure 5.7 E**), the signal slowly decays to return to baseline within 20 to 30 s of odour exposure (**Figure 5.7 B**).

Similarly to ACh release, DA release seems to also be increased as the odour is turned off after 60 s (**Figure 5.7 B**), which is likely to be due to transient changes in airflow speed after the odour is turned off.



**Figure 5.7: DA is release at onset and offset of odour presentation.**

**A:** Top, schematic of the genotype used for the experiment. GRAB<sub>DA2m</sub> is expressed in all KCs labelled by OK107-GAL4. The recording plane is shown in red and is located on the horizontal lobe of the mushroom body. Bottom, example of field of view during recording and ROI used for analysis. The dotted line represents the midline.

**B:** Left, mean ( $\pm$  SEM)  $\Delta F/F_0$  during 60 s of odour presentation. Right, zoom on the first 10 s of odour presentation, when the peak occurs. In the whole panel,  $n = 9$  flies undergoing the protocol once each with either MCH or OCT as an odour.

**C:** Peak  $\Delta F/F_0$  of odour response within the first 10 s of odour exposure. Grey bars represent the mean. One-sample Wilcoxon, theoretical median = 0,  $p$ -value = 0.0039.

**F:** AUC of odour response taken over the first 10 s of odour exposure. Grey bars represent the mean. One-sample Wilcoxon, theoretical median = 0,  $p$ -value = 0.0039.

**G:** AUC of odour response taken over the whole 60 s of odour exposure. Grey bars represent the mean. One-sample Wilcoxon, theoretical median = 0,  $p$ -value = 0.0195.

### 5.3.2. Exploring modalities for fine tuning of DA release from aversive

#### DANs

##### 5.3.2.1. DA release evoked by shocks is not impacted by consequent odour presentation

To test whether ACh has an effect on DA release in neurons responsive to aversive stimuli, we designed our protocol (**Figure 5.8 A**) so that we can first test for DA release to foot shocks in the absence of an odour and therefore in the absence of ACh in the environment. Next, we present an odour as a control for the odour responsiveness of the DANs, since DA is released upon odour onset (**section 5.3.2**). This step allows us to control for the fact that neither the antennae nor neurons in the odour processing pathway have been damaged during the surgery. Indeed, if there is no response from DA neurons it suggests that the odour information might not have been detected or relayed properly, and we cannot be sure the odour presentation has evoked ACh release from KCs. We provided shock stimulus first and odour stimulus second to avoid confound of potential associative learning occurring between the odour and the shocks (Yarali and Gerber 2010; Gerber et al. 2014). Last, we present an odour at the same time as providing foot shocks so ACh is released due to the odour stimulation, and DA is also released during each electric foot shock. We can therefore compare, within the same animal DA release evoked by foot shock in the presence and in the absence of ACh in the vicinity of the DA terminals.

To achieve this, we expressed GRAB<sub>DA2m</sub> in a DAN innervating the  $\gamma$ 1-pedc compartment and which is known to be responsive to foot shock (Aso et al. 2010; Villar et al. 2022) and that is also involved in aversive learning. We imaged the response to shock (**Figure 5.8 B and C**) and odour stimuli using the protocol described above. The implication of  $\gamma$ 1-ped

in aversive learning strongly suggest that some plasticity between KCs and DA could occur in this compartment learning.

We first analysed the response to odours (OCT or MCH), as we used that stimulus as a quality control of our recordings. If no increase in  $\Delta F/F_0$  was observed upon odour onset, we did not keep data from this fly, as we couldn't exclude the fact that its antennae or other brain regions were damaged and therefore potentially the odour information was not relayed to the MB and no ACh would be released from KCs.

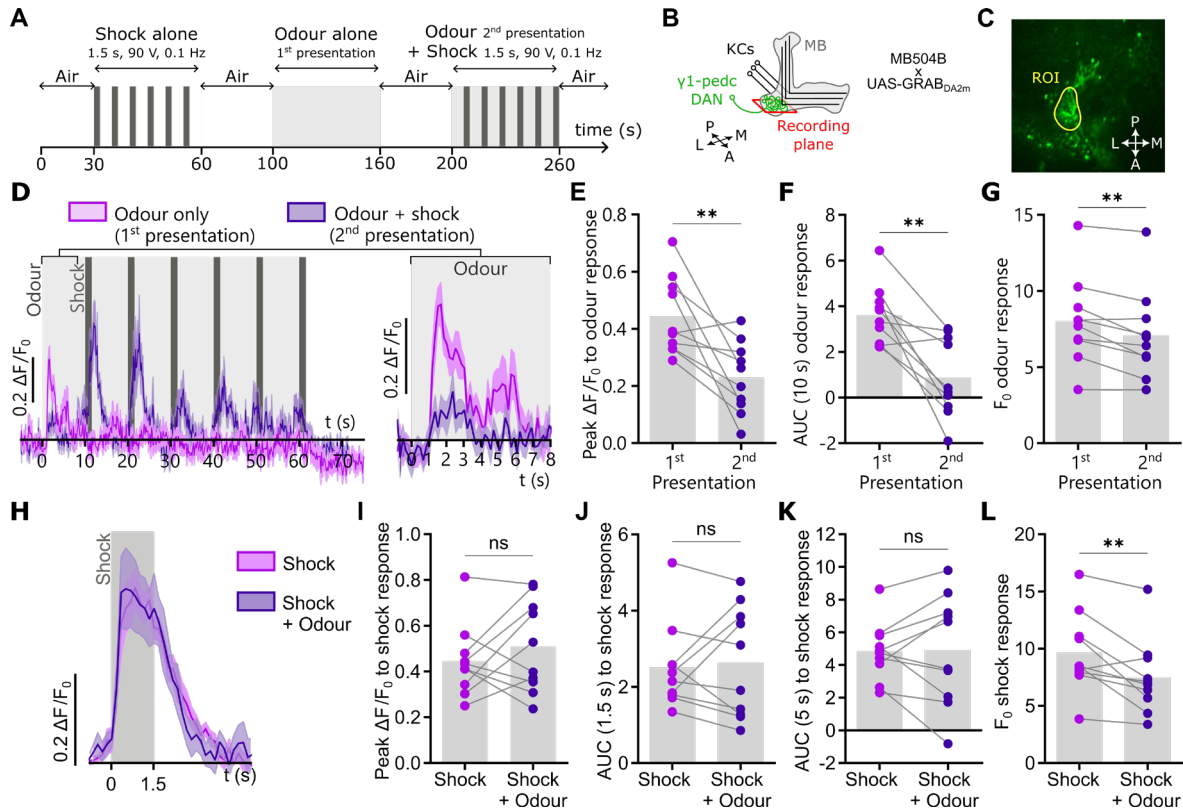
Similarly to what we observed when imaging the whole horizontal lobe of the MB (**section 5.3.1.3**), we found that for the first odour presentation  $\Delta F/F_0$  increases upon odour onset in the  $\gamma 1$ -pedc compartment (**Figure 5.8 D, E and F**). The signal returns to baseline within 10 s after odour onset (**Figure 5.8 D**). Finally, we observed no increase in  $\Delta F/F_0$  in response to the termination of the odour, which is likely to be a result of fine tuning to the set up to limit airflow fluctuation upon odour switches.

The second odour presentation did not elicit a strong peak in  $\Delta F/F_0$  at odour onset (**Figure 5.8 D**). The response to the second presentation of the odour was smaller in amplitude (peak  $\Delta F/F_0$  **Figure 5.8 E**) and in AUC (**Figure 5.8 F**) compared to the first presentation. We only compared AUC between first and second presentation over the first 10 s succeeding the odour onset because this is when the rise in the signal is the greatest (see **Figure 5.7 B** and **Figure 5.8 D**), and because shock are also presented during the second odour presentation, which causes the  $\Delta F/F_0$  to rise periodically in response to the shocks, therefore, the comparing the AUC over 60 s of odour presentation would be unfit.

Next, we assessed whether the shock response (DA release) would be affected by the presence of ACh (trigger by odour presentation). We compared the increase in  $\Delta F/F_0$  evoked by the shock stimulus alone (Shock only condition), and the shocks given while an odour was presented (Shock + Odour condition). Each  $n$  corresponds to an average of all shock responses from a fly in a certain condition (shock only or shock + odour). We expect to see a change of DA release when ACh is coincidentally released.

We found no difference in the peak  $\Delta F/F_0$  between the shock only and shock + odour condition (**Figure 5.8 H and I**). No statistical difference in area under the curve (AUC) taken over 1.5 s (length of the shock) or 5 s (time for signal to return to baseline) were found between the two conditions either (**Figure 5.8 H, J and K**).

A small decrease in the  $F_0$  was found the response to shock in the shock + odour condition compared to the shock only condition (**Figure 5.8 L**). However, a decay in  $F_0$  was also found for the response to odour in the shock + odour condition (which is the second odour presentation) compared to the first odour presentation (**Figure 5.8 G**). This suggests that there is a general decay in  $F_0$  which is likely to be due to bleaching, rather than plasticity decrease baseline levels of DA release.



**Figure 5.8: Shock responses from  $\gamma 1$ -pedc DAN remains unchanged whether or not an odour is concurrently delivered.**

**A:** Schematic of the protocol used. Electric foot shock given were 1.5 s long, 90 V, and pretended at a rate of 0.1 Hz. During concurrent odour delivery, the start of the electric foot shock sequence was delayed by 10 s to allow for odour response to go back to baseline before first shock. Dark grey shaded areas represent shock times, and light grey shaded areas represent times where an odour is presented to the fly.

**B:** Schematic of the genotype used for the experiment. GRAB<sub>DA2m.4</sub> is expressed in the  $\gamma 1$ -pedc DAN (green) labelled by MB504B-GAL4. The recording plane is shown in red.

**C:** Example of field of view during recording and ROI used for analysis. Contrasts have been increased to allow for better visualisation.

**D:** Left, mean ( $\pm$  SEM)  $\Delta F/F_0$  during odour presentation alone (1<sup>st</sup> presentation, pink) or odour + shock presentation (2<sup>nd</sup> presentation, purple). Right, zoom on the first 8 s of odour presentation, where the main response occurs. In the whole panel,  $n = 10$  flies undergoing the protocol once each with either MCH or OCT as an odour.

**E:** Peak  $\Delta F/F_0$  of odour response within the first 10 s of odour exposure. Grey bars represent the mean. Paired t-test,  $p$ -value = 0.0050.

**F:** AUC of odour response taken over the first 10 s of odour exposure. Grey bars represent the mean. Paired t-test,  $p$ -value = 0.0016.

**G:**  $F_0$  of odour response. Grey bars represent the mean. Wilcoxon test,  $p$ -value = 0.0059.

**H:** Mean ( $\pm$  SEM)  $\Delta F/F_0$  during shock alone (pink) or shock + odour presentation (purple). The shade area indicated the time of the shock.

**I:** Peak  $\Delta F/F_0$  of shock response. Grey bars represent the mean. Wilcoxon test,  $p$ -value = 0.3223.

**J:** AUC of shock response taken over the shock duration (1.5 s). Grey bars represent the mean. Wilcoxon test,  $p$ -value > 0.9999.

**K:** AUC of shock response taken over the 5 s until signal returns to baseline. Grey bars represent the mean. Paired t-test,  $p$ -value = 0.8992.

**L:**  $F_0$  of shock response. Grey bars represent the mean. Wilcoxon test,  $p$ -value = 0.0098.



### 5.3.2.2. Internal state alters DA release evoked by foot shocks

Next, we wondered if altering the internal state of the flies could reveal differences in responses to shocks in the presence or absence of ACh in the environment. In hungry *Drosophila*, aversively reinforcing dopaminergic neurons (DANs) are inhibited to provide part of the hunger-dependent control of the expression of food-seeking memory (Hirano et al. 2013; Plačais and Preat 2013). However, inhibiting part of the reinforcement system in food-deprived flies could potentially undermines its ability to function in aversive learning and provide relevant teaching signals. Furthermore, data from the lab (Meschi et al. 2024) has shown that hunger enhances aversive learning. We therefore first tested how hunger impact naïve response to shocks only, and next tested whether starved flies have different responses to shock only and shock presented at the same time as an odour.

#### *i. DA release evoked by foot shocks is heightened from baseline in starved animals*

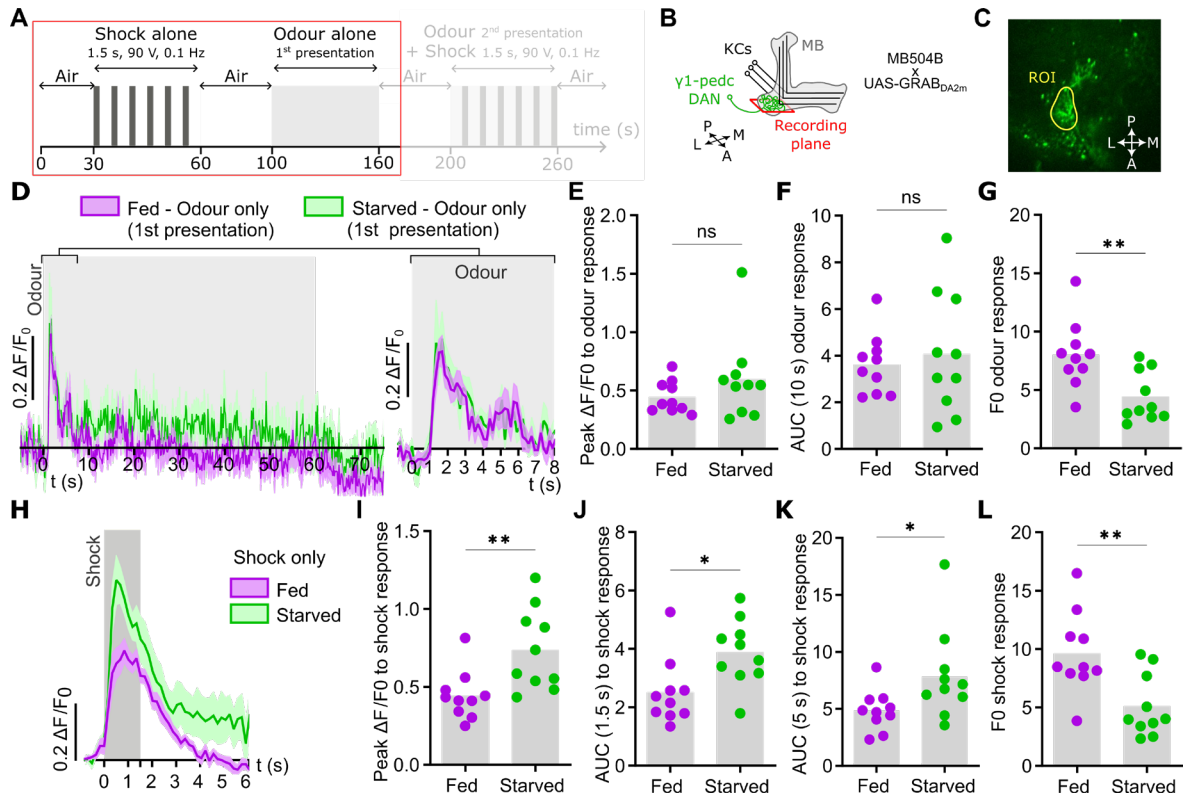
In this section, we first compare the odour and shock responses of fed and hungry animals in order to assess the impact of internal state – here hunger – on DA release in response to these behaviourally relevant stimulations (**Figure 5.9 A**).

We expressed GRAB<sub>DA2m</sub> in the  $\gamma 1$ -pedc DAN (**Figure 5.9 B and C**) known to be responsive to foot shock (Villar et al. 2022), involved aversive learning and in the expression of aversive memories relevant to the current internal state of the animal, in particular hunger (Krashes et al. 2009; Plačais and Preat 2013). Hungry flies were starved on 1% agar for 15 to 19 h before recording, and fed flies were fed *ad libitum* until the experiment.

We found that responses to the first presentation of the odour are the same between fed and starved animals (**Figure 5.9 D, E and F**). Indeed, both peak  $\Delta F/F_0$  and AUC were not significantly different between the two states (**Figure 5.9 E and F**). This indicates that in this specific MB compartment DA release in response to odour stimuli does not vary depending on the state of hunger of the fly.

Next, we compared signals evoked by electric foot shocks, and we found that peak  $\Delta F/F_0$  and AUC at 1.5 s and 5 s were increased in starved animals compared to when the animal is satiated (**Figure 5.9 H, I, J and K**). Therefore, shocks trigger larger DA release events when the animal is food deprived compared to fed animals.

Finally, we also noticed that the baseline fluorescence  $F_0$  was lower in food deprived flies compared to the fed flies for both odour response (**Figure 5.9 G**) and shock response (**Figure 5.9 L**). This suggest that upon starvation, the  $\gamma 1$ -pedc DAN is releases less DA in a tonic manner compared to when the fly is satiated. This lower  $F_0$  found in starved animals did not affect the amplitude of the response to odours compared to fed animals and is therefore unlikely to be the driving force for the increased response to shock observed in starved animals.



**Figure 5.9 : Foot shock elicits higher DA release in food-deprived animals compared to satiated animals despite odour response to be the same.**

**A:** Schematic of the protocol used. In this panel, only the naïve response to foot shock and the first odour presentation are compared between fed and starved animals. Electric foot shock given were 1.5 s long, 90 V, and pretended at a rate of 0.1 Hz. Dark grey shaded areas represent shock times, and light grey shaded areas represent times where an odour is presented to the fly.

**B:** Schematic of the genotype used for the experiment. GRAB<sub>DA2m.4</sub> is expressed in the  $\gamma 1$ -pedc DAN (green) labelled by MB504B-GAL4. The recording plane is shown in red.

**C:** Example of field of view during recording and ROI used for analysis. Contrasts have been increased to allow for better visualisation.

**D:** Left, mean ( $\pm$  SEM)  $\Delta F/F_0$  during odour presentation alone (1<sup>st</sup> presentation) in fed flies (pink) and starved food-deprived animals (light green). Right, zoom on the first 8 s of odour presentation, where the main response occurs. In the whole panel,  $n = 10$  fed flies were used, and  $n = 10$  food deprived flies. Each fly underwent the protocol only once either MCH or OCT as an odour.

**E:** Peak  $\Delta F/F_0$  of odour response within the first 10 s of odour exposure. Grey bars represent the mean. Mann-Whitney test,  $p$ -value = 0.4040.

**F:** AUC of odour response taken over the first 10 s of odour exposure. Grey bars represent the mean. Unpaired t-test,  $p$ -value = 0.6271.

**G:**  $F_0$  of odour response. Grey bars represent the mean. Unpaired t-test,  $p$ -value = 0.0050.

**H:** Mean ( $\pm$  SEM)  $\Delta F/F_0$  during shock alone in fed (pink) or starved (light green) animals. The shade area indicated the time of the shock.

**I:** Peak  $\Delta F/F_0$  of shock response. Grey bars represent the mean. Mann-Whitney test,  $p$ -value = 0.0031.

**J:** AUC of shock response taken over the shock duration (1.5 s). Grey bars represent the mean. Mann-Whitney test,  $p$ -value = 0.0152.

**K:** AUC of shock response taken over the 5 s until signal returns to baseline. Grey bars represent the mean. Mann-Whitney test,  $p$ -value = 0.0243.

**L:**  $F_0$  of shock response. Grey bars represent the mean. Unpaired t-test,  $p$ -value = 0.0046.

This data has been included in Meschi et al., 2024 which demonstrated that hunger enhances aversive olfactory learning and that adipokinetic hormone (AKH) signalling – the fly functional homolog of the vertebrate glucagon – is required for aversive learning in both satiated and hungry states. The improved efficacy for aversive learning in food-deprived flies can be attributed to their hunger-mediated elevated response to shocks as shown in **Figure 5.9**.

Next, we hypothesised that starvation, by enhancing the response to shock and improving olfactory learning, could be a way for us to reveal the potential effect of ACh – released upon odour delivery – in modulating DA release evoked by electric foot shocks.

*ii. In starved animals, DA release evoked by shocks is lowered when shocks are provided in coincidence with an odour stimulus*

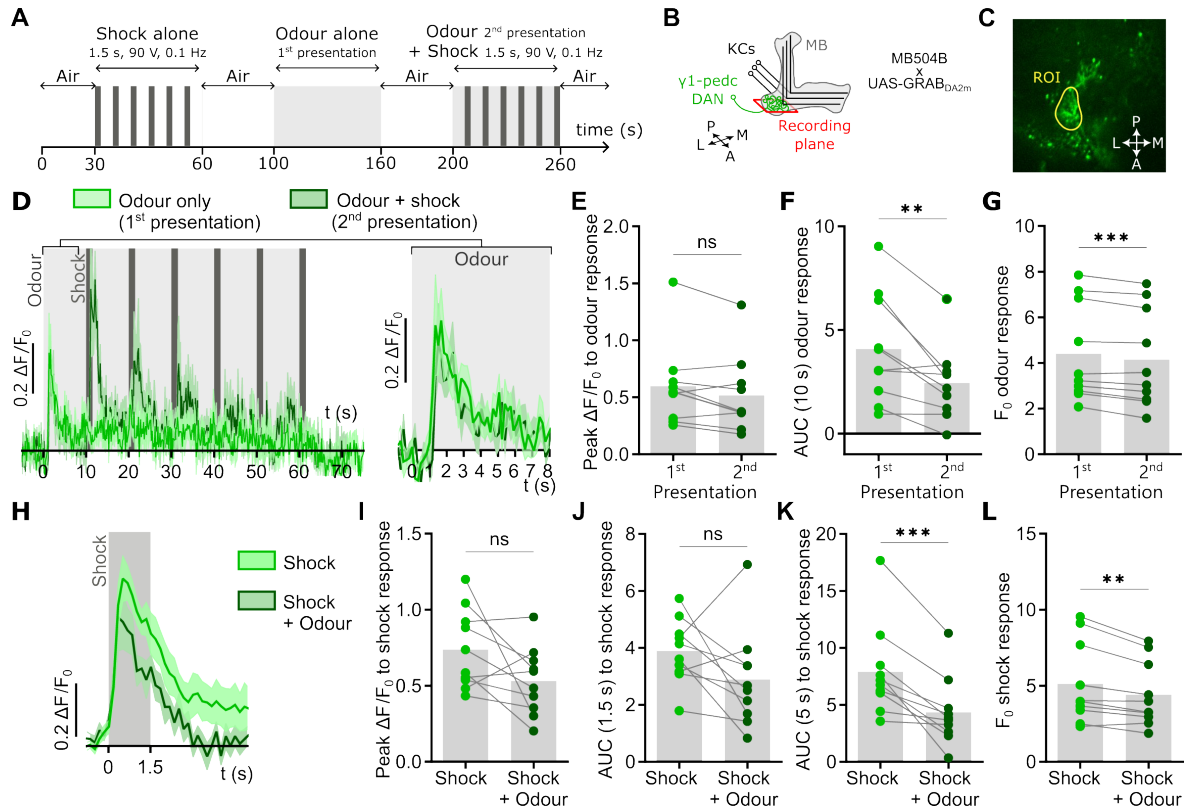
Since starvation increases the amplitude of DA transients in response to shocks compared to satiated animals, we wondered whether this heightened sensitivity for shock could reveal an action of ACh onto the DA terminals. Again, we expressed GRAB<sub>DA2m</sub> in the  $\gamma$ 1-pedc DAN (**Figure 5.10 B and C**), and all flies were starved on 1% agar for 15 to 19 h before recording. We exposed each fly to the protocol described in **section 5.2.3** and **5.3.2.1**.

First, we compared the odour responses during first and second presentation (**Figure 5.10 D**). Although the  $\Delta F/F_0$  peak value did not show significant decrease between the first and the second presentation (**Figure 5.10 E**) as would be expected from results in fed flies

(**Figure 5.8 D and E**), a statistically significant reduction in AUC was found for the second odour presentation compared to the first one (**Figure 5.10 F**). Since no differences were found in the odour response to the 1<sup>st</sup> presentation between food-deprived and satiated animals in **Figure 5.9 D, E and F**, this could suggest that adaptation is handled differently depending on the hunger state of the animal, since the decrease in release at the second presentation can only be read out when measuring AUC and not  $\Delta F/F_0$  peak for food-deprived animals.

Secondly, we compared responses to shocks in the absence (shock alone) or presence (shock + odour) of an odour, which causes ACh release in the environment of the DA axons. We found that although there is a trend towards a decrease in DA response to shocks in the presence of an odour (**Figure 5.10 H**) both for the peak  $\Delta F/F_0$  (**Figure 5.10 H**) and the AUC at 1.5 s (**Figure 5.10 J**), these metrics did not reveal a statistically significant decrease. However, the AUC at 5 s was statistically reduced in the shock + odour condition compared to the shock alone condition (**Figure 5.10 K**).

Similarly to what was observed for fed animals (**Figure 5.8 L and G**), a small decrease in the  $F_0$  was found the response to shock in the shock + odour condition compared to the shock only condition (**Figure 5.10 L**) and for the response to odour in the shock + odour (second presentation) condition compared to the odour only condition (first presentation) (**Figure 5.10 G**). This suggests that there is a general slight decay in  $F_0$  over the 200 s of recording which is likely to be due to bleaching, rather than plasticity decrease baseline levels of DA release.



**Figure 5.10: Shock responses in food-deprived animals from  $\gamma 1$ -pedc DAN seems lowered upon coincident odour delivery.**

**A:** Schematic of the protocol used. Electric foot shock given were 1.5 s long, 90 V, and pretended at a rate of 0.1 Hz. During concurrent odour delivery, the start of the electric foot shock sequence was delayed by 10 s to allow for odour response to go back to baseline before first shock. Dark grey shaded areas represent shock times, and light grey shaded areas represent times where an odour is presented to the fly.

**B:** Schematic of the genotype used for the experiment. GRAB<sub>DA2m.4</sub> is expressed in the  $\gamma 1$ -pedc DAN (green) labelled by MB504B-GAL4. The recording plane is shown in red.

**C:** Example of field of view during recording and ROI used for analysis. Contrasts have been increased to allow for better visualisation.

**D:** Left, mean ( $\pm$  SEM)  $\Delta F/F_0$  during odour presentation alone (1<sup>st</sup> presentation, light green) or odour + shock presentation (2<sup>nd</sup> presentation, dark green). Right, zoom on the first 8 s of odour presentation, where the main response occurs. In the whole panel,  $n = 9$  flies undergoing the protocol once each with either MCH or OCT as an odour.

**E:** Peak  $\Delta F/F_0$  of odour response within the first 10 s of odour exposure. Grey bars represent the mean. Wilcoxon test,  $p$ -value = 0.0645.

**F:** AUC of odour response taken over the first 10 s of odour exposure. Grey bars represent the mean. Paired  $t$ -test,  $p$ -value = 0.0069.

**G:**  $F_0$  of odour response. Grey bars represent the mean. Paired  $t$ -test,  $p$ -value = 0.0010.

**H:** Mean ( $\pm$  SEM)  $\Delta F/F_0$  during shock alone (light green) or shock + odour presentation (dark green). The shade area indicated the time of the shock.

**I:** Peak  $\Delta F/F_0$  of shock response. Grey bars represent the mean. Paired  $t$ -test,  $p$ -value = 0.0696.

**J:** AUC of shock response taken over the shock duration (1.5 s). Grey bars represent the mean. Paired  $t$ -test,  $p$ -value = 0.1212.

**K:** AUC of shock response taken over the 5 s until signal returns to baseline. Grey bars represent the mean. Paired  $t$ -test,  $p$ -value = 0.0007.

**L:**  $F_0$  of shock response. Grey bars represent the mean. Paired  $t$ -test,  $p$ -value = 0.0050.

Altogether this data suggests that in food deprived animals, responses to shocks seem to be decreased by the presence of ACh in the environment released by the KCs from odour presentation. In particular, the width of the transient seems affected as neither peak  $\Delta F/F_0$  nor AUC over shorter period (1.5 s, length of the shock) revealed a significant decrease, but only AUC taken over a longer period of time (5 s) has shown a drastic decrease when an odour is presented in coincidence with the electric foot shocks. Therefore, this could indicate that when flies are food-deprived ACh has an inhibitory effect of DA release in response to foot shocks.

## 5.4. Conclusion and discussion

In this chapter, we explored the potentially conserved mechanism governing axo-axonal modulation of DA release by ACh in the centre for olfactory learning of *Drosophila melanogaster*, and its potential behavioural relevance. The mushroom body is known to exhibit strong DAN to KC connections which are necessary for the formation and retrieval of associative learning, however little is known the reciprocal KC to DAN connections. We decided to focus on the MB compartment involved in aversive learning, the  $\gamma$ 1-pedc compartment, within which nAChRs on DA axons are believed to be necessary for normal expression of aversive memory (Cervantes-Sandoval et al. 2017). We assessed whether ACh modulates DA release in the presence or absence of an odour (which drives ACh release through KCs) using GRAB<sub>ACh</sub> or GRAB<sub>DA</sub> in live *Drosophila*.

We first established the release patterns of both ACh and DA in response to odour presentations. We confirmed that ACh was released from KCs upon odour delivery, and showed that, to a lesser extent, DANs also released DA in response to an odour presentation. We also confirmed that the DAN innervating the  $\gamma$ 1-pedc compartment release DA in response to foot shock which was expected from work using Ca<sup>2+</sup> sensors in that compartment (Villar et al. 2022).

Next, we investigated whether the DA release in response to shocks alone would be different from the DA release in response to shocks given on a background of odour delivery – and therefore when ACh is present in the environment. We found that DA release was similar whether or not ACh was present in the environment. We next explored whether internal state could affect the ACh-DA interaction and found that hunger enhances shock response in the  $\gamma$ 1-pedc compartment, but it does not alter the response to odours. In hungry flies, coincident ACh release seemed decrease DA release in response to shock, suggesting



there might be state-specific modulation of DA release by ACh in the  $\gamma 1$ -pedc compartment of the mushroom body. This effect could be mediated via nAChRs present at DA axons, but we could not demonstrate or test their involvement in the present study.

#### 5.4.1. ACh release plasticity in odour responses.

We found that 10 s exposure to the same odour twice did not significantly reduce ACh release upon the second presentation. This suggests that habituation to a specific odour is mild in this context. However, over the 1 min long presentation, ACh release isn't sustained at initial levels throughout the exposure, suggesting that ACh does adapt on longer time scales. However, we did not test whether flies habituate to a second prolonged odour exposure, which therefore might be a confounding effect in this study. Indeed, if ACh release is decreased upon second 1 min exposure of the same odour, it is possible that in fed animals, we do not observe an effect of ACh on DA release because very little ACh is actually being released. Further experiments testing for habituation would be needed, as well as a potentially shorter exposition time to odour in general, to limit adaptation. A possible alternative would be to expose the flies to shock+ odour *before* testing their response to odour only.

We investigated impact of altering internal state on DA release to shocks and odour in the  $\gamma 1$ -pedc compartment. We found that DA response to the first presentation of an odour is similar between starved and fed animals (**Figure 5.9 D**). However, in fed animals, we found a strong habituation as no DA release was seen upon 2<sup>nd</sup> odour presentation (**Figure 5.8 D**). On the contrary, in hungry animals, the second odour presentation elicits similar DA release

levels as the first presentation (**Figure 5.10 D**) which highlights an interesting state-dependent plasticity in the dynamics of DA release.

Finally, it would be interesting to test whether the DA release evoked by odour presentation is (at least partly) mediated by ACh release through activation of nAChRs present on DANs. Any plasticity – such as habituation or its state-dependence for example – could also involve differential ACh release, and potential effect of ACh on DA release.

#### **5.4.2. No difference in shock response in the presence or absence of ACh in the environment in fed flies: technical limitations or biological process?**

We found that DA release evoked by shock did not change in the presence of ACh in the environment. This could be due, as discussed above before, to a potential lack of ACh release following prolonged exposure to an odour (1 min) due to habituation. It could also be due to the slow frame rate used in these experiments. Indeed, despite a lot of optimisations, we were still limited to 6 Hz for these experiments. As demonstrated in **Chapter 4**, in mice the effect of ACh on DA release through nAChRs occurs within ms of one another when stimulation is synchronised across the circuit. In the context of *in vivo* recordings in flies, where activity across the network is not as highly synchronised as on brain slices, the lack of temporal resolution could prevent us from seeing more subtle changes in the DA release.

The lack of phenotype observed could also be due to the lack of precision with which shocks are detected in our set up. Indeed, the shock delivery varies greatly from shock to shock as all shocks are not necessarily *felt* by the fly because they can avoid shocks by changing their leg placement on the grid. Although I implemented a way to detect the current when it travels through the fly, I could only extract visual approximate of how much current the fly receives, and not actual current values. Therefore, the intensity of the shock could not

be used as a parameter to normalise their response. Plus, since they can move their legs on the platform, the length of the shock could also vary. Taken together, these factors could greatly hinder our capacity to compare the signal properly between conditions in relation the amount of current the fly actually receives.

Despite these experimental caveats, we revealed that in hungry flies, DA release in response to foot shock was lowered when an odour was delivered at the same time. Since DA release does not habituate to the second odour presentation in hungry animals, the lower DA release in the shock+ odour condition could potentially be due to DA vesicle depletion, which we have not tested for. Finally, further experiments are needed to test the involvement of nAChRs in this potential axo-axonal cholinergic modulation of DA release. For instance, the use of RNAi for specific nAChRs subunits expressed in DA neurons could help assess whether this effect is mediated through nAChRs, and give clarity on what nAChR-subunit might be involved in this effect.

# 6

## General Discussion

Midbrain dopaminergic neurons project their extensively branched axons to the striatum where DA transmission is involved in shaping associative learning and RPE, sensory-motor processing and goal-directed behaviour amongst many other functions. In addition to being driven through APs coming from DA cell bodies in the midbrain, DA release is also highly modulated locally from neurotransmitters present within the striatum, and in particular by ACh released from ChIs. Unravelling mechanisms through which DA transmission is governed is therefore critical to the understand how DA can shape behaviours. Furthermore, understanding these mechanisms in healthy animals could provide insight on how they could be dysregulated in disease, and potentially help identify new targets for therapies.

The main aim of this body of work was to explore the downstream mechanisms by which ACh shapes DA release through nAChRs. We hypothesised that nAChRs exert their dual effect – either promoting or hindering DA release – by modifying the DA axonal membrane excitability. In this context, we first explored the potential role of VGKCs (**chapter 3**) in the mechanisms downstream of nAChR activation. We tested whether activation of nAChRs at DA axons provoked a prolonged inactivation of VGKCs that incapacitated the membrane from producing subsequent spikes, leaving an apparent inhibition in DA release. Although altering VGKCs function did affect DA release, our data suggested that VGKCs are unlikely to be supporting the dual effect of nAChR on DA release. Next, in **chapter 4**, we investigated the role of VGSCs. We found that nAChR activation provokes a prolonged depolarisation at DA axonal membrane, and that lower availability of VGSCs following nAChRs activation limits the amplitude of subsequent population spikes during a pulse-train stimulation, which in turn limits subsequent  $\text{Ca}^{2+}$  entry and consequently also limits DA

release. In **chapter 5**, we streamlined a novel approach to study the ACh-DA interaction by investigating these potentially highly conserved mechanisms in another simple brain of *Drosophila melanogaster*. The MB possess similar functions to the basal ganglia in multiple regards, and hosts relevant ACh-DA connections whose behavioural relevance remain to be confirmed. Our preliminary data suggest a potential internal-state dependence of axo-axonal cholinergic modulation of DA release within the MB. In this chapter, I will discuss the main findings presented in the experimental chapters and their implications.

## 6.1. Revisiting the involvement of VGKCs in the nAChR-mediated control of DA release

In this thesis, we investigated the potential role two types of voltage-gated ion channels in the mechanisms modulating DA release downstream of nAChR activation. We first explored the potential role of VGKCs (**chapter 3**) and next assessed the role of VGSCs (**chapter 4**). Although, our experiments from **chapter 3** blocking VGKCs in the presence or absence of nAChR control were inconclusive, our experiments our work in **chapter 4** suggested that activity of nAChRs triggers a prolonged depolarisation at DA axons, which limits the pool of VGSCs available to evoke DA release by pulse-train stimulation. Thus, in the light of these findings, the role of VGKCs in the nAChR-mediated modulation of DA release needs to be revisited. Indeed, blockage of VGKCs could have impacted the initial spike produced by electrical stimulation corresponding to the direct activation of DA axon, but it may have also changed the dynamics of the prolonged depolarisation caused by the activation of nAChRs at DA axons. Both of these effects might be confounding factors that we could not disentangle by the sole use of FCV in **chapter 3**, but may now be re-assessed

using the voltage imaging technique set up in **chapter 4**. Differences in VGKCs present on the DA axonal membrane could also be the cause of the regional differences observed in the shape of the voltage sensor signal in **chapter 4**. Plus, specific VGKCs could be involved in mediating the duration of the prolonged depolarisation.

## **6.2. Potential *in vivo* implications of the prolonged depolarisation mediated by nAChRs activation at DA axons**

### **6.2.1. The controversial role of nAChRs on DA release *in vivo***

In *ex vivo* striatal mouse brain slices, DA and ACh strongly and bidirectionally regulate each other. Synchronised activity of ChIs activates nAChR receptors located on DA axons which depolarises the axons (sometimes reaching spiking threshold), and consequently triggering subsequent DA release (Threlfell et al. 2012; Cacheo et al. 2012; Liu et al. 2022; Kramer et al. 2022). However, subsequent DA release is hindered by nAChR activation (Rice and Cragg 2004; Zhang and Sulzer 2004; Zhang and Cragg 2017). In turn, DA has been demonstrated to exert an inhibitory effect on ACh release mainly through D<sub>2</sub>Rs located on ChIs (Chuhma et al. 2014; Straub et al. 2014). This inhibitory connection has been hypothesised to mediate the pause-response exhibited in by ChIs in response to reward stimulus (Goldberg and Reynolds 2011; Gallo et al. 2022), but studies have found that this pause can also be induced by glutamatergic afferents to the ChIs (Zhang et al. 2018b).

However, the study of the behavioural relevance of these interactions *in vivo*, and the identification of when, where and how they potentially occur has revealed challenging, in part due to the complexity of the striatal network. The D<sub>2</sub>R-mediated inhibition of ACh release has been demonstrated to occur both *ex vivo* (Maurice et al. 2004; Deng et al. 2007) and *in vivo*

where it is involved in decision making and learning (Aosaki et al. 1994; Gallo et al. 2022; Chantranupong et al. 2023). However, studies investigating nAChRs effects on DA release *in vivo* depict a much more complex picture. Indeed, some studies reveal a role for nAChRs as promoting DA release (Kramer et al. 2022; Mohebi et al. 2023). Kramer et al. 2022 demonstrates using patch-clamp at DA axons of mice (DMS) or macaques (Putamen) that application of  $\beta 2$ -containing nAChR antagonist DH $\beta$ E abolishes spontaneous and evoked axonal excitatory postsynaptic potentials (axEPSPs) (Kramer et al. 2022), suggesting that nAChRs at exert an excitatory effect on DA axons. Mohebi et al. demonstrated that in NAcC of unrestrained rats, optogenetic activation of ChIs could evoke DA release measured by GRAB<sub>DA</sub>. This study also highlighted interesting co-increase of both ACh and DA release upon receiving a cue associated with unexpected reward or ramping in both ACh and DA release during approach to a potential reward. During these ‘release’ ramps, no changes in firing rate occurred at the cell bodies of DA neurons, indicating a local control for this increased release, likely through nAChR activation, suggesting a key role for ChIs and local nAChR-mediate DA release in motivated behaviours (Mohebi et al. 2023).

Other studies however have attributed an inhibiting effect of nAChRs on striatal DA release *in vivo* (Collins et al. 2016; Zhang et al. 2025). Using FCV in NAcC of male rats, Collins et al. (Collins et al. 2016) found that antagonising nAChRs increased cue evoked DA release following Pavlovian conditioning and unexpected reward response and cue, which contrasts with the findings of Mohebi et al., 2023 in motivated behaviours. Finally, recent work using GRAB<sub>DA</sub> to report DA release in DLS of anaesthetised mice showed an increase in basal DA tone upon DH $\beta$ E application, suggesting that nAChRs inhibit tonic DA release (Zhang et al. 2025). Although the authors also drive APs from midbrain DA cell bodies using electrical stimulation, they do not comment on how potential changes in tonic DA release affect the



DA response to their stimulations and therefore the effect on phasic release remains to be investigated.

Finally, studies have also reported an absence of correlation between release patterns of ACh and DA (Krok et al. 2023). Using GRAB sensors to detect release of both ACh and DA, they demonstrated that coordination between DA and ACh release arises from extra-striatal sources suggesting no dependence of DA release on nAChR activation. Other work by Chantranupong et al. 2023 also reported that preventing ACh release did not affect DA transient in response to reward stimulus (Chantranupong et al. 2023), also suggesting no role for nAChR modulation of phasic DA release.

Taken together, these studies do not come to a consensus for the potential role of nAChR activation on striatal DA axons *in vivo*. Although these studies have covered different regions of the striatum and different tasks, most of them used the same tools to monitor ACh and DA release namely the GRAB sensors and occasionally FCV. As suggested in this thesis, nAChR effect on DA axonal activity and DA release occurs within few milliseconds. Therefore, FCV and GRAB imaging might not possess the sensitivity and temporal resolution necessary to investigate these processes. Nevertheless, these studies highlight the importance of nACh and DA signalling in striatal processing in multiple behavioural contexts. Further investigations are needed to elucidate the role of these interactions *in vivo*.

### **6.2.2. Time-course and kinetics of nAChR-mediated depolarisation at a population of striatal DA axons**

In Chapter 4, we found that in *ex vivo* mouse brain slices, activation of nAChRs at DA axons leads to a prolonged depolarisation likely to be due to either Na<sup>+</sup> entry through the cation-permeable pore of nAChR or to asynchronous spiking across the DA axonal

population within our ROI or due to both (as discussed in **section 4.4**). We found that this prolonged depolarisation arises approximately 3 to 4 ms after stimulation and lasts approximately 150 ms. However, transposition of these time-courses to *in vivo* scenarios might not be that simple. Indeed, we perform our experiments at a temperature of 32°C which is lower than mammalian body temperatures that are closer to 36-37°C. Since kinetics of biological processes heavily rely on the temperature of their environment, it is highly probable that the time-courses described in this study are modified *in vivo*. Although increasing temperatures to body temperature usually shortens time-courses of biological processes, the amplitude of currents involved might be affected in various manners. A study using whole cell patch-clamp recording has reported that currents evoked by ACh through  $\alpha 7$ -containing nAChRs could be reduced by raising the temperature from 22°C to 37° (Sitzia et al. 2011). Therefore, the translation of our data to *in vivo* scenarios needs to be taken with care, since time-courses as well as amplitude of these process might be different.

### 6.2.3. What can we infer about desensitisation of nAChRs?

In **Chapter 4 (section 4.3.1.1iii)** we attempted to investigate the time-course of the nAChRs -mediated prolonged depolarisation and probe the duration over which these channels are desensitised, *ie.* unable to be activated despite presence of ACh in the environment. However, our study was limited by strong STD of ACh release evoked by electrical stimulation in striatal slice. Because of this confound, we were unable to assess the time-course over which the nAChRs are desensitised. In the literature, very little information about recovery from desensitisation is available for  $\alpha 4\beta 2$ -containing nAChRs (Dani et al. 2000; Giniatullin et al. 2005), and would therefore require further investigation.

Nevertheless, let's consider that the time over which nAChRs are desensitised corresponds at the very least to the duration of the prolonged depolarisation (approximately 150 ms). Given that *in vivo* ChIs tonically fire at rates between 3 and 10 Hz (Goldberg and Reynolds 2011), it would indicate that nAChR could be constantly desensitised by ACh *in vivo* and therefore effectively unable to play a role in the modulation of DA release. However, optogenetic activation of ChIs (anaesthetised mice or in awake rats) evokes DA release from striatal DA axons (Cachope et al. 2012; Mohebi et al. 2023), suggesting that nAChRs are not all desensitised in these contexts. Thus, tonic ACh release *in vivo* might not be sufficient to activate *all* nAChRs present at DA axons as opposed to evoked ACh release.

Although the desensitisation kinetics of nAChRs remain unclear, it seems unlikely that *in vivo* nAChRs would be mainly in a desensitised state and thereby not do not exert a modulatory effect on DA release. More studies would be needed to determine both the desensitisation time-course of the nAChRs present in the striatum and the role they play on tonic/basal DA release.

#### **6.2.4. The involvement of VGSCs in nAChR-mediated STD of DA release could also restrict signal propagation at DA axons**

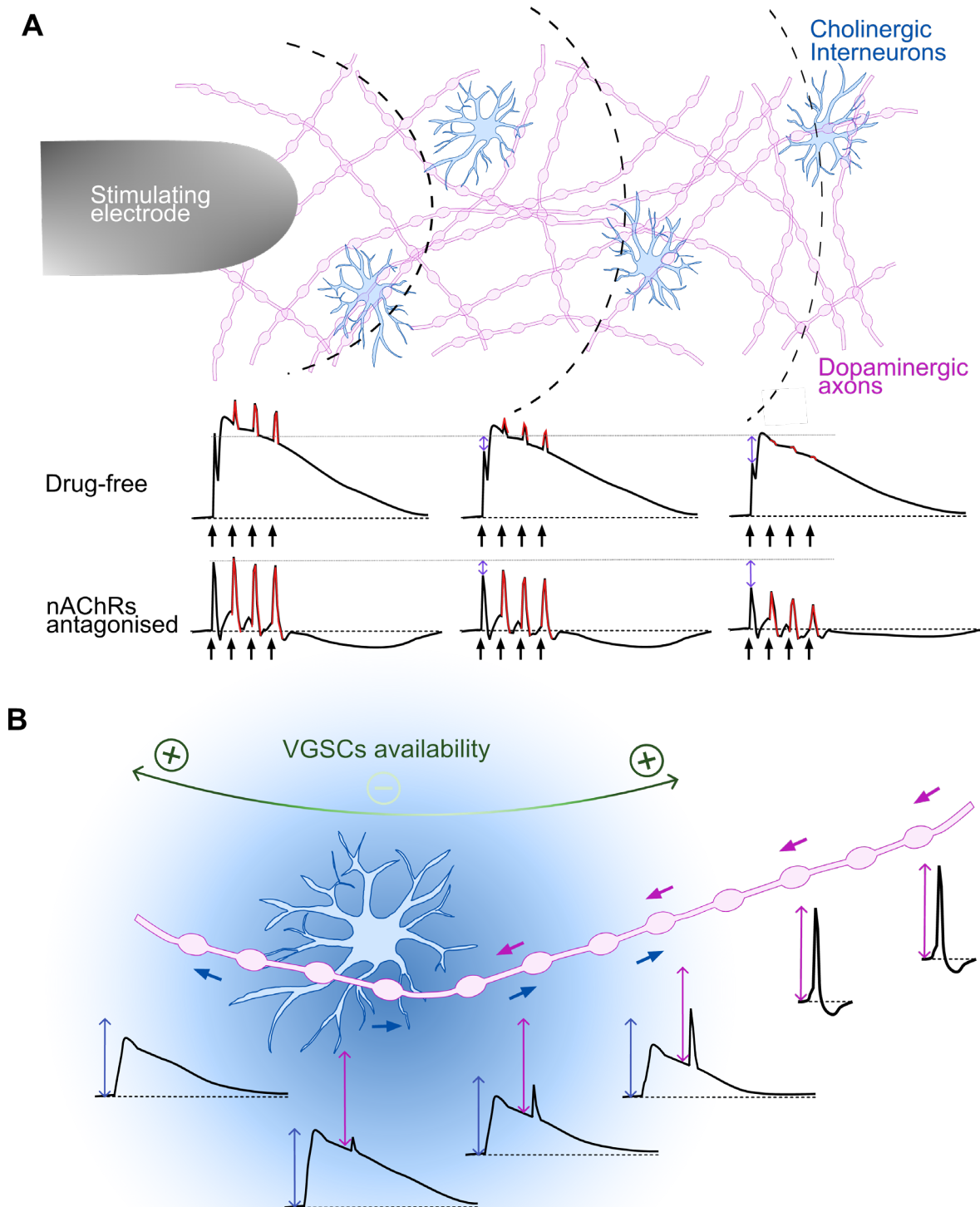
We found that upon nAChRs activation, nAChRs evoke a prolonged depolarisation which first supports DA release but also compromise the pool of VGSCs available to be recruited for the formation of a subsequent population spike during pulse trains, effectively hindering release at subsequent pulses.

VGSCs are known to be involved in the upstroke of AP and are necessary for its faithful propagation along axons (Debanne 2004; Rama et al. 2018). Thus, our data might

suggest that, upon repeated stimulation (through pulse-trains) nAChR activity might effectively locally restrict the propagation of AP along the DA axons by limiting the availability of VGSCs (**Figure 6.1 A**). This could affect incoming APs generated at the cell body, and activity of nAChRs could therefore act as a local filter (**Figure 6.1 B**). In addition, DA axons are extremely branched and enriched in varicosities, which can render faithful propagation of APs difficult.

This hypothesis seems contradictory with the results found by Matityahu et al. 2023 (Matityahu et al. 2023) which suggested that nAChR activation spatially extended the range of DA release evoked by a single pulse electrical stimulation and read out using GRAB<sub>DA2m</sub> in striatal mouse brain slices. Although the spatial range of DA release seems initially extended by single pulse electrical stimulation, data from **chapter 4** suggests that within the range of nAChR activation, VGSCs are highly unavailable to propagate any further stimulation. Together these data can be reconciled by considering that synchronous activation of nAChRs initially extends the spatial range of DA release, but this ultimately will also limit faithful propagation incoming APs (**Figure 6.1 A**). Finally, the experiments in Matityahu et al. 2023 engage all neurons in the vicinity of the stimulating electrode in a highly synchronous manner, which is not realistic *in vivo* and it is therefore possible that the extended range of DA release is highly exaggerated by the experimental parameters.

Further *ex vivo* experiments using the voltage sensor and imaging at different ROIs located at increasing distances from the stimulating electrode could help understand the dynamics of signal propagation in the presence (drug-free condition) or absence (DH $\beta$ E condition) of nAChR control. Further investigation *in vivo* would be needed to test whether AP generated at the cell bodies of DA neurons in the midbrain could indeed be attenuated by local ChI activity.



**Figure 6.1: Schematic representing potential scenarios in which signal propagation is altered by nAChR activity on DA axons.**

**A:** Propagation of signal in a population of DA axons evoked by electrical stimulation in mouse brain slices. Electrical stimulation activates all cells in its vicinity in a synchronous manner. The dotted curved lines represent the propagation of the electric signal in general, and below are the corresponding *hypothetical* traces reported by voltage sensors expressed in DA neurons in drug-free condition or when nAChRs are antagonised. Initial spike height is reduced with increasing distance from stimulating electrode both when nAChRs are active

and when they are not. However, the height of the subsequent population spikes decays faster with increasing distance from the stimulating electrode when nAChRs are active partly because they are initially smaller but also because the unavailability of VGSCs (due to prolonged depolarisation cause by nAChRs) prevents them from spreading further. The amplitude of the nAChR prolonged depolarisation might also be reduced with increasing distance from the stimulating electrode.

**B:** Hypothetical voltage sensor imaging along a DA axon when activity at ChIs and DA axons is asynchronous. A ChI releases ACh (represented by the blue hue) which provoked a prolong depolarisation at DA axons, and few ms later an AP is being transmitted along a DA axon (from right to left). When the AP propagation reaches the part of the axons that underwent nAChRs activity, VGSCs are unavailable to support the propagation of that AP along the axon. The blue and pink arrows adjacent to the hypothetical voltage sensor traces represent the initial height of the nAChR-mediated prolonged depolarisation (at its highest point) and the AP amplitude respectively.

---

### 6.3. Dopamine and ACh in Parkinson's disease

Parkinson's disease (PD), affecting approximately 6 million individuals worldwide, is the second most common neurodegenerative disease (Rocca 2018).

PD patients can exhibit a triad of sometimes highly debilitating motor symptoms, such a resting tremor, bradykinesia and rigidity, as well as many non-motor symptoms including loss of smell, sleep disturbances and dementia (Samii et al. 2004; Park and Stacy 2009). Although this disease is mostly considered an idiopathic disorder, rare genetically inherited forms exists (Singleton et al. 2003). The hallmark of PD is the degeneration of DA neurons whose cell bodies originate from the SNc in the midbrain which result in denervation in the DLS (Hornykiewicz 1998; Sulzer and Surmeier 2013). A second common feature found in PD patients are the Lewy bodies, constituted by the accumulation of protein aggregates – mainly comprised of  $\alpha$ -synuclein- in the cytoplasm of SNc DA neurons (Spillantini et al. 1998). Although the role of these aggregates, and whether or not the cell death precedes their formation is unclear, they have been shown to disrupt presynaptic function including DA release (Anwar et al. 2011; Janezic et al. 2013). DA denervation to the DLS provokes aberrant downstream signalling, disrupting basal ganglia activity that leads to the motor symptoms of PD (Graybiel et al. 1994a).

Historically, the ACh-DA interaction within the striatum has been seen as an antagonising one, from the realisation that motor symptoms in PD can be treated by dopaminergic drugs (such as L-DOPA), or anti-cholinergic agents to partly restore striatal output to other basal ganglia nuclei (Barbeau 1962). This led to the DA-ACh imbalance theory, whereby DA was believed to mainly exert a powerful inhibitory effect on ChIs – and henceforth ACh release. This would create strong disinhibition in PD, from the loss of DA neurons, leaving the striatum in a hypercholinergic state. More recent studies – conducted in a mouse model for early PD – have nuanced this view and suggested that reinstating healthy burst firing patterns in ChIs in a model of PD – therefore enhancing ACh release – could also alleviate motor-impairments (Cai et al. 2021). The authors suggested it would in turn enhance DA through activity at nAChRs. Furthermore, the aberrant ChI activity observed in PD was shown to arise from downregulation of metabotropic Glutamate receptor 1 (mGluR1) due to the loss of DA terminals which co-release glutamate (Cai et al. 2021). Further investigation on the nature of the dysregulated interaction between ACh and DA release in the striatum, and in particular the potential involvement of nAChRs would be necessary. In particular, we could apply the use of our voltage sensor in mouse models of PD to interrogate whether the membrane voltage of DA axons behaves differently from healthy axons, and if the nAChR-mediated effects on DA axons are similar in healthy animals compared to PD models.

## 6.4. Conclusion

Overall, the data presented in this thesis outlined the potential mechanism through which nAChRs exert their dual activation on striatal DA release by either promoting or preventing DA release and described the crucial involvement of VGSCs in this process. These findings

were enabled by the new use of the GEVI ASAP5f, which allow monitoring of the membrane voltage of a group of DA axons within the mouse striatum. Finally, parallel work on *Drosophila melanogaster* suggests a potential state-dependent modulation of DA release by ACh within the  $\gamma$ 1-pedc compartment of the MB.



## References

- Abeliovich A, Schmitz Y, Fariñas I, et al (2000) Mice lacking alpha-synuclein display functional deficits in the nigrostriatal dopamine system. *Neuron* 25:239–252. [https://doi.org/10.1016/s0896-6273\(00\)80886-7](https://doi.org/10.1016/s0896-6273(00)80886-7)
- Aimon S, Katsuki T, Jia T, et al (2019) Fast near-whole-brain imaging in adult *Drosophila* during responses to stimuli and behavior. *PLoS Biology* 17:e2006732. <https://doi.org/10.1371/journal.pbio.2006732>
- Albuquerque EX, Pereira EFR, Alkondon M, Rogers SW (2009) Mammalian Nicotinic Acetylcholine Receptors: From Structure to Function. *Physiol Rev* 89:73–120. <https://doi.org/10.1152/physrev.00015.2008>
- Alkondon M, Pereira EF, Eisenberg HM, Albuquerque EX (1999) Choline and selective antagonists identify two subtypes of nicotinic acetylcholine receptors that modulate GABA release from CA1 interneurons in rat hippocampal slices. *J Neurosci* 19:2693–2705. <https://doi.org/10.1523/JNEUROSCI.19-07-02693.1999>
- Anderegg A, Poulin J-F, Awatramani R (2015) Molecular Heterogeneity of Midbrain Dopaminergic Neurons – Moving Toward Single Cell Resolution. *FEBS Lett* 589:3714–3726. <https://doi.org/10.1016/j.febslet.2015.10.022>
- Anwar S, Peters O, Millership S, et al (2011) Functional Alterations to the Nigrostriatal System in Mice Lacking All Three Members of the Synuclein Family. *J Neurosci* 31:7264–7274. <https://doi.org/10.1523/JNEUROSCI.6194-10.2011>
- Aoki S, Liu AW, Zucca A, et al (2015) Role of Striatal Cholinergic Interneurons in Set-Shifting in the Rat. *J Neurosci* 35:9424–9431. <https://doi.org/10.1523/JNEUROSCI.0490-15.2015>
- Aosaki T, Graybiel AM, Kimura M (1994) Effect of the Nigrostriatal Dopamine System on Acquired Neural Responses in the Striatum of Behaving Monkeys. *Science* 265:412–415. <https://doi.org/10.1126/science.8023166>
- Apicella P, Legallet E, Trouche E (1997) Responses of tonically discharging neurons in the monkey striatum to primary rewards delivered during different behavioral states. *Exp Brain Res* 116:456–466. <https://doi.org/10.1007/PL00005773>
- Armstrong-James M, Millar J, Kruk ZL (1980) Quantification of noradrenaline iontophoresis. *Nature* 288:181–183. <https://doi.org/10.1038/288181a0>
- Arroyo S (2007) Rofinamide. *Neurotherapeutics* 4:155–162. <https://doi.org/10.1016/j.nurt.2006.11.006>
- Aso Y, Grübel K, Busch S, et al (2009) The Mushroom Body of Adult *Drosophila* Characterized by GAL4 Drivers. *Journal of Neurogenetics* 23:156–172. <https://doi.org/10.1080/01677060802471718>

- Aso Y, Hattori D, Yu Y, et al (2014a) The neuronal architecture of the mushroom body provides a logic for associative learning. *Elife* 3:e04577. <https://doi.org/10.7554/eLife.04577>
- Aso Y, Herb A, Ogueta M, et al (2012) Three Dopamine Pathways Induce Aversive Odor Memories with Different Stability. *PLoS Genet* 8:e1002768. <https://doi.org/10.1371/journal.pgen.1002768>
- Aso Y, Sitaraman D, Ichinose T, et al (2014b) Mushroom body output neurons encode valence and guide memory-based action selection in *Drosophila*. *Elife* 3:e04580. <https://doi.org/10.7554/eLife.04580>
- Aso Y, Siwanowicz I, Bräcker L, et al (2010) Specific Dopaminergic Neurons for the Formation of Labile Aversive Memory. *Current Biology* 20:1445–1451. <https://doi.org/10.1016/j.cub.2010.06.048>
- Assous M (2021) Striatal cholinergic transmission. Focus on nicotinic receptors' influence in striatal circuits. *Eur J Neurosci* 53:2421–2442. <https://doi.org/10.1111/ejn.15135>
- Augustine GJ (1990) Regulation of transmitter release at the squid giant synapse by presynaptic delayed rectifier potassium current. *The Journal of Physiology* 431:343–364. <https://doi.org/10.1113/jphysiol.1990.sp018333>
- Avshalumov MV, Patel JC, Rice ME (2008) AMPA Receptor-Dependent H<sub>2</sub>O<sub>2</sub> Generation in Striatal Medium Spiny Neurons But Not Dopamine Axons: One Source of a Retrograde Signal That Can Inhibit Dopamine Release. *Journal of Neurophysiology*. <https://doi.org/10.1152/jn.90548.2008>
- Avvisati R, Kaufmann A-K, Young CJ, et al (2024) Distributional coding of associative learning in discrete populations of midbrain dopamine neurons. *Cell Rep* 43:114080. <https://doi.org/10.1016/j.celrep.2024.114080>
- Bäckman CM, Malik N, Zhang Y, et al Characterization of a mouse strain expressing Cre recombinase from the 3' untranslated region of the dopamine transporter locus. <https://doi.org/10.1002/dvg.20228>
- Balleine BW, Delgado MR, Hikosaka O (2007) The Role of the Dorsal Striatum in Reward and Decision-Making. *J Neurosci* 27:8161–8165. <https://doi.org/10.1523/JNEUROSCI.1554-07.2007>
- Barbeau A (1962) The Pathogenesis of Parkinson's Disease: A New Hypothesis. *Can Med Assoc J* 87:802–807
- Barnes S, Hille B (1988) Veratridine modifies open sodium channels. *Journal of General Physiology* 91:421–443. <https://doi.org/10.1085/jgp.91.3.421>

- Baukrowitz T, Yellen G (1995) Modulation of K<sup>+</sup> current by frequency and external [K<sup>+</sup>]: A tale of two inactivation mechanisms. *Neuron* 15:951–960. [https://doi.org/10.1016/0896-6273\(95\)90185-X](https://doi.org/10.1016/0896-6273(95)90185-X)
- Beaulieu J-M, Gainetdinov RR (2011) The physiology, signaling, and pharmacology of dopamine receptors. *Pharmacol Rev* 63:182–217. <https://doi.org/10.1124/pr.110.002642>
- Berke JD (2018) What does dopamine mean? *Nat Neurosci* 21:787–793. <https://doi.org/10.1038/s41593-018-0152-y>
- Berry JA, Cervantes-Sandoval I, Chakraborty M, Davis RL (2015) Sleep facilitates memory by blocking dopamine neuron mediated forgetting. *Cell* 161:1656–1667. <https://doi.org/10.1016/j.cell.2015.05.027>
- Bérubé-Carrière N, Guay G, Fortin GM, et al (2012) Ultrastructural characterization of the mesostriatal dopamine innervation in mice, including two mouse lines of conditional VGLUT2 knockout in dopamine neurons. *European Journal of Neuroscience* 35:527–538. <https://doi.org/10.1111/j.1460-9568.2012.07992.x>
- Block ER, Nuttle J, Balcita-Pedicino JJ, et al (2015) Brain Region-Specific Trafficking of the Dopamine Transporter. *J Neurosci* 35:12845–12858. <https://doi.org/10.1523/JNEUROSCI.1391-15.2015>
- Bonnevie T, Zaghoul KA (2019) The Subthalamic Nucleus: Unravelling New Roles and Mechanisms in the Control of Action. *Neuroscientist* 25:48–64. <https://doi.org/10.1177/1073858418763594>
- Bonsi P, Cuomo D, Ding J, et al (2007) Endogenous Serotonin Excites Striatal Cholinergic Interneurons via the Activation of 5-HT<sub>2C</sub>, 5-HT<sub>6</sub>, and 5-HT<sub>7</sub> Serotonin Receptors: Implications for Extrapyrmidal Side Effects of Serotonin Reuptake Inhibitors. *Neuropsychopharmacol* 32:1840–1854. <https://doi.org/10.1038/sj.npp.1301294>
- Bostock H, Sears TA, Sherratt RM (1981) The effects of 4-aminopyridine and tetraethylammonium ions on normal and demyelinated mammalian nerve fibres. *The Journal of Physiology* 313:301–315. <https://doi.org/10.1113/jphysiol.1981.sp013666>
- Bouabid S, Zhang L, Vu M-AT, et al (2025) Distinct spatially organized striatum-wide acetylcholine dynamics for the learning and extinction of Pavlovian associations. 2024.07.10.602947
- Brand AH, Perrimon N (1993) Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* 118:401–415. <https://doi.org/10.1242/dev.118.2.401>
- Brill-Weil SG, Kramer PF, Yanez A, et al (2024) Presynaptic GABAA receptors control integration of nicotinic input onto dopaminergic axons in the striatum. *bioRxiv* 2024.06.25.600616. <https://doi.org/10.1101/2024.06.25.600616>

- Brimblecombe KR, Cragg SJ (2015) Substance P Weights Striatal Dopamine Transmission Differently within the Striosome-Matrix Axis. *J Neurosci* 35:9017–9023. <https://doi.org/10.1523/JNEUROSCI.0870-15.2015>
- Brimblecombe KR, Cragg SJ (2017) The Striosome and Matrix Compartments of the Striatum: A Path through the Labyrinth from Neurochemistry toward Function. *ACS Chem Neurosci* 8:235–242. <https://doi.org/10.1021/acscchemneuro.6b00333>
- Brimblecombe KR, Threlfell S, Dautan D, et al (2018) Targeted Activation of Cholinergic Interneurons Accounts for the Modulation of Dopamine by Striatal Nicotinic Receptors. *eNeuro* 5:. <https://doi.org/10.1523/ENEURO.0397-17.2018>
- Brody DL, Yue DT (2000) Release-Independent Short-Term Synaptic Depression in Cultured Hippocampal Neurons. *J Neurosci* 20:2480–2494. <https://doi.org/10.1523/JNEUROSCI.20-07-02480.2000>
- Brown MTC, Tan KR, O'Connor EC, et al (2012) Ventral tegmental area GABA projections pause accumbal cholinergic interneurons to enhance associative learning. *Nature* 492:452–456. <https://doi.org/10.1038/nature11657>
- Burke CJ, Huetteroth W, Oswald D, et al (2012) Layered reward signaling through octopamine and dopamine in *Drosophila*. *Nature* 492:433–437. <https://doi.org/10.1038/nature11614>
- Burnett CJ, Li C, Webber E, et al (2016) Hunger-driven motivational state competition. *Neuron* 92:187–201. <https://doi.org/10.1016/j.neuron.2016.08.032>
- Cachope R, Mateo Y, Mathur BN, et al (2012) Selective activation of cholinergic interneurons enhances accumbal phasic dopamine release: setting the tone for reward processing. *Cell Rep* 2:33–41. <https://doi.org/10.1016/j.celrep.2012.05.011>
- Cai X, Liu C, Tsutsui-Kimura I, et al (2024) Dopamine dynamics are dispensable for movement but promote reward responses. *Nature* 635:406–414. <https://doi.org/10.1038/s41586-024-08038-z>
- Cai Y, Nielsen BE, Boxer EE, et al (2021) Loss of nigral excitation of cholinergic interneurons contributes to parkinsonian motor impairments. *Neuron* 109:1137–1149.e5. <https://doi.org/10.1016/j.neuron.2021.01.028>
- Caillé I, Dumartin B, Bloch B (1996) Ultrastructural localization of D1 dopamine receptor immunoreactivity in rat striatonigral neurons and its relation with dopaminergic innervation. *Brain Research* 730:17–31. [https://doi.org/10.1016/0006-8993\(96\)00424-6](https://doi.org/10.1016/0006-8993(96)00424-6)
- Calabresi P, Picconi B, Tozzi A, et al (2014) Direct and indirect pathways of basal ganglia: a critical reappraisal. *Nat Neurosci* 17:1022–1030. <https://doi.org/10.1038/nn.3743>

- Carvelli L, McDonald PW, Blakely RD, DeFelice LJ (2004) Dopamine transporters depolarize neurons by a channel mechanism. *Proceedings of the National Academy of Sciences* 101:16046–16051. <https://doi.org/10.1073/pnas.0403299101>
- Centonze D, Grande C, Saulle E, et al (2003) Distinct Roles of D1 and D5 Dopamine Receptors in Motor Activity and Striatal Synaptic Plasticity. *J Neurosci* 23:8506–8512. <https://doi.org/10.1523/JNEUROSCI.23-24-08506.2003>
- Cervantes-Sandoval I, Phan A, Chakraborty M, Davis RL (2017) Reciprocal synapses between mushroom body and dopamine neurons form a positive feedback loop required for learning. *eLife* 6:e23789. <https://doi.org/10.7554/eLife.23789>
- Chamaon K, Schulz R, Smalla K-H, et al (2000) Neuronal nicotinic acetylcholine receptors of *Drosophila melanogaster*: the  $\alpha$ -subunit D $\alpha$ 3 and the  $\beta$ -type subunit ARD co-assemble within the same receptor complex. *FEBS Letters* 482:189–192. [https://doi.org/10.1016/S0014-5793\(00\)02057-3](https://doi.org/10.1016/S0014-5793(00)02057-3)
- Chamberland S, Yang HH, Pan MM, et al (2017) Fast two-photon imaging of subcellular voltage dynamics in neuronal tissue with genetically encoded indicators. *eLife* 6:e25690. <https://doi.org/10.7554/eLife.25690>
- Chantranupong L, Beron CC, Zimmer JA, et al (2023) Dopamine and glutamate regulate striatal acetylcholine in decision-making. *Nature* 621:577–585. <https://doi.org/10.1038/s41586-023-06492-9>
- Chesselet MF, Reisine TD (1983) Somatostatin regulates dopamine release in rat striatal slices and cat caudate nuclei. *J Neurosci* 3:232–236. <https://doi.org/10.1523/JNEUROSCI.03-01-00232.1983>
- Choquet D, Korn H (1992) Mechanism of 4-aminopyridine action on voltage-gated potassium channels in lymphocytes. *J Gen Physiol* 99:217–240. <https://doi.org/10.1085/jgp.99.2.217>
- Chuhma N, Mingote S, Moore H, Rayport S (2014) Dopamine Neurons Control Striatal Cholinergic Neurons via Regionally Heterogeneous Dopamine and Glutamate Signaling. *Neuron* 81:901–912. <https://doi.org/10.1016/j.neuron.2013.12.027>
- Claridge-Chang A, Roorda RD, Vrontou E, et al (2009) Writing memories with light-addressable reinforcement circuitry. *Cell* 139:405–415. <https://doi.org/10.1016/j.cell.2009.08.034>
- Clements M (2008) Control of striatal dopamine transmission by cocaine and nicotine: acetylcholine and dopamine interactions. Thesis D.Phil.--University of Oxford.
- Cognigni P, Felsenberg J, Waddell S (2018) Do the right thing: neural network mechanisms of memory formation, expression and update in *Drosophila*. *Curr Opin Neurobiol* 49:51–58. <https://doi.org/10.1016/j.conb.2017.12.002>

- Cohn R, Morantte I, Ruta V (2015) Coordinated and Compartmentalized Neuromodulation Shapes Sensory Processing in *Drosophila*. *Cell* 163:1742–1755. <https://doi.org/10.1016/j.cell.2015.11.019>
- Collins AL, Aitken TJ, Greenfield VY, et al (2016) Nucleus Accumbens Acetylcholine Receptors Modulate Dopamine and Motivation. *Neuropsychopharmacol* 41:2830–2838. <https://doi.org/10.1038/npp.2016.81>
- Condon MD, Platt NJ, Zhang Y-F, et al (2019) Plasticity in striatal dopamine release is governed by release-independent depression and the dopamine transporter. *Nature Communications* 10:4263. <https://doi.org/10.1038/s41467-019-12264-9>
- Contant C, Umbriaco D, Garcia S, et al (1996) Ultrastructural characterization of the acetylcholine innervation in adult rat neostriatum. *Neuroscience* 71:937–947. [https://doi.org/10.1016/0306-4522\(95\)00507-2](https://doi.org/10.1016/0306-4522(95)00507-2)
- Corringer PJ, Le Novère N, Changeux JP (2000) Nicotinic receptors at the amino acid level. *Annu Rev Pharmacol Toxicol* 40:431–458. <https://doi.org/10.1146/annurev.pharmtox.40.1.431>
- Cragg SJ (2003) Variable Dopamine Release Probability and Short-Term Plasticity between Functional Domains of the Primate Striatum. *J Neurosci* 23:4378–4385. <https://doi.org/10.1523/JNEUROSCI.23-10-04378.2003>
- Cragg SJ, Rice ME (2004) DANCING past the DAT at a DA synapse. *Trends in Neurosciences* 27:270–277. <https://doi.org/10.1016/j.tins.2004.03.011>
- Croset V, Treiber CD, Waddell S (2018) Cellular diversity in the *Drosophila* midbrain revealed by single-cell transcriptomics. *Elife* 7:. <https://doi.org/10.7554/eLife.34550>
- Czarniak N, Kamińska J, Matowicka-Karna J, Koper-Lenkiewicz OM (2023) Cerebrospinal Fluid—Basic Concepts Review. *Biomedicines* 11:1461. <https://doi.org/10.3390/biomedicines11051461>
- Dani JA (2015) Neuronal Nicotinic Acetylcholine Receptor Structure and Function and Response to Nicotine. *Int Rev Neurobiol* 124:3–19. <https://doi.org/10.1016/bs.irn.2015.07.001>
- Dani JA, Mayer ML (1995) Structure and function of glutamate and nicotinic acetylcholine receptors. *Curr Opin Neurobiol* 5:310–317. [https://doi.org/10.1016/0959-4388\(95\)80043-3](https://doi.org/10.1016/0959-4388(95)80043-3)
- Dani JA, Radcliffe KA, Pidoplichko VI (2000) Variations in desensitization of nicotinic acetylcholine receptors from hippocampus and midbrain dopamine areas. *European Journal of Pharmacology* 393:31–38. [https://doi.org/10.1016/S0014-2999\(00\)00003-0](https://doi.org/10.1016/S0014-2999(00)00003-0)

- Debanne D (2004) Information processing in the axon. *Nat Rev Neurosci* 5:304–316. <https://doi.org/10.1038/nrn1397>
- Decker E, Dani J (1990) Calcium permeability of the nicotinic acetylcholine receptor: the single-channel calcium influx is significant. *J Neurosci* 10:3413–3420. <https://doi.org/10.1523/JNEUROSCI.10-10-03413.1990>
- Deeks ED, Scott LJ (2006) Rofinamide. *CNS Drugs* 20:751–760. <https://doi.org/10.2165/00023210-200620090-00007>
- Delignat-Lavaud B, Kano J, Ducrot C, et al (2023) Synaptotagmin-1-dependent phasic axonal dopamine release is dispensable for basic motor behaviors in mice. *Nat Commun* 14:4120. <https://doi.org/10.1038/s41467-023-39805-7>
- Deng P, Zhang Y, Xu ZC (2007) Involvement of Ih in Dopamine Modulation of Tonic Firing in Striatal Cholinergic Interneurons. *J Neurosci* 27:3148–3156. <https://doi.org/10.1523/JNEUROSCI.5535-06.2007>
- Descarries L, Gisiger V, Steriade M (1997) Diffuse transmission by acetylcholine in the CNS. *Prog Neurobiol* 53:603–625. [https://doi.org/10.1016/s0301-0082\(97\)00050-6](https://doi.org/10.1016/s0301-0082(97)00050-6)
- Descarries L, Watkins KC, Garcia S, et al (1996) Dual character, asynaptic and synaptic, of the dopamine innervation in adult rat neostriatum: A quantitative autoradiographic and immunocytochemical analysis. *Journal of Comparative Neurology* 375:167–186. [https://doi.org/10.1002/\(SICI\)1096-9861\(19961111\)375:2<167::AID-CNE1>3.0.CO;2-0](https://doi.org/10.1002/(SICI)1096-9861(19961111)375:2<167::AID-CNE1>3.0.CO;2-0)
- Ding J, Guzman JN, Tkatch T, et al (2006) RGS4-dependent attenuation of M4 autoreceptor function in striatal cholinergic interneurons following dopamine depletion. *Nat Neurosci* 9:832–842. <https://doi.org/10.1038/nn1700>
- Ding JB, Guzman JN, Peterson JD, et al (2010) Thalamic Gating of Corticostriatal Signaling by Cholinergic Interneurons. *Neuron* 67:294–307. <https://doi.org/10.1016/j.neuron.2010.06.017>
- Dodge FA (1963) A Study of Ionic Permeability Changes Underlying Excitation in Myelinated nerve Fibers of the Frog. Ph.D. thesis, The Rockefeller University
- Dodson PD, Dreyer JK, Jennings KA, et al (2016) Representation of spontaneous movement by dopaminergic neurons is cell-type selective and disrupted in parkinsonism. *Proceedings of the National Academy of Sciences* 113:E2180–E2188. <https://doi.org/10.1073/pnas.1515941113>
- Doig NM, Magill PJ, Apicella P, et al (2014) Cortical and Thalamic Excitation Mediate the Multiphasic Responses of Striatal Cholinergic Interneurons to Motivationally Salient Stimuli. *J Neurosci* 34:3101–3117. <https://doi.org/10.1523/JNEUROSCI.4627-13.2014>

- Dreyer JK, Herrik KF, Berg RW, Hounsgaard JD (2010) Influence of Phasic and Tonic Dopamine Release on Receptor Activation. *J Neurosci* 30:14273–14283. <https://doi.org/10.1523/JNEUROSCI.1894-10.2010>
- DSM-IVR APA (2013) Diagnostic and Statistical Manual of Mental Disorders (DSM-5®). American Psychiatric Association
- Duquenoy L, O'Connor B (2024) Voltage sensor imaging in a population of dopaminergic axons in the mouse striatum. *protocols.io*. <https://doi.org/dx.doi.org/10.17504/protocols.io.rm7vzxp6rgx1/v1>
- Dylla KV, Raiser G, Galizia CG, Szyszka P (2017) Trace Conditioning in *Drosophila* Induces Associative Plasticity in Mushroom Body Kenyon Cells and Dopaminergic Neurons. *Front Neural Circuits* 11:. <https://doi.org/10.3389/fncir.2017.00042>
- Eichler K, Li F, Litwin-Kumar A, et al (2017) The complete connectome of a learning and memory centre in an insect brain. *Nature* 548:175–182. <https://doi.org/10.1038/nature23455>
- Evans SW, Shi D-Q, Chavarha M, et al (2023) A positively tuned voltage indicator for extended electrical recordings in the brain. *Nat Methods* 20:1104–1113. <https://doi.org/10.1038/s41592-023-01913-z>
- Exley R, Clements MA, Hartung H, et al (2008)  $\alpha$ 6-Containing Nicotinic Acetylcholine Receptors Dominate the Nicotine Control of Dopamine Neurotransmission in Nucleus Accumbens. *Neuropsychopharmacol* 33:2158–2166. <https://doi.org/10.1038/sj.npp.1301617>
- Exley R, Clements MA, Hartung H, et al (2013) Striatal dopamine transmission is reduced after chronic nicotine with a decrease in  $\alpha$ 6-nicotinic receptor control in nucleus accumbens. *European Journal of Neuroscience* 38:3036–3043. <https://doi.org/10.1111/ejn.12298>
- Exley R, Cragg SJ (2008) Presynaptic nicotinic receptors: a dynamic and diverse cholinergic filter of striatal dopamine neurotransmission. *Br J Pharmacol* 153:S283–S297. <https://doi.org/10.1038/sj.bjp.0707510>
- Exley R, Maubourguet N, David V, et al (2011) Distinct contributions of nicotinic acetylcholine receptor subunit  $\alpha$ 4 and subunit  $\alpha$ 6 to the reinforcing effects of nicotine. *Proc Natl Acad Sci U S A* 108:7577–7582. <https://doi.org/10.1073/pnas.1103000108>
- Exley R, McIntosh JM, Marks MJ, et al (2012a) Striatal  $\alpha$ 5 Nicotinic Receptor Subunit Regulates Dopamine Transmission in Dorsal Striatum. *J Neurosci* 32:2352–2356. <https://doi.org/10.1523/JNEUROSCI.4985-11.2012>
- Exley R, McIntosh JM, Marks MJ, et al (2012b) Striatal  $\alpha$ 5 nicotinic receptor subunit regulates dopamine transmission in dorsal striatum. *J Neurosci* 32:2352–2356. <https://doi.org/10.1523/JNEUROSCI.4985-11.2012>



- Farassat N, Costa KM, Stojanovic S, et al (2019) In vivo functional diversity of midbrain dopamine neurons within identified axonal projections. *eLife* 8:e48408. <https://doi.org/10.7554/eLife.48408>
- Felsenberg J, Barnstedt O, Cognigni P, et al (2017) Re-evaluation of learned information in *Drosophila*. *Nature* 544:240–244. <https://doi.org/10.1038/nature21716>
- Felsenberg J, Jacob PF, Walker T, et al (2018) Integration of Parallel Opposing Memories Underlies Memory Extinction. *Cell*. <https://doi.org/10.1016/j.cell.2018.08.021>
- Fieber LA, Adams DJ (1991) Acetylcholine-evoked currents in cultured neurones dissociated from rat parasympathetic cardiac ganglia. *J Physiol* 434:215–237
- Fleming W, Lee J, Briones BA, et al (2022) Cholinergic interneurons mediate cocaine extinction in male mice through plasticity across medium spiny neuron subtypes. *Cell Rep* 39:110874. <https://doi.org/10.1016/j.celrep.2022.110874>
- Floresco SB, West AR, Ash B, et al (2003) Afferent modulation of dopamine neuron firing differentially regulates tonic and phasic dopamine transmission. *Nat Neurosci* 6:968–973. <https://doi.org/10.1038/nn1103>
- Folkers E, Waddell S, Quinn WG (2006) The *Drosophila* radish gene encodes a protein required for anesthesia-resistant memory. *Proc Natl Acad Sci U S A* 103:17496–17500. <https://doi.org/10.1073/pnas.0608377103>
- Fucile S (2004) Ca<sup>2+</sup> permeability of nicotinic acetylcholine receptors. *Cell Calcium* 35:1–8. <https://doi.org/10.1016/j.ceca.2003.08.006>
- Galili DS, Dylla KV, Lüdke A, et al (2014) Converging Circuits Mediate Temperature and Shock Aversive Olfactory Conditioning in *Drosophila*. *Current Biology* 24:1712–1722. <https://doi.org/10.1016/j.cub.2014.06.062>
- Gallo EF, Greenwald J, Yeisley J, et al (2022) Dopamine D2 receptors modulate the cholinergic pause and inhibitory learning. *Mol Psychiatry* 27:1502–1514. <https://doi.org/10.1038/s41380-021-01364-y>
- Geiger JRP, Jonas P (2000) Dynamic Control of Presynaptic Ca<sup>2+</sup> Inflow by Fast-Inactivating K<sup>+</sup> Channels in Hippocampal Mossy Fiber Boutons. *Neuron* 28:927–939. [https://doi.org/10.1016/S0896-6273\(00\)00164-1](https://doi.org/10.1016/S0896-6273(00)00164-1)
- Gerber B, Yarali A, Diegelmann S, et al (2014) Pain-relief learning in flies, rats, and man: basic research and applied perspectives. *Learn Mem* 21:232–252. <https://doi.org/10.1101/lm.032995.113>
- Giniatullin R, Nistri A, Yakel JL (2005) Desensitization of nicotinic ACh receptors: shaping cholinergic signaling. *Trends in Neurosciences* 28:371–378. <https://doi.org/10.1016/j.tins.2005.04.009>

- Goldberg JA, Reynolds JNJ (2011) Spontaneous firing and evoked pauses in the tonically active cholinergic interneurons of the striatum. *Neuroscience* 198:27–43. <https://doi.org/10.1016/j.neuroscience.2011.08.067>
- González C, Baez-Nieto D, Valencia I, et al (2012) K<sup>+</sup> Channels: Function-Structural Overview. In: *Comprehensive Physiology*. John Wiley & Sons, Ltd, pp 2087–2149
- Grace AA, Bunney BS (1984a) The control of firing pattern in nigral dopamine neurons: burst firing. *J Neurosci* 4:2877–2890
- Grace AA, Bunney BS (1984b) The control of firing pattern in nigral dopamine neurons: single spike firing. *J Neurosci* 4:2866–2876. <https://doi.org/10.1523/JNEUROSCI.04-11-02866.1984>
- Gras C, Herzog E, Bellenchi GC, et al (2002) A third vesicular glutamate transporter expressed by cholinergic and serotonergic neurons. *J Neurosci* 22:5442–5451. <https://doi.org/10.1523/JNEUROSCI.22-13-05442.2002>
- Graybiel AM, Aosaki T, Flaherty AW, Kimura M (1994a) The Basal Ganglia and Adaptive Motor Control. *Science* 265:1826–1831. <https://doi.org/10.1126/science.8091209>
- Graybiel AM, Aosaki T, Flaherty AW, Kimura M (1994b) The Basal Ganglia and Adaptive Motor Control. *Science* 265:1826–1831. <https://doi.org/10.1126/science.8091209>
- Guo J-Z, Tredway TL, Chiappinelli VA (1998) Glutamate and GABA Release Are Enhanced by Different Subtypes of Presynaptic Nicotinic Receptors in the Lateral Geniculate Nucleus. *J Neurosci* 18:1963–1969. <https://doi.org/10.1523/JNEUROSCI.18-06-01963.1998>
- Handler A, Graham TGW, Cohn R, et al (2019) Distinct Dopamine Receptor Pathways Underlie the Temporal Sensitivity of Associative Learning. *Cell* 178:60-75.e19. <https://doi.org/10.1016/j.cell.2019.05.040>
- Hao YA, Lee S, Roth RH, et al (2024) A fast and responsive voltage indicator with enhanced sensitivity for unitary synaptic events. *Neuron* 112:3680-3696.e8. <https://doi.org/10.1016/j.neuron.2024.08.019>
- Harpsøe K, Hald H, Timmermann DB, et al (2013) Molecular Determinants of Subtype-selective Efficacies of Cytisine and the Novel Compound NS3861 at Heteromeric Nicotinic Acetylcholine Receptors. *J Biol Chem* 288:2559–2570. <https://doi.org/10.1074/jbc.M112.436337>
- Hille B (2001) *Ionic channels of excitable membranes*, Third Edition, Third Edition. Sinauer Associates, Inc.
- Hirano Y, Masuda T, Naganos S, et al (2013) Fasting Launches CRTC to Facilitate Long-Term Memory Formation in *Drosophila*. *Science* 339:443–446. <https://doi.org/10.1126/science.1227170>

- Hodgkin AL (1958) Ionic movements and electrical activity in giant nerve fibres. *Proceedings of the Royal Society of London Series B - Biological Sciences, The Croonian Lecture 148*:1–37. <https://doi.org/10.1098/rspb.1958.0001>
- Hodgkin AL, Huxley AF (1952a) Currents carried by sodium and potassium ions through the membrane of the giant axon of *Loligo*. *J Physiol* 116:449–472
- Hodgkin AL, Huxley AF (1952b) A quantitative description of membrane current and its application to conduction and excitation in nerve. *J Physiol* 117:500–544
- Hodgkin AL, Huxley AF (1952c) The dual effect of membrane potential on sodium conductance in the giant axon of *Loligo*. *J Physiol* 116:497–506
- Hodgkin AL, Huxley AF (1952d) The components of membrane conductance in the giant axon of *Loligo*. *J Physiol* 116:473–496
- Hodgkin AL, Katz B (1949) The effect of sodium ions on the electrical activity of the giant axon of the squid. *J Physiol* 108:37–77
- Honegger KS, Campbell RAA, Turner GC (2011) Cellular-resolution population imaging reveals robust sparse coding in the *Drosophila* mushroom body. *J Neurosci* 31:11772–11785. <https://doi.org/10.1523/JNEUROSCI.1099-11.2011>
- Hornykiewicz O (1998) Biochemical aspects of Parkinson's disease. *Neurology* 51:S2–S9. [https://doi.org/10.1212/WNL.51.2\\_Suppl\\_2.S2](https://doi.org/10.1212/WNL.51.2_Suppl_2.S2)
- Howe MW, Dombeck DA (2016) Rapid signalling in distinct dopaminergic axons during locomotion and reward. *Nature* 535:505–510. <https://doi.org/10.1038/nature18942>
- Howe WM, Young DA, Bekheet G, Kozak R (2016) Nicotinic receptor subtypes differentially modulate glutamate release in the dorsal medial striatum. *Neurochem Int* 100:30–34. <https://doi.org/10.1016/j.neuint.2016.08.009>
- Hu Y, Flockhart I, Vinayagam A, et al (2011) An integrative approach to ortholog prediction for disease-focused and other functional studies. *BMC Bioinformatics* 12:357. <https://doi.org/10.1186/1471-2105-12-357>
- Huetteroth W, Perisse E, Lin S, et al (2015) Sweet Taste and Nutrient Value Subdivide Rewarding Dopaminergic Neurons in *Drosophila*. *Curr Biol* 25:751–758. <https://doi.org/10.1016/j.cub.2015.01.036>
- Humayun MJ, Wadhwa R (2022) *Rufinamide*. In: StatPearls. StatPearls Publishing, Treasure Island (FL)
- Hyland BI, Reynolds JNJ, Hay J, et al (2002) Firing modes of midbrain dopamine cells in the freely moving rat. *Neuroscience* 114:475–492. [https://doi.org/10.1016/s0306-4522\(02\)00267-1](https://doi.org/10.1016/s0306-4522(02)00267-1)

- Ikemoto S (2007) Dopamine reward circuitry: two projection systems from the ventral midbrain to the nucleus accumbens-olfactory tubercle complex. *Brain Res Rev* 56:27–78. <https://doi.org/10.1016/j.brainresrev.2007.05.004>
- Ingram SL, Prasad BM, Amara SG (2002) Dopamine transporter-mediated conductances increase excitability of midbrain dopamine neurons. *Nat Neurosci* 5:971–978. <https://doi.org/10.1038/nn920>
- Ito I, Ong RC, Raman B, Stopfer M (2008) Sparse odor representation and olfactory learning. *Nat Neurosci* 11:1177–1184. <https://doi.org/10.1038/nn.2192>
- Ito K, Suzuki K, Estes P, et al (1998) The organization of extrinsic neurons and their implications in the functional roles of the mushroom bodies in *Drosophila melanogaster* Meigen. *Learn Mem* 5:52–77
- Izzo PN, Bolam JP (1988) Cholinergic synaptic input to different parts of spiny striatonigral neurons in the rat. *Journal of Comparative Neurology* 269:219–234. <https://doi.org/10.1002/cne.902690207>
- Jacob PF, Waddell S (2020) Spaced Training Forms Complementary Long-Term Memories of Opposite Valence in *Drosophila*. *Neuron* 106:977–991.e4. <https://doi.org/10.1016/j.neuron.2020.03.013>
- Janezic S, Threlfell S, Dodson PD, et al (2013) Deficits in dopaminergic transmission precede neuron loss and dysfunction in a new Parkinson model. *Proc Natl Acad Sci U S A* 110:E4016–E4025. <https://doi.org/10.1073/pnas.1309143110>
- Jing M, Li Y, Zeng J, et al (2020) An optimized acetylcholine sensor for monitoring in vivo cholinergic activity. *Nat Methods* 17:1139–1146. <https://doi.org/10.1038/s41592-020-0953-2>
- Jing M, Zhang P, Wang G, et al (2018) A genetically encoded fluorescent acetylcholine indicator for in vitro and in vivo studies. *Nat Biotechnol* 36:726–737. <https://doi.org/10.1038/nbt.4184>
- Jones AK, Brown LA, Sattelle DB (2007) Insect nicotinic acetylcholine receptor gene families: from genetic model organism to vector, pest and beneficial species. *Invert Neurosci* 7:67–73. <https://doi.org/10.1007/s10158-006-0039-6>
- Jørgensen SH, Ejdrup AL, Lycas MD, et al (2023) Behavioral encoding across timescales by region-specific dopamine dynamics. *Proceedings of the National Academy of Sciences* 120:e2215230120. <https://doi.org/10.1073/pnas.2215230120>
- Katz B, Miledi R (1967) A study of synaptic transmission in the absence of nerve impulses. *J Physiol* 192:407–436
- Kawaguchi Y (1993) Physiological, morphological, and histochemical characterization of three classes of interneurons in rat neostriatum. *J Neurosci* 13:4908–4923. <https://doi.org/10.1523/JNEUROSCI.13-11-04908.1993>

- Khaliq ZM, Bean BP (2010) Pacemaking in Dopaminergic Ventral Tegmental Area Neurons: Depolarizing Drive from Background and Voltage-Dependent Sodium Conductances. *J Neurosci* 30:7401–7413. <https://doi.org/10.1523/JNEUROSCI.0143-10.2010>
- Khammy MM, Kim S, Bentzen BH, et al (2018) 4-Aminopyridine: a pan voltage-gated potassium channel inhibitor that enhances Kv7.4 currents and inhibits noradrenaline-mediated contraction of rat mesenteric small arteries. *Br J Pharmacol* 175:501–516. <https://doi.org/10.1111/bph.14097>
- Kilfeather P, Khoo JH, Wagner K, et al (2024) Single-cell spatial transcriptomic and translational profiling of dopaminergic neurons in health, aging, and disease. *Cell Rep* 43:113784. <https://doi.org/10.1016/j.celrep.2024.113784>
- Kim DM, Nimigean CM (2016) Voltage-Gated Potassium Channels: A Structural Examination of Selectivity and Gating. *Cold Spring Harb Perspect Biol* 8:a029231. <https://doi.org/10.1101/cshperspect.a029231>
- Kim Y-K, Saver M, Simon J, et al (2018) Repetitive aggressive encounters generate a long-lasting internal state in *Drosophila melanogaster* males. *Proc Natl Acad Sci U S A* 115:1099–1104. <https://doi.org/10.1073/pnas.1716612115>
- Kimura M, Rajkowski J, Evarts E (1984) Tonicly discharging putamen neurons exhibit set-dependent responses. *Proc Natl Acad Sci USA* 81:4998–5001. <https://doi.org/10.1073/pnas.81.15.4998>
- Kita H, Chang HT, Kitai ST (1983) Pallidal inputs to subthalamus: Intracellular analysis. *Brain Research* 264:255–265. [https://doi.org/10.1016/0006-8993\(83\)90823-5](https://doi.org/10.1016/0006-8993(83)90823-5)
- Kitai ST, Deniau JM (1981) Cortical inputs to the subthalamus: intracellular analysis. *Brain Research* 214:411–415. [https://doi.org/10.1016/0006-8993\(81\)91204-X](https://doi.org/10.1016/0006-8993(81)91204-X)
- Klink R, d'Exaerde A de K, Zoli M, Changeux J-P (2001) Molecular and Physiological Diversity of Nicotinic Acetylcholine Receptors in the Midbrain Dopaminergic Nuclei. *J Neurosci* 21:1452–1463. <https://doi.org/10.1523/JNEUROSCI.21-05-01452.2001>
- Knowlton BJ, Mangels JA, Squire LR (1996) A Neostriatal Habit Learning System in Humans. *Science* 273:1399–1402. <https://doi.org/10.1126/science.273.5280.1399>
- Kondo S, Takahashi T, Yamagata N, et al (2020) Neurochemical Organization of the *Drosophila* Brain Visualized by Endogenously Tagged Neurotransmitter Receptors. *Cell Reports* 30:284–297.e5. <https://doi.org/10.1016/j.celrep.2019.12.018>
- Kosillo P, Zhang Y-F, Threlfell S, Cragg SJ (2016) Cortical Control of Striatal Dopamine Transmission via Striatal Cholinergic Interneurons. *Cereb Cortex* 26:4160–4169. <https://doi.org/10.1093/cercor/bhw252>

- Kramer PF, Brill-Weil SG, Cummins AC, et al (2022) Synaptic-like axo-axonal transmission from striatal cholinergic interneurons onto dopaminergic fibers. *Neuron* 110:2949–2960.e4. <https://doi.org/10.1016/j.neuron.2022.07.011>
- Kramer PF, Twedell EL, Shin JH, et al (2020) Axonal mechanisms mediating  $\gamma$ -aminobutyric acid receptor type A (GABA-A) inhibition of striatal dopamine release. *eLife* 9:e55729. <https://doi.org/10.7554/eLife.55729>
- Krashes MJ, DasGupta S, Vreede A, et al (2009) A Neural Circuit Mechanism Integrating Motivational State with Memory Expression in *Drosophila*. *Cell* 139:416–427. <https://doi.org/10.1016/j.cell.2009.08.035>
- Krashes MJ, Waddell S (2011a) *Drosophila* Appetitive Olfactory Conditioning. *Cold Spring Harb Protoc* 2011:pdb.prot5609. <https://doi.org/10.1101/pdb.prot5609>
- Krashes MJ, Waddell S (2011b) *Drosophila* Aversive Olfactory Conditioning. *Cold Spring Harb Protoc* 2011:pdb.prot5608. <https://doi.org/10.1101/pdb.prot5608>
- Krok AC, Maltese M, Mistry P, et al (2023) Intrinsic dopamine and acetylcholine dynamics in the striatum of mice. *Nature* 621:543–549. <https://doi.org/10.1038/s41586-023-05995-9>
- Lacey MG, Mercuri NB, North RA (1989) Two cell types in rat substantia nigra zona compacta distinguished by membrane properties and the actions of dopamine and opioids. *J Neurosci* 9:1233–1241. <https://doi.org/10.1523/JNEUROSCI.09-04-01233.1989>
- Lambe EK, Picciotto MR, Aghajanian GK (2003) Nicotine Induces Glutamate Release from Thalamocortical Terminals in Prefrontal Cortex. *Neuropsychopharmacol* 28:216–225. <https://doi.org/10.1038/sj.npp.1300032>
- Lammel S, Hetzel A, Häckel O, et al (2008) Unique Properties of Mesoprefrontal Neurons within a Dual Mesocorticolimbic Dopamine System. *Neuron* 57:760–773. <https://doi.org/10.1016/j.neuron.2008.01.022>
- Lansdell SJ, Millar NS (2002) D $\beta$ 3, an atypical nicotinic acetylcholine receptor subunit from *Drosophila*: molecular cloning, heterologous expression and coassembly. *Journal of Neurochemistry* 80:1009–1018. <https://doi.org/10.1046/j.0022-3042.2002.00789.x>
- Liao S, Liu T, Yang R, et al (2022) Structure and Function of Sodium Channel Nav1.3 in Neurological Disorders. *Cell Mol Neurobiol* 43:575–584. <https://doi.org/10.1007/s10571-022-01211-w>
- Lim SAO, Kang UJ, McGehee DS (2014) Striatal cholinergic interneuron regulation and circuit effects. *Front Synaptic Neurosci* 6:. <https://doi.org/10.3389/fnsyn.2014.00022>

- Lin L, Li K, Tian B, et al (2022a) Two Novel Functional Mutations in Promoter Region of SCN3B Gene Associated with Atrial Fibrillation. *Life (Basel)* 12:1794. <https://doi.org/10.3390/life12111794>
- Lin S, Oswald D, Chandra V, et al (2014) Neural correlates of water reward in thirsty *Drosophila*. *Nat Neurosci* 17:1536–1542. <https://doi.org/10.1038/nn.3827>
- Lin Y-C, Lai Y-C, Lin T-H, et al (2022b) Selective stabilization of the intermediate inactivated Na<sup>+</sup> channel by the new-generation anticonvulsant rufinamide. *Biochemical Pharmacology* 197:114928. <https://doi.org/10.1016/j.bcp.2022.114928>
- Lin Y-C, Lai Y-C, Lin T-H, et al (2022c) Selective stabilization of the intermediate inactivated Na<sup>+</sup> channel by the new-generation anticonvulsant rufinamide. *Biochemical Pharmacology* 197:114928. <https://doi.org/10.1016/j.bcp.2022.114928>
- Liu C, Cai X, Ritzau-Jost A, et al (2022) An action potential initiation mechanism in distal axons for the control of dopamine release. *Science* 375:1378–1385. <https://doi.org/10.1126/science.abn0532>
- Liu C, Goel P, Kaeser PS (2021) Spatial and temporal scales of dopamine transmission. *Nat Rev Neurosci* 22:345–358. <https://doi.org/10.1038/s41583-021-00455-7>
- Liu C, Kershberg L, Wang J, et al (2018) Dopamine Secretion Is Mediated by Sparse Active Zone-like Release Sites. *Cell* 172:706–718.e15. <https://doi.org/10.1016/j.cell.2018.01.008>
- Llinás R, Walton K, Bohr V (1976) Synaptic transmission in squid giant synapse after potassium conductance blockage with external 3- and 4-aminopyridine. *Biophysical Journal* 16:83–86. [https://doi.org/10.1016/S0006-3495\(76\)85664-0](https://doi.org/10.1016/S0006-3495(76)85664-0)
- Lopes EF, Roberts BM, Siddorn RE, et al (2019) Inhibition of Nigrostriatal Dopamine Release by Striatal GABAA and GABAB Receptors. *J Neurosci* 39:1058–1065. <https://doi.org/10.1523/JNEUROSCI.2028-18.2018>
- Lopez GC, Lerner TN (2025) How dopamine enables learning from aversion. *Current Opinion in Behavioral Sciences* 61:101476. <https://doi.org/10.1016/j.cobeha.2024.101476>
- Lozovaya N, Eftekhari S, Cloarec R, et al (2018) GABAergic inhibition in dual-transmission cholinergic and GABAergic striatal interneurons is abolished in Parkinson disease. *Nat Commun* 9:1422. <https://doi.org/10.1038/s41467-018-03802-y>
- Mansvelder HD, Keath JR, McGehee DS (2002) Synaptic mechanisms underlie nicotine-induced excitability of brain reward areas. *Neuron* 33:905–919. [https://doi.org/10.1016/s0896-6273\(02\)00625-6](https://doi.org/10.1016/s0896-6273(02)00625-6)

- Mansvelder HD, McGehee DS (2000) Long-Term Potentiation of Excitatory Inputs to Brain Reward Areas by Nicotine. *Neuron* 27:349–357. [https://doi.org/10.1016/S0896-6273\(00\)00042-8](https://doi.org/10.1016/S0896-6273(00)00042-8)
- Marshall D, Soliakov L, Redfern P, Wonnacott S (1996) Tetrodotoxin-sensitivity of nicotine-evoked dopamine release from rat striatum. *Neuropharmacology* 35:1531–1536. [https://doi.org/10.1016/S0028-3908\(96\)00117-7](https://doi.org/10.1016/S0028-3908(96)00117-7)
- Matamalas M, Götz J, Bertran-Gonzalez J (2016) Quantitative Imaging of Cholinergic Interneurons Reveals a Distinctive Spatial Organization and a Functional Gradient across the Mouse Striatum. *PLoS One* 11:e0157682. <https://doi.org/10.1371/journal.pone.0157682>
- Matityahu L, Gilin N, Sarpong GA, et al (2023) Acetylcholine waves and dopamine release in the striatum. *Nat Commun* 14:6852. <https://doi.org/10.1038/s41467-023-42311-5>
- Matityahu L, Hobel ZB, Berkowitz N, et al (2024) Synchronous activation of striatal cholinergic interneurons induces local serotonin release. *bioRxiv* 2024.11.03.621726. <https://doi.org/10.1101/2024.11.03.621726>
- Matsuda W, Furuta T, Nakamura KC, et al (2009) Single Nigrostriatal Dopaminergic Neurons Form Widely Spread and Highly Dense Axonal Arborizations in the Neostriatum. *J Neurosci* 29:444–453. <https://doi.org/10.1523/JNEUROSCI.4029-08.2009>
- Maurice N, Mercer J, Chan CS, et al (2004) D2 Dopamine Receptor-Mediated Modulation of Voltage-Dependent Na<sup>+</sup> Channels Reduces Autonomous Activity in Striatal Cholinergic Interneurons. *J Neurosci* 24:10289–10301. <https://doi.org/10.1523/JNEUROSCI.2155-04.2004>
- McCormack K, Joiner WI, Heinemann SH (1994) A characterization of the activating structural rearrangements in voltage-dependent *Shaker* K<sup>+</sup> channels. *Neuron* 12:301–315. [https://doi.org/10.1016/0896-6273\(94\)90273-9](https://doi.org/10.1016/0896-6273(94)90273-9)
- McFarland K, Lapish CC, Kalivas PW (2003) Prefrontal glutamate release into the core of the nucleus accumbens mediates cocaine-induced reinstatement of drug-seeking behavior. *J Neurosci* 23:3531–3537. <https://doi.org/10.1523/JNEUROSCI.23-08-03531.2003>
- McGregor MM, Nelson AB (2019) Circuit Mechanisms of Parkinson’s Disease. *Neuron* 101:1042–1056. <https://doi.org/10.1016/j.neuron.2019.03.004>
- Meschi E, Duquenoy L, Otto N, et al (2024) Compensatory enhancement of input maintains aversive dopaminergic reinforcement in hungry *Drosophila*. *Neuron* 112:2315–2332.e8. <https://doi.org/10.1016/j.neuron.2024.04.035>



- Miyasaka A, Kanda T, Nonaka N, et al (2025) Sequential transitions of male sexual behaviors driven by dual acetylcholine-dopamine dynamics. *Neuron* 0: <https://doi.org/10.1016/j.neuron.2025.01.032>
- Mohebi A, Collins VL, Berke JD (2023) Accumbens cholinergic interneurons dynamically promote dopamine release and enable motivation. *eLife* 12:e85011. <https://doi.org/10.7554/eLife.85011>
- Morgenstern NA, Isidro AF, Israely I, Costa RM (2022) Pyramidal tract neurons drive amplification of excitatory inputs to striatum through cholinergic interneurons. *Science Advances* 8:eabh4315. <https://doi.org/10.1126/sciadv.abh4315>
- Morris G, Arkadir D, Nevet A, et al (2004) Coincident but Distinct Messages of Midbrain Dopamine and Striatal Tonically Active Neurons. *Neuron* 43:133–143. <https://doi.org/10.1016/j.neuron.2004.06.012>
- Mulle C, Choquet D, Korn H, Changeux J-P (1992) Calcium influx through nicotinic receptor in rat central neurons: Its relevance to cellular regulation. *Neuron* 8:135–143. [https://doi.org/10.1016/0896-6273\(92\)90115-T](https://doi.org/10.1016/0896-6273(92)90115-T)
- Nair-Roberts RG, Chatelain-Badie SD, Benson E, et al (2008) Stereological estimates of dopaminergic, GABAergic and glutamatergic neurons in the ventral tegmental area, substantia nigra and retrorubral field in the rat. *Neuroscience* 152:1024–1031. <https://doi.org/10.1016/j.neuroscience.2008.01.046>
- Nosaka D, Wickens JR (2022) Striatal Cholinergic Signaling in Time and Space. *Molecules* 27:1202. <https://doi.org/10.3390/molecules27041202>
- Okray Z, Jacob PF, Stern C, et al (2023) Multisensory learning binds neurons into a cross-modal memory engram. *Nature* 617:777–784. <https://doi.org/10.1038/s41586-023-06013-8>
- O'Neill B, Patel JC, Rice ME (2017) Characterization of Optically and Electrically Evoked Dopamine Release in Striatal Slices from Digenic Knock-in Mice with DAT-Driven Expression of Channelrhodopsin. *ACS Chem Neurosci* 8:310–319. <https://doi.org/10.1021/acscchemneuro.6b00300>
- Otoom S, Tian L-M, A. Alkadhi K (1998) Veratridine-treated brain slices: a cellular model for epileptiform activity. *Brain Research* 789:150–156. [https://doi.org/10.1016/S0006-8993\(98\)00026-2](https://doi.org/10.1016/S0006-8993(98)00026-2)
- Otto N, Pleijzier MW, Morgan IC, et al (2020) Input Connectivity Reveals Additional Heterogeneity of Dopaminergic Reinforcement in *Drosophila*. *Current Biology* 30:3200–3211.e8. <https://doi.org/10.1016/j.cub.2020.05.077>
- Park A, Stacy M (2009) Non-motor symptoms in Parkinson's disease. *J Neurol* 256:293–298. <https://doi.org/10.1007/s00415-009-5240-1>

- Pascoli V, Terrier J, Hiver A, Lüscher C (2015) Sufficiency of Mesolimbic Dopamine Neuron Stimulation for the Progression to Addiction. *Neuron* 88:1054–1066. <https://doi.org/10.1016/j.neuron.2015.10.017>
- Pascual A, Pr  at T (2001) Localization of long-term memory within the *Drosophila* mushroom body. *Science* 294:1115–1117. <https://doi.org/10.1126/science.1064200>
- Patriarchi T, Cho JR, Merten K, et al (2018) Ultrafast neuronal imaging of dopamine dynamics with designed genetically encoded sensors. *Science* 360:eaat4422. <https://doi.org/10.1126/science.aat4422>
- Pavlovsky A, Schor J, Pla  ais P-Y, Preat T (2018) A GABAergic Feedback Shapes Dopaminergic Input on the *Drosophila* Mushroom Body to Promote Appetitive Long-Term Memory. *Current Biology* 28:1783-1793.e4. <https://doi.org/10.1016/j.cub.2018.04.040>
- Pereira DB, Schmitz Y, M  esz  aros J, et al (2016) Fluorescent false neurotransmitter reveals functionally silent dopamine vesicle clusters in the striatum. *Nat Neurosci* 19:578–586. <https://doi.org/10.1038/nn.4252>
- Perisse E, Oswald D, Barnstedt O, et al (2016) Aversive Learning and Appetitive Motivation Toggle Feed-Forward Inhibition in the *Drosophila* Mushroom Body. *Neuron* 90:1086–1099. <https://doi.org/10.1016/j.neuron.2016.04.034>
- Phelps PE, Houser CR, Vaughn JE (1985) Immunocytochemical localization of choline acetyltransferase within the rat neostriatum: A correlated light and electron microscopic study of cholinergic neurons and synapses. *Journal of Comparative Neurology* 238:286–307. <https://doi.org/10.1002/cne.902380305>
- Pickel VM, Beckley SC, Joh TH, Reis DJ (1981) Ultrastructural immunocytochemical localization of tyrosine hydroxylase in the neostriatum. *Brain Research* 225:373–385. [https://doi.org/10.1016/0006-8993\(81\)90843-X](https://doi.org/10.1016/0006-8993(81)90843-X)
- Pissadaki EK, Bolam JP (2013) The energy cost of action potential propagation in dopamine neurons: clues to susceptibility in Parkinson’s disease. *Front Comput Neurosci* 7:. <https://doi.org/10.3389/fncom.2013.00013>
- Pla  ais P-Y, Preat T (2013) To Favor Survival Under Food Shortage, the Brain Disables Costly Memory. *Science* 339:440–442. <https://doi.org/10.1126/science.1226018>
- Poorthuis RB, Bloem B, Schak B, et al (2013) Layer-Specific Modulation of the Prefrontal Cortex by Nicotinic Acetylcholine Receptors. *Cerebral Cortex* 23:148–161. <https://doi.org/10.1093/cercor/bhr390>
- Poppi LA, Ho-Nguyen KT, Shi A, et al (2021) Recurrent Implication of Striatal Cholinergic Interneurons in a Range of Neurodevelopmental, Neurodegenerative, and Neuropsychiatric Disorders. *Cells* 10:907. <https://doi.org/10.3390/cells10040907>

- Poulin J-F, Caronia G, Hofer C, et al (2018) Mapping projections of molecularly defined dopamine neuron subtypes using intersectional genetic approaches. *Nat Neurosci* 21:1260–1271. <https://doi.org/10.1038/s41593-018-0203-4>
- Poulin J-F, Zou J, Drouin-Ouellet J, et al (2014) Defining midbrain dopaminergic neuron diversity by single-cell gene profiling. *Cell Rep* 9:930–943. <https://doi.org/10.1016/j.celrep.2014.10.008>
- Pyakurel P, Shin M, Venton BJ (2018) Nicotinic acetylcholine receptor (nAChR) mediated dopamine release in larval *Drosophila melanogaster*. *Neurochem Int* 114:33–41. <https://doi.org/10.1016/j.neuint.2017.12.012>
- Quick MW, Lester RAJ (2002) Desensitization of neuronal nicotinic receptors. *Journal of Neurobiology* 53:457–478. <https://doi.org/10.1002/neu.10109>
- Quinn DM (1987) Acetylcholinesterase: enzyme structure, reaction dynamics, and virtual transition states. *Chem Rev* 87:955–979. <https://doi.org/10.1021/cr00081a005>
- Rama S, Zbili M, Debanne D (2018) Signal propagation along the axon. *Current Opinion in Neurobiology* 51:37–44. <https://doi.org/10.1016/j.conb.2018.02.017>
- Redgrave P, Costa RM (2021) Chapter 38: The Basal Ganglia. In: Kandel ER, Koester JD, Mack SH, Siegelbaum SA (eds) *Principle of Neural Science*, 6th Edition. McGraw Hill Medical
- Rice ME, Cragg SJ (2004) Nicotine amplifies reward-related dopamine signals in striatum. *Nat Neurosci* 7:583–584. <https://doi.org/10.1038/nn1244>
- Rice ME, Nicholson Charles (1989) Measurement of nanomolar dopamine diffusion using low-noise perfluorinated ionomer-coated carbon fiber microelectrodes and high-speed cyclic voltammetry. *Analytical Chemistry* 61:1805–1810. <https://doi.org/10.1021/ac00192a005>
- Rice ME, Patel JC, Cragg SJ (2011) Dopamine release in the basal ganglia. *Neuroscience* 198:112–137. <https://doi.org/10.1016/j.neuroscience.2011.08.066>
- Roberts BM, Doig NM, Brimblecombe KR, et al (2020) GABA uptake transporters support dopamine release in dorsal striatum with maladaptive downregulation in a parkinsonism model. *Nat Commun* 11:4958. <https://doi.org/10.1038/s41467-020-18247-5>
- Robinson BG, Cai X, Wang J, et al (2019) RIM is essential for stimulated but not spontaneous somatodendritic dopamine release in the midbrain. *eLife* 8:e47972. <https://doi.org/10.7554/eLife.47972>
- Rocca WA (2018) The burden of Parkinson's disease: a worldwide perspective. *The Lancet Neurology* 17:928–929. [https://doi.org/10.1016/S1474-4422\(18\)30355-7](https://doi.org/10.1016/S1474-4422(18)30355-7)

- Rodríguez-López C, Clascá F, Prensa L (2017) The Mesoaccumbens Pathway: A Retrograde Labeling and Single-Cell Axon Tracing Analysis in the Mouse. *Front Neuroanat* 11:25. <https://doi.org/10.3389/fnana.2017.00025>
- Roeper J (2013) Dissecting the diversity of midbrain dopamine neurons. *Trends in Neurosciences* 36:336–342. <https://doi.org/10.1016/j.tins.2013.03.003>
- Rosenthal JS, Yuan Q (2021) Constructing and Tuning Excitatory Cholinergic Synapses: The Multifaceted Functions of Nicotinic Acetylcholine Receptors in *Drosophila* Neural Development and Physiology. *Front Cell Neurosci* 15:720560. <https://doi.org/10.3389/fncel.2021.720560>
- Samii A, Nutt JG, Ransom BR (2004) Parkinson's disease. *The Lancet* 363:1783–1793. [https://doi.org/10.1016/S0140-6736\(04\)16305-8](https://doi.org/10.1016/S0140-6736(04)16305-8)
- Schloss P, Betz H, Schroder C, Gundelfinger ED (1991) Neuronal Nicotinic Acetylcholine Receptors in *Drosophila*: Antibodies Against an  $\alpha$ -Like and a Non- $\alpha$ -Subunit Recognize the Same High-Affinity  $\alpha$ -Bungarotoxin Binding Complex. *Journal of Neurochemistry* 57:1556–1562. <https://doi.org/10.1111/j.1471-4159.1991.tb06351.x>
- Schloss P, Mayser W, Gundelfinger ED, Betz H (1992) Cross-linking of 125I- $\alpha$ -bungarotoxin to *Drosophila* head membranes identifies a 42 kDa toxin binding polypeptide. *Neuroscience Letters* 145:63–66. [https://doi.org/10.1016/0304-3940\(92\)90204-K](https://doi.org/10.1016/0304-3940(92)90204-K)
- Schultz W (1986) Responses of midbrain dopamine neurons to behavioral trigger stimuli in the monkey. *Journal of Neurophysiology* 56:1439–1461. <https://doi.org/10.1152/jn.1986.56.5.1439>
- Schultz W (2016) Dopamine reward prediction error coding. *Dialogues Clin Neurosci* 18:23–32. <https://doi.org/10.31887/DCNS.2016.18.1/wschultz>
- Schultz W, Dayan P, Montague PR (1997) A Neural Substrate of Prediction and Reward. *Science* 275:1593–1599. <https://doi.org/10.1126/science.275.5306.1593>
- Schulz JM, Reynolds JNJ (2013) Pause and rebound: sensory control of cholinergic signaling in the striatum. *Trends Neurosci* 36:41–50. <https://doi.org/10.1016/j.tins.2012.09.006>
- Schulz R, Bertrand S, Chamaon K, et al (2000) Neuronal Nicotinic Acetylcholine Receptors from *Drosophila*. *Journal of Neurochemistry* 74:2537–2546. <https://doi.org/10.1046/j.1471-4159.2000.0742537.x>
- Schulz R, Sawruk E, Mülhardt C, et al (1998) D $\alpha$ 3, a New Functional  $\alpha$  Subunit of Nicotinic Acetylcholine Receptors from *Drosophila*. *Journal of Neurochemistry* 71:853–862. <https://doi.org/10.1046/j.1471-4159.1998.71020853.x>

- Sellings LHL, Clarke PBS (2003) Segregation of amphetamine reward and locomotor stimulation between nucleus accumbens medial shell and core. *J Neurosci* 23:6295–6303. <https://doi.org/10.1523/JNEUROSCI.23-15-06295.2003>
- Semelidou O, Acevedo SF, Skoulakis EM (2018) Temporally specific engagement of distinct neuronal circuits regulating olfactory habituation in *Drosophila*. *eLife* 7:e39569. <https://doi.org/10.7554/eLife.39569>
- Senapati B, Tsao C-H, Juan Y-A, et al (2019) A neural mechanism for deprivation state-specific expression of relevant memories in *Drosophila*. *Nat Neurosci* 22:2029–2039. <https://doi.org/10.1038/s41593-019-0515-z>
- Shang Y, Claridge-Chang A, Sjulson L, et al (2007) Excitatory Local Circuits and Their Implications for Olfactory Processing in the Fly Antennal Lobe. *Cell* 128:601–612. <https://doi.org/10.1016/j.cell.2006.12.034>
- Sharott A, Doig NM, Mallet N, Magill PJ (2012) Relationships between the Firing of Identified Striatal Interneurons and Spontaneous and Driven Cortical Activities In Vivo. *J Neurosci* 32:13221–13236. <https://doi.org/10.1523/JNEUROSCI.2440-12.2012>
- Shin JH, Adrover MF, Wess J, Alvarez VA (2015) Muscarinic regulation of dopamine and glutamate transmission in the nucleus accumbens. *Proc Natl Acad Sci U S A* 112:8124–8129. <https://doi.org/10.1073/pnas.1508846112>
- Siju KP, De Backer J-F, Grunwald Kadow IC (2021) Dopamine modulation of sensory processing and adaptive behavior in flies. *Cell Tissue Res* 383:207–225. <https://doi.org/10.1007/s00441-020-03371-x>
- Siju KP, Štih V, Aimon S, et al (2020) Valence and State-Dependent Population Coding in Dopaminergic Neurons in the Fly Mushroom Body. *Current Biology* 30:2104–2115.e4. <https://doi.org/10.1016/j.cub.2020.04.037>
- Singleton AB, Farrer M, Johnson J, et al (2003)  $\alpha$ -Synuclein Locus Triplication Causes Parkinson's Disease. *Science* 302:841–841. <https://doi.org/10.1126/science.1090278>
- Sitzia F, Brown JT, Randall AD, Dunlop J (2011) Voltage- and Temperature-Dependent Allosteric Modulation of  $\alpha 7$  Nicotinic Receptors by PNU120596. *Front Pharmacol* 2:81. <https://doi.org/10.3389/fphar.2011.00081>
- Specht CG, Schoepfer R (2001) Deletion of the alpha-synuclein locus in a subpopulation of C57BL/6J inbred mice. *BMC Neurosci* 2:11. <https://doi.org/10.1186/1471-2202-2-11>
- Spillantini MG, Crowther RA, Jakes R, et al (1998)  $\alpha$ -Synuclein in filamentous inclusions of Lewy bodies from Parkinson's disease and dementia with Lewy bodies. *Proc Natl Acad Sci U S A* 95:6469–6473

- Steinbusch HW (1981) Distribution of serotonin-immunoreactivity in the central nervous system of the rat-cell bodies and terminals. *Neuroscience* 6:557–618.  
[https://doi.org/10.1016/0306-4522\(81\)90146-9](https://doi.org/10.1016/0306-4522(81)90146-9)
- Steiner H, Tseng KY (2016) *Handbook of Basal Ganglia Structure and Function*. Academic Press
- St-Pierre F, Marshall JD, Yang Y, et al (2014) High-fidelity optical reporting of neuronal electrical activity with an ultrafast fluorescent voltage sensor. *Nat Neurosci* 17:884–889. <https://doi.org/10.1038/nn.3709>
- Straub C, Tritsch NX, Hagan NA, et al (2014) Multiphasic Modulation of Cholinergic Interneurons by Nigrostriatal Afferents. *J Neurosci* 34:8557–8569.  
<https://doi.org/10.1523/JNEUROSCI.0589-14.2014>
- Sulzer D, Cragg SJ, Rice ME (2016) Striatal dopamine neurotransmission: regulation of release and uptake. *Basal Ganglia* 6:123–148.  
<https://doi.org/10.1016/j.baga.2016.02.001>
- Sulzer D, Surmeier DJ (2013) Neuronal vulnerability, pathogenesis, and Parkinson's disease. *Mov Disord* 28:41–50. <https://doi.org/10.1002/mds.25095>
- Sun F, Zeng J, Jing M, et al (2018) A Genetically Encoded Fluorescent Sensor Enables Rapid and Specific Detection of Dopamine in Flies, Fish, and Mice. *Cell* 174:481–496.e19.  
<https://doi.org/10.1016/j.cell.2018.06.042>
- Sun F, Zhou J, Dai B, et al (2020) Next-generation GRAB sensors for monitoring dopaminergic activity in vivo. *Nat Methods* 17:1156–1166.  
<https://doi.org/10.1038/s41592-020-00981-9>
- Sutro J (1986) Kinetics of veratridine action on Na channels of skeletal muscle. *J Gen Physiol* 87:1–24
- Szydlowski SN, Dorocic IP, Planert H, et al (2013) Target Selectivity of Feedforward Inhibition by Striatal Fast-Spiking Interneurons. *J Neurosci* 33:1678–1683.  
<https://doi.org/10.1523/JNEUROSCI.3572-12.2013>
- Takemura S, Aso Y, Hige T, et al (2017) A connectome of a learning and memory center in the adult *Drosophila* brain. *eLife* 6:e26975. <https://doi.org/10.7554/eLife.26975>
- Tanaka NK, Tanimoto H, Ito K (2008) Neuronal assemblies of the *Drosophila* mushroom body. *Journal of Comparative Neurology* 508:711–755.  
<https://doi.org/10.1002/cne.21692>
- Tanimura A, Du Y, Kondapalli J, et al (2019) Cholinergic Interneurons Amplify Thalamostriatal Excitation of Striatal Indirect Pathway Neurons in Parkinson's Disease Models. *Neuron* 101:444–458.e6.  
<https://doi.org/10.1016/j.neuron.2018.12.004>

- Tempel BL, Bonini N, Dawson DR, Quinn WG (1983) Reward learning in normal and mutant *Drosophila*. *Proc Natl Acad Sci U S A* 80:1482–1486
- Tepper JM, Bolam JP (2004) Functional diversity and specificity of neostriatal interneurons. *Current Opinion in Neurobiology* 14:685–692. <https://doi.org/10.1016/j.conb.2004.10.003>
- Tepper JM, Koós T, Ibanez-Sandoval O, et al (2018) Heterogeneity and Diversity of Striatal GABAergic Interneurons: Update 2018. *Front Neuroanat* 12:. <https://doi.org/10.3389/fnana.2018.00091>
- Threlfell S, Clements MA, Khodai T, et al (2010) Striatal muscarinic receptors promote activity dependence of dopamine transmission via distinct receptor subtypes on cholinergic interneurons in ventral versus dorsal striatum. *J Neurosci* 30:3398–3408. <https://doi.org/10.1523/JNEUROSCI.5620-09.2010>
- Threlfell S, Cragg SJ (2011) Dopamine signaling in dorsal versus ventral striatum: the dynamic role of cholinergic interneurons. *Front Syst Neurosci* 5:11. <https://doi.org/10.3389/fnsys.2011.00011>
- Threlfell S, Lalic T, Platt NJ, et al (2012) Striatal Dopamine Release Is Triggered by Synchronized Activity in Cholinergic Interneurons. *Neuron* 75:58–64. <https://doi.org/10.1016/j.neuron.2012.04.038>
- Trantham-Davidson H, Neely LC, Lavin A, Seamans JK (2004) Mechanisms Underlying Differential D1 versus D2 Dopamine Receptor Regulation of Inhibition in Prefrontal Cortex. *J Neurosci* 24:10652–10659. <https://doi.org/10.1523/JNEUROSCI.3179-04.2004>
- Tully T, Quinn WG (1985) Classical conditioning and retention in normal and mutant *Drosophila melanogaster*. *J Comp Physiol* 157:263–277. <https://doi.org/10.1007/BF01350033>
- Turner GC, Bazhenov M, Laurent G (2008) Olfactory Representations by *Drosophila* Mushroom Body Neurons. *Journal of Neurophysiology* 99:734–746. <https://doi.org/10.1152/jn.01283.2007>
- Uchigashima M, Ohtsuka T, Kobayashi K, Watanabe M (2016) Dopamine synapse is a neuroligin-2-mediated contact between dopaminergic presynaptic and GABAergic postsynaptic structures. *Proceedings of the National Academy of Sciences* 113:4206–4211. <https://doi.org/10.1073/pnas.1514074113>
- Ulbricht W (1998) Effects of veratridine on sodium currents and fluxes. *Rev Physiol Biochem Pharmacol* 133:1–54. <https://doi.org/10.1007/BFb0000612>
- Vernino S, Rogers M, Radcliffe KA, Dani JA (1994) Quantitative measurement of calcium flux through muscle and neuronal nicotinic acetylcholine receptors. *J Neurosci* 14:5514–5524. <https://doi.org/10.1523/JNEUROSCI.14-09-05514.1994>

- Vietti-Michelina S (2022) Investigating the mechanisms underlying cholinergic regulation of dopamine release. Thesis D.Phil. University of Oxford.
- Vilaró MT, Palacios JoséM, Mengod G (1990) Localization of m5 muscarinic receptor mRNA in rat brain examined by in situ hybridization histochemistry. *Neuroscience Letters* 114:154–159. [https://doi.org/10.1016/0304-3940\(90\)90064-G](https://doi.org/10.1016/0304-3940(90)90064-G)
- Villar ME, Pavão-Delgado M, Amigo M, et al (2022) Differential coding of absolute and relative aversive value in the *Drosophila* brain. *Current Biology* 32:4576-4592.e5. <https://doi.org/10.1016/j.cub.2022.08.058>
- Villette V, Chavarha M, Dimov IK, et al (2019) Ultrafast Two-Photon Imaging of a High-Gain Voltage Indicator in Awake Behaving Mice. *Cell* 179:1590-1608.e23. <https://doi.org/10.1016/j.cell.2019.11.004>
- Vogt K, Aso Y, Hige T, et al (2016) Direct neural pathways convey distinct visual information to *Drosophila* mushroom bodies. *eLife* 5:e14009. <https://doi.org/10.7554/eLife.14009>
- Voorn P, Vanderschuren LJMJ, Groenewegen HJ, et al (2004) Putting a spin on the dorsal–ventral divide of the striatum. *Trends in Neurosciences* 27:468–474. <https://doi.org/10.1016/j.tins.2004.06.006>
- Waddell S (2010) Dopamine reveals neural circuit mechanisms of fly memory. *Trends Neurosci* 33:457–464. <https://doi.org/10.1016/j.tins.2010.07.001>
- Waddell S (2013) Reinforcement signalling in *Drosophila*; dopamine does it all after all. *Current Opinion in Neurobiology* 23:324–329. <https://doi.org/10.1016/j.conb.2013.01.005>
- Wang L, Shang S, Kang X, et al (2014a) Modulation of dopamine release in the striatum by physiologically relevant levels of nicotine. *Nat Commun* 5:3925. <https://doi.org/10.1038/ncomms4925>
- Wang L, Zhang X, Xu H, et al (2014b) Temporal components of cholinergic terminal to dopaminergic terminal transmission in dorsal striatum slices of mice. *The Journal of Physiology* 592:3559–3576. <https://doi.org/10.1113/jphysiol.2014.271825>
- Wang Y, Toyoshima O, Kunitatsu J, et al (2021) Tonic firing mode of midbrain dopamine neurons continuously tracks reward values changing moment-by-moment. *eLife* 10:e63166. <https://doi.org/10.7554/eLife.63166>
- Watabe-Uchida M, Zhu L, Ogawa SK, et al (2012) Whole-Brain Mapping of Direct Inputs to Midbrain Dopamine Neurons. *Neuron* 74:858–873. <https://doi.org/10.1016/j.neuron.2012.03.017>
- Weiner DM, Levey AI, Brann MR (1990) Expression of muscarinic acetylcholine and dopamine receptor mRNAs in rat basal ganglia. *Proceedings of the National Academy of Sciences* 87:7050–7054. <https://doi.org/10.1073/pnas.87.18.7050>



- White KE, Humphrey DM, Hirth F (2010) The Dopaminergic System in the Aging Brain of *Drosophila*. *Front Neurosci* 4:. <https://doi.org/10.3389/fnins.2010.00205>
- Xiao Y, Yang J, Ji W, et al (2021) A- and D-type potassium currents regulate axonal action potential repolarization in midbrain dopamine neurons. *Neuropharmacology* 185:108399. <https://doi.org/10.1016/j.neuropharm.2020.108399>
- Yan Z, Surmeier DJ (1996) Muscarinic (m2/m4) receptors reduce N- and P-type Ca<sup>2+</sup> currents in rat neostriatal cholinergic interneurons through a fast, membrane-delimited, G-protein pathway. *J Neurosci* 16:2592–2604. <https://doi.org/10.1523/JNEUROSCI.16-08-02592.1996>
- Yarali A, Gerber B (2010) A Neurogenetic Dissociation between Punishment-, Reward-, and Relief-Learning in *Drosophila*. *Front Behav Neurosci* 4:. <https://doi.org/10.3389/fnbeh.2010.00189>
- Yeh JZ, Oxford GS, Wu CH, Narahashi T (1976) Dynamics of aminopyridine block of potassium channels in squid axon membrane. *Journal of General Physiology* 68:519–535. <https://doi.org/10.1085/jgp.68.5.519>
- Yetnikoff L, Lavezzi HN, Reichard RA, Zahm DS (2014) An update on the connections of the ventral mesencephalic dopaminergic complex. *Neuroscience* 282:23–48. <https://doi.org/10.1016/j.neuroscience.2014.04.010>
- Yin HH, Knowlton BJ (2006) The role of the basal ganglia in habit formation. *Nat Rev Neurosci* 7:464–476. <https://doi.org/10.1038/nrn1919>
- Yung KKL, Bolam JP, Smith AD, et al (1995) Immunocytochemical localization of D 1 and D 2 dopamine receptors in the basal ganglia of the rat: Light and electron microscopy. *Neuroscience* 65:709–730. [https://doi.org/10.1016/0306-4522\(94\)00536-E](https://doi.org/10.1016/0306-4522(94)00536-E)
- Zell V, Teuns G, Needham AS, et al (2023) Characterization of Selective M5 Acetylcholine Muscarinic Receptor Modulators on Dopamine Signaling in the Striatum. *The Journal of Pharmacology and Experimental Therapeutics* 387:226–234. <https://doi.org/10.1124/jpet.123.001737>
- Zhang H, Sulzer D (2004) Frequency-dependent modulation of dopamine release by nicotine. *Nat Neurosci* 7:581–582. <https://doi.org/10.1038/nn1243>
- Zhang X, Bi R, Zhang P, Gan Y (2018a) Veratridine modifies the gating of human voltage-gated sodium channel Nav1.7. *Acta Pharmacol Sin* 39:1716–1724. <https://doi.org/10.1038/s41401-018-0065-z>
- Zhang Y-F, Cragg SJ (2017) Pauses in Striatal Cholinergic Interneurons: What is Revealed by Their Common Themes and Variations? *Front Syst Neurosci* 11:. <https://doi.org/10.3389/fnsys.2017.00080>

- Zhang Y-F, Luan P, Qiao Q, et al (2025) An axonal brake on striatal dopamine output by cholinergic interneurons. *Nat Neurosci* 1–12. <https://doi.org/10.1038/s41593-025-01906-5>
- Zhang Y-F, Reynolds JNJ, Cragg SJ (2018b) Pauses in Cholinergic Interneuron Activity Are Driven by Excitatory Input and Delayed Rectification, with Dopamine Modulation. *Neuron* 98:918-925.e3. <https://doi.org/10.1016/j.neuron.2018.04.027>