

The Dynamic Landscape of Cortical Synaptic Inhibition

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Declaration

I, Richard Joseph Burman, declare that this thesis titled, '*The Dynamic Landscape of Cortical Synaptic Inhibition*', and the work presented in it are wholly my own apart from certain sections that were performed in collaboration. Here I declare the following contributions made by others to the data presented in this thesis:

- **Figure 6.1F** - Ms Tara Diviney, a MSc Neuroscience student who completed her Hilary Term 2020 project rotation under my co-supervision, contributed electrophysiological data
- **Figure 6.3** - Ms Gemma Gothard, a fellow DPhil Scholar in the Akerman Research Group, performed the *in utero* electroporation of the optogenetic construct

I further confirm that all the data I present in this thesis is entirely original. None of the results contained within this thesis have been published at the time of its original submission.

I shall be telling this with a sigh
Somewhere ages and ages hence:
Two roads diverged in a wood, and I—
I took the one less traveled by,
And that has made all the difference.

The Road Not Taken, Robert Frost (1874–1963)

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Abstract

Fast synaptic inhibition shapes cellular responses and network activity in the mammalian cortex. This inhibition is principally mediated by the activation of ionotropic GABA-A receptors (GABA_ARs), which are permeable to chloride (Cl⁻). For this reason, GABA_AR function is intrinsically linked to processes that regulate the distribution of Cl⁻ on either side of the neuronal membrane. Despite their fundamental role, the manner by which synaptic GABA_ARs function in the intact cortex has remained unknown. In this thesis, I explore how GABA_AR-mediated synaptic signalling operates across different brain states and neuronal cell types in the mammalian cortex. To achieve this aim, I establish *in vivo* gramicidin perforated patch-clamp recordings, which enable me to study GABA_AR function in the living rodent cortex, whilst preserving the integrity of the transmembrane Cl⁻ gradient. By combining this method with pharmacological manipulations, I confirm that Cl⁻ co-transporter proteins play a key role in establishing the equilibrium potential for GABA_ARs ($E_{\text{GABA}_A\text{R}}$) *in vivo*. I then use *in vivo* gramicidin perforated patch-clamp recording to reveal that GABA_AR synaptic signalling is predominately shunting in the awake cortex. This results from relatively depolarised $E_{\text{GABA}_A\text{R}}$ values, which arise due to the high levels of local network activity in the awake state. I further show that the animal's sleep-wake history also contributes in setting $E_{\text{GABA}_A\text{R}}$ in the awake cortex, consistent with evidence that sleep-wake history can modulate the activity of Cl⁻ co-transporter proteins. To extend my studies into different neuronal cell types, I then establish a novel optogenetic method for probing intracellular Cl⁻ regulation. By using a light-activated Cl⁻ channel, I reveal differences in both the steady-state Cl⁻ levels and Cl⁻ extrusion capacities of two genetically-defined neuronal populations in the cortex, pyramidal neurons and parvalbumin-expressing interneurons. Taken together, the findings of this thesis support the idea that the 'landscape' within which GABA_ARs function is highly dynamic, depending on both the cortical state and neuronal cell type. This has important implications for how GABA_AR function should be conceptualised within the cortex.

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List of Abbreviations

ACR	anion-conducting rhodopsins
ACSF	artificial cerebrospinal fluid
AE	anion exchanger
AIS	axon initial segment
Alexa594	Alexa Fluor dye 594
AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
An.	anaesthetised cortex
AP	action potential
Aw.	awake cortex
BSA	bovine serum albumin
CCC	cation-chloride co-transporters
ChR2	channelrhodopsin 2
Ca²⁺	calcium
ChABC	chondroitinase ABC
Cl⁻	chloride
[Cl⁻]_e	extracellular concentration of chloride
[Cl⁻]_i	intracellular concentration of chloride
DF_{GABA}	driving force for GABA signalling
DF_{GABA_AR}	driving force for the GABA-A receptor
E_{Cl}	equilibrium potential for chloride
E_e	equilibrium potential for excitatory synaptic signalling
E_{GABA}	equilibrium potential for GABA signalling
E_{GABA_AR}	equilibrium potential for the GABA-A receptor
E_{HCO₃}	equilibrium potential for bicarbonate

$E_i / E_{\text{Inhibition}}$	equilibrium potential for inhibitory synaptic signalling
E_{Leak}	equilibrium potential for the leak conductance
E_{Syn}	equilibrium potential for synaptic signalling
eNpHR3.0	halorhodopsin
EYFP	enhanced-yellow fluorescent protein
f_s	sampling frequency
FRET	fluorescence resonance energy transfer
GABA	gamma (γ) aminobutyric acid
GABA_AR	GABA-A receptor
GABA_BR	GABA-B receptor
Gad	glutamate decarboxylase
g_e	conductance of excitatory synaptic channels
g_i	conductance of inhibitory synaptic channels
g_{Leak}	conductance of membrane leak channels
g_m	total membrane conductance
GHK	Goldman–Hodgkin–Katz
GlyR	glycine receptor
HCO_3^-	bicarbonate
$[\text{HCO}_3^-]_e$	extracellular concentration of bicarbonate
$[\text{HCO}_3^-]_i$	intracellular concentration of bicarbonate
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IC	current-clamp
IP	intraperitoneal
K^+	potassium
$[\text{K}^+]_e$	extracellular concentration of potassium
KCC2	potassium-chloride cotransporter
LJP	liquid junction potential
Na^+	sodium
$\text{Na}^+ / \text{K}^+ \text{ ATPase}$	sodium-potassium ATPase
NBQX	2,3-dioxo-6-nitro-7-sulfamoyl-benzo[f]quinoxaline
NCBE	sodium-chloride-bicarbonate exchanger
NKCC1	sodium-potassium-chloride cotransporter

NMDA	N-methyl-D-aspartate
NGS	normal goat serum
NREM	non-rapid eye movement sleep
PFA	paraformaldehyde
PBS	phosphate buffered saline
PV	parvalbumin
R	rested cortex
R+	rested cortex with NBQX
REM	rapid eye movement sleep
R_i	input resistance
R_m	membrane resistance
R_s	series resistance
RMP	resting membrane potential
SC	subcutaneous
SCN	suprachiasmatic nucleus
SD	sleep-deprived cortex
SD+	sleep-deprived cortex with NBQX
SEM	standard error of the mean
stGtACR2	soma-targeted Guillardia theta anion-conducting channelrhodopsin
SST	somatostatin
TdTomato	tandem tomato fluorescent reporter
V_m	resting membrane potential
VC	voltage-clamp

To Mum and Dad,
I dedicate this to you.

Chapter 1

Introduction

The overall aim of this thesis is to investigate cortical synaptic inhibition under *in vivo* conditions and, in particular, the dynamic regulation of intracellular chloride Cl^- levels that determine how inhibitory synapses influence neuronal activity. In order to set the context, I, therefore, need to first introduce a series of principles that underpin the experimental work. This includes introducing basic principles of cortical organisation, how *in vivo* cortex exhibits different ‘states’ that are characterised by different patterns of network activity, and how this is thought to impact the behaviour of individual neurons within the cortical network. I will then turn my attention to the principles that underpin synaptic inhibition in cortical neurons and the central role that is played by Cl^- regulatory mechanisms. I will introduce the biophysical principles that link Cl^- regulation to synaptic inhibition and will then review what the field has learned, primarily from *in vitro* studies, about Cl^- regulatory processes in neurons. Finally, I will introduce the key experimental techniques that the field has used to investigate Cl^- regulatory mechanisms.

1.1 Cortical network states and the impact of neuronal activity

The mammalian cortex is the pinnacle of evolutionary design. The complex microarchitecture of this grey matter is able to generate enormous computational power. At its core, the cortex is made up of interconnected neural circuits that are comprised of a series of different neuronal cell types (Cajal, 1911; Zeng and Sanes, 2017). This general

architecture is replicated across the cortex and, owing to its biological success, has largely been conserved between multiple mammalian species (Harris and Shepherd, 2015; Hodge et al., 2019). The computational prowess of the cortex is a feature of its hierarchical design (Douglas and Martin, 2007). Neurons are arranged into neural circuits, which are then interconnected to form cortical networks (Douglas and Martin, 2004). At each of these levels, information is received, processed and distributed, with the end result being expressed as a response in the form of action.

Cortical circuits are further defined by their precise patterns of connections, which occur at synapses (Shepard, 2004). These electrochemical junctions can be either excitatory or inhibitory, depending on whether they initiate or repress a neuron's ability to generate action potentials. A single excitatory neuron in the visual cortex of the adult mouse brain can receive anywhere between 5220 and 6472 synaptic inputs, with the majority of these inputs coming from neurons in the immediate surroundings (Wildenberg et al., 2021). The activation of synapses varies across temporal and spatial dimensions, and the integration of these synaptic inputs ultimately determines whether the neuron reaches the threshold to generate an action potential and thus provide an output to the rest of the network.

1.1.1 Cortical networks exhibit different 'states'

The ability of the cortex to perform different computational tasks depends on the operational mode that the cortical networks occupy. These modes, or 'states', reflect global cortical dynamics and often correlate with different behavioural states in the animal. For example, sleep and wakefulness are considered two highly distinct states, which occur physiologically and are associated with very different behaviours (Brown et al., 2012; Hobson and Pace-Schott, 2002; Jones, 2005). While asleep, an animal will not respond to incoming stimuli and will be motionless with its eyes closed. By contrast, during wakefulness, an animal will have its eyes open to allow it to actively engage with its environment through movement and by responding to incoming stimuli. However, cortical states are not always physiological, but can also be induced. Anaesthesia for example, forces an animal into a sleep-like state, during which the animal cannot engage with its local environment or respond to incoming stimuli (Brown et al., 2010).

Cortical states can be distinguished based on differences in the amplitude, frequency and synchronicity of the predominant electrical activity (Harris and Thiele, 2011; Hobson and Pace-Schott, 2002). For example, in the awake brain, the activity within cortical networks is typically low-amplitude, high-frequency and appears globally desynchronised (Destexhe et al., 1999; Moruzzi and Magoun, 1949; Rheinberger and Jasper, 1937; Steriade, McCormick and Sejnowski, 1993). By contrast, as the cortex transitions towards a sleep state, cortical networks become increasingly more synchronised with activity decreasing in frequency and increasing in amplitude (Jouvet, 1967; Loomis et al., 1935; Steriade, 2000; Steriade, McCormick and Sejnowski, 1993).

The patterns of activity within each cortical state are not static and can further vary across shorter timescales (Harris and Thiele, 2011). For example, as the cortex progresses through the sleep cycle, each stage is defined by distinct changes in cortical activity (Brown et al., 2012; Steriade et al., 2001). During non-rapid eye movement (NREM) sleep, there is the emergence of slow-wave activity (0.5-4Hz) in which cortical networks fluctuate between periods of increased neuronal spiking and silence (Crunelli and Hughes, 2010; Nir et al., 2011; Steriade et al., 2001; Timofeev et al., 2001). Such highly synchronised activity, consisting of so-called up states and down states, can also be reproduced through various anaesthetic agents (Steriade, Nunez and Amzica, 1993). However, upon entering the rapid-eye movement (REM) stage of the sleep cycle, cortical networks become less synchronised and generate electrical activity that is lower in amplitude and higher in frequency, similar to what is observed in the awake cortex (Aserinsky and Kleitman, 1953; Park and Weber, 2020; Steriade et al., 1996, 1990, 2001). During wakefulness, the level of electrical activity depends on whether the cortical network is activated during periods of arousal (lower amplitude, higher frequency of ~ 20 -100Hz) or deactivated during periods of quiescence (higher amplitude, lower frequency of ~ 1 -10Hz) (Ayaz et al., 2019; Fernandez et al., 2017; McGinley et al., 2015; Nestvogel and McCormick, 2022; Poulet and Crochet, 2019; Poulet and Petersen, 2008; Zaghera et al., 2013). Furthermore, during periods of increased arousal and attention, as seen during performing a specific task, high frequency activity (between 30-90Hz) can become highly synchronised across cortical regions (Destexhe et al., 1999; Engel and Singer, 2001; Steriade et al., 1996).

The transition between cortical states, specifically sleep and wakefulness, is controlled through an interaction between environmental cues, such as light, and the activity of subcortical brain areas that oscillate across different time scales (Hobson and Pace-Schott, 2002; Jones, 2005; Lin, 2000; Pace-Schott and Hobson, 2002; Schwartz and Klerman, 2019). Circadian rhythms are mediated by the suprachiasmatic nucleus (SCN), the so-called 'Master Clock', which lies within the hypothalamus and are responsible for influencing the tendency to sleep at specific times of day according to the light-dark cycle (Klein et al., 1991; Reppert and Weaver, 2001). The activity of neurons in the SCN are genetically controlled by so-called 'clock genes' whose expression oscillates across a diurnal or 24-hour cycle (Albrecht, 2002; Reppert and Weaver, 2001). This central circadian influence then interacts with the homeostatic need for sleep, or sleep pressure, which is determined by the period that the animal has been awake (Aeschbach et al., 2001; Dijk et al., 1987). The need for sleep is driven by an activity-dependent accumulation of endogenous substrates, or 'somnogens' (Pace-Schott and Hobson, 2002). These sleep-driving substrates include, but are not limited to, an accumulation of adenosine, which is thought to be responsible for disinhibiting neurons in the ventrolateral preoptic nucleus of the hypothalamus and to facilitate the transition into sleep (Porkka-Heiskanen et al., 1997; Strecker et al., 2000).

The interaction between circadian rhythms (also known as 'Process C') and sleep homeostasis (also known as 'Process S') are part of Borbély's two-process model of sleep regulation (Borbély, 1982; Borbély et al., 2016). The combined output of these processes is a coordinated activation of various neuromodulatory systems that either promote wakefulness or sleep (Jones, 2005). For example, during wakefulness there is increased firing of serotonergic (Jacobs and Fornal, 1999), cholinergic (Steriade et al., 1990), dopaminergic (Maloney et al., 2002) and noradrenergic (Aston-Jones and Bloom, 1981) neurons from different nuclei within the brainstem. The activity of thalamus is another important contributor in determining the activity of local cortical networks across different states (Hobson and Pace-Schott, 2002; Poulet and Crochet, 2019). This is especially evident during NREM sleep, when the thalamus acts in unison with cortex to generate slow-wave activity that consists of cortical up and down states (Steriade, 2000; Steriade, McCormick and Sejnowski, 1993). In addition, thalamic inputs during wakefulness contribute towards the cortex becoming activated during periods of

arousal (Poulet et al., 2012). In summary, whilst I have only been able to introduce general concepts, the above examples illustrate the diversity of cortical states, the fact that they emerge from interactions between subcortical and cortical circuits, and that all ultimately expressed through the activity of the individual neurons that comprise cortical networks.

1.1.2 Cortical neurons reflect different network states

The bioelectric potential of a neuron comes from the distribution of ions on either side of the neuron's selectively-permeable membrane (Hille, 2001c; Johnson and Wu, 1995b). This creates a potential difference in charge across extracellular and intracellular compartments. As ion-selective channels in the membrane open and close, the distribution of charge changes and, in doing so, alters the membrane potential of the neuron. The amount of ionic flux that can occur is determined by the number of open channels in the membrane. This is more commonly referred to as total membrane conductance (g_m), which is the inverse of the membrane resistance (R_m) (**Equation 1.1**).

$$g_m = \frac{1}{R_m} \quad (1.1)$$

Embedded within the neuronal membrane are different channels that are selectively permeable to different ions. Leak channels are constitutively active and largely reflect the membrane permeability to potassium ions (K^+). Beyond the contribution of leak channels, the opening of membrane channels is determined by incoming synaptic inputs. At the synapse, neurotransmitters are released from the presynaptic terminal and bind to receptors on the postsynaptic membrane, which modulate a specific ion channel either directly (i.e. ionotropic receptors) or indirectly by first binding to a G-protein coupled receptor (i.e. metabotropic receptors). Through this binding process, the channel opens and permits ionic flux and the resulting flow of charge, or current, affects the electrical activity of the postsynaptic neuron. Following Ohm's law, a neuron's response to an incoming synaptic current (I_{syn}), measured as a change in its membrane potential (ΔV), is controlled by the state of its R_m (**Equation 1.2**).

$$\Delta V_m = I_{\text{Syn}} \times R_m \quad (1.2)$$

The level of synaptic conductance also directly affects the resting membrane potential (V_m). This is evident in the conductance-based model of the neuronal membrane, which relates the conductance and reversal potential of different channels (Hille, 2001a; Hodgkin and Huxley, 1952; Johnson and Wu, 1995a). The reversal potential or equilibrium of the leak (E_{Leak}) and synaptic (E_{Syn}) channels is a specific voltage at which there is no flow of charge. Using the chord conductance equation (**Equation 1.3**), it is possible to estimate the neuron's V_m at any instant by combining the conductances from both the leak (g_{Leak}) and synapse-activated channels (g_{Syn}), multiplied by their respective equilibrium potentials (Destexhe et al., 2003).

$$V_m = \frac{(g_{\text{Leak}} \times E_{\text{Leak}}) + (g_{\text{Syn}} \times E_{\text{Syn}})}{(g_{\text{Leak}} + g_{\text{Syn}})} \quad (1.3)$$

As the leak conductance (g_{Leak}) is unlikely to change across cortical states, the varying levels of synaptic conductance (g_{Syn}) are more likely to dynamically alter V_m . This biophysical property plays an important role in setting the function of different channels, as will be explained below.

The amount of synaptic input that a neuron is receiving has a direct role in determining the conductance of the neuronal membrane (Destexhe et al., 2003). As most of the synaptic input that a cortical neuron receives comes from its immediate network, when the activity of the local population increases, the neuron will receive a bombardment of synaptic inputs (Tsodyks et al., 1999). In this way, the activity that varies across cortical states sets the conductance of individual neurons within the cortical network (**Figure 1.1**). In the awake cortex, in which there is a high level of background synaptic activity, cortical neurons have a large number of open membrane channels. This causes a lower R_m and means that the neuron operates with a higher membrane conductance g_m (Destexhe and Paré, 1999; Woody and Gruen, 1978).

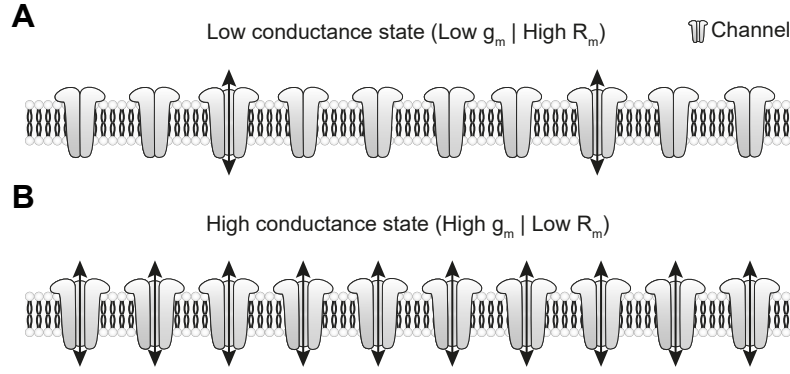


Figure 1.1: Synaptic activity sets the conductance state of the neuronal membrane. **A**, During periods of decreased synaptic activity, fewer membrane channels are open, which limits the movement of charge across the neuronal membrane. This leads to a state characterised by low membrane conductance (g_m) and high membrane resistance (R_m). **B**, When synaptic activity increases, g_m also increases as there are more channels open to permit the flow of charge (low R_m).

1.1.3 Network activity consists of balanced excitatory and inhibitory synaptic conductances

Different synaptic inputs have opposing effects on the electrical activity of the postsynaptic membrane. Excitatory synapses facilitate the generation of action potentials whilst their inhibitory counterparts repress them. These excitatory and inhibitory processes are mediated through specific ion channels, each with its own conductance and reversal potential. By incorporating this into the conductance-based model (**Equation 1.3**), it is evident that the amount of both excitation and inhibition a neuron receives contributes towards setting V_m (**Equation 1.4**).

$$V_m = \frac{(g_{\text{Leak}} \times E_{\text{Leak}}) + (g_e \times E_e) + (g_i \times E_i)}{(g_{\text{Leak}} + g_e + g_i)} \quad (1.4)$$

Experimental studies have shown that periods of intense synaptic activity in the cortex involves coincident excitatory and inhibitory synaptic inputs (Callaway, 1998, 2004; Felleman and Van Essen, 1991; Isaacson and Scanziani, 2011; Rockland and Pandya, 1979). In feed-forward circuits, activation of excitatory neurons drives inhibitory neurons that then suppress a distant population of excitatory neurons. In this way feed-forward inhibition helps refine the temporal fidelity and expand the dynamic range of cortical networks (Pouille et al., 2009; Pouille and Scanziani, 2001). By contrast,

in feedback circuits, excitatory neurons activate inhibitory neurons that then directly inhibit the same population of excitatory neurons and thereby prevent runaway excitation (Isaacson and Scanziani, 2011). The interplay between these two ‘inhibitory motifs’ demonstrate how excitation and inhibition are tightly linked to one another, whereby excitatory actions generate corresponding inhibitory actions (van Vreeswijk and Sompolinsky, 1996). However, whilst these opposing processes occur at the same time, their relative contribution to membrane conductance varies across different cortical states (**Figure 1.2**). This is especially evident during highly active states such as seen during wakefulness, where inhibitory conductances are dominant (Anderson, Carandini and Ferster, 2000; Borg-Graham et al., 1998; Galarreta and Hestrin, 1998; Rudolph et al., 2007).

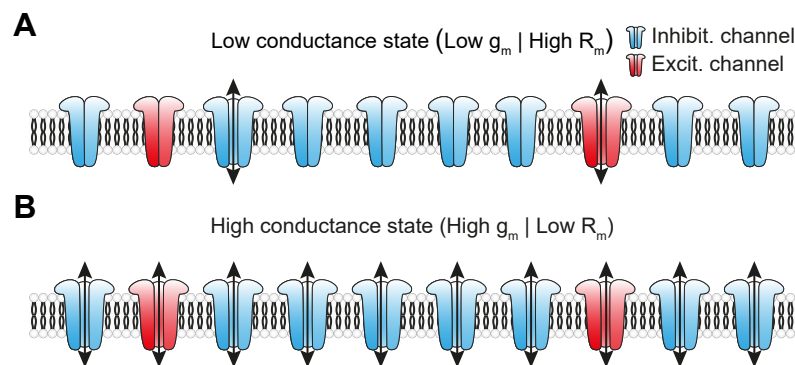


Figure 1.2: High conductance states are dominated by synaptic inhibition. **A**, Channels linked to excitatory ('Excit. Channel', red) and inhibitory ('Inhibit. Channel', blue) channels coexist on the neuronal membrane. **B**, During high conductance states the contribution of inhibitory conductances predominate over excitatory conductances (at a ratio of 5:1 according to Destexhe and Paré (2003))

The dominance of inhibition reflects its role in optimising cortical processing (Cardin, 2018; Herstel and Wierenga, 2021; Isaacson and Scanziani, 2011). Inhibition modulates neuronal gain and, in doing so, is able to refine responses to external stimuli (Grienberger et al., 2017; Haider et al., 2013; Liu et al., 2011; Poo and Isaacson, 2009; Wehr and Zador, 2003). In addition to modulating neuronal responsiveness, inhibition has also been implicated in driving oscillatory activity and establishing network connectivity within the cortex (Atallah et al., 2012; Cardin et al., 2009; Sohal et al., 2009). The manner in which synaptic inhibition is able to perform these important functions is closely related to the function of the receptor through which its effects are deployed.

1.2 Synaptic inhibition depends upon the dynamic regulation of chloride

1.2.1 Fast synaptic inhibition is mediated via the GABA-A receptor

The principal mechanism by which synaptic inhibition operates in the brain is through the binding of the neurotransmitter γ -Aminobutyric acid (GABA) to its ligand-specific receptors (Bormann, 2000). GABA's prospective targets can either be the ionotropic GABA-A receptor (GABA_AR) or the metabotropic GABA-B receptor (GABA_BR). A key difference between these receptors is the speed of their inhibitory action, with the GABA_AR being responsible for mediating fast synaptic inhibition in the cortex.

The GABA_AR is a pentameric membrane channel that can exist in different forms according to its constitutive subunits (**Figure 1.3**). Each of these different configurations of the GABA_AR has important implications for its positioning within the synapse, as well as its sensitivity to different endogenous and exogenous modulators (Olsen and Sieghart, 2009). The most common form of the GABA_AR consists of two α_1 subunits, two β_2 subunits and a γ_2 subunit. This configuration of the GABA_AR is expressed within the synaptic cleft and facilitates phasic inhibition (Brickley et al., 1999; Farrant and Nusser, 2005).

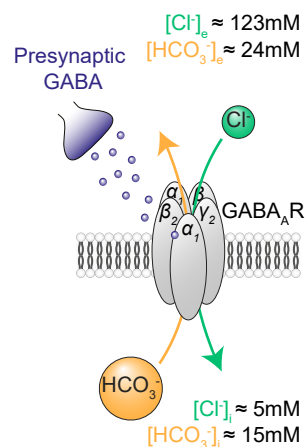


Figure 1.3: The GABA-A receptor is primarily permeable to chloride. The GABA_AR is made up of five different protein subunits and is expressed on the postsynaptic membrane. After being activated by GABA, the channel opens and allows Cl⁻ and HCO₃⁻ ions to flow down their respective transmembrane gradients. Figure is adapted from Burman and colleagues (2022).

The function of the GABA_AR is determined by the opposing flux of chloride (Cl⁻) and bicarbonate (HCO₃⁻) ions (Bormann et al., 1987; Constanti and Nistri, 1976; Kaila and

Voipio, 1987; Takeuchi and Takeuchi, 1967). The permeability of the channel to these ions is not proportional as it has a strong bias for Cl^- over HCO_3^- in a ratio of 4:1 (Kaila, 1994). Due to this bias, GABA_AR function is largely determined by Cl^- flux.

Upon the binding of GABA, the GABA_AR opens and permits a flow of charge across the neuronal membrane. This generates a current (I_{GABAAR}) where the magnitude is determined by the amount of charge allowed to travel through the channel, or conductance (g_{GABAAR}), and the driving force (DF_{GABAAR}). This is highlighted in **Equation 1.5**.

$$I_{\text{GABAAR}} = g_{\text{GABAAR}} \times DF_{\text{GABAAR}} \quad (1.5)$$

The DF_{GABAAR} describes the net electromotive force acting on the ions passing through the GABA_AR . The size and direction of the DF_{GABAAR} is determined by the relationship between the V_m and the equilibrium potential of the GABA_AR (E_{GABAAR} , **Equation 1.6**). As a neuron's V_m is continuously varying, there are continual variations in the DF_{GABAAR} . Alternatively, the DF_{GABAAR} can also be calculated by using the E_{Leak} which is reflective of the resting membrane potential (RMP, **Equation 1.7**).

$$DF_{\text{GABAAR}} = V_m - E_{\text{GABAAR}} \quad (1.6)$$

$$DF_{\text{GABAAR}} = E_{\text{Leak}}(\text{or RMP}) - E_{\text{GABAAR}} \quad (1.7)$$

The equilibrium potential for the GABA_AR (E_{GABAAR}) is the voltage of the neuronal membrane at which there is no net flux of ions through the GABA_AR (Hille, 2001b; Johnson and Wu, 1995b). It is also known as the reversal potential because it is the membrane voltage at which the direction of ion flow changes, or reverses, through the GABA_AR . At the E_{GABAAR} , the diffusion force set by the transmembrane ionic gradient is balanced by the opposing electrostatic forces between Cl^- ions (**Figure 1.4**).

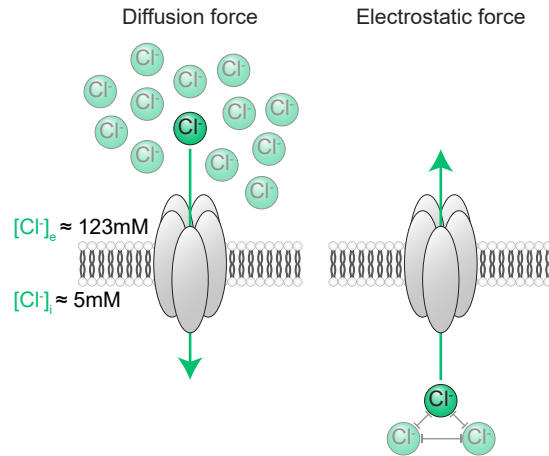


Figure 1.4: The equilibrium potential for the GABA_AR is determined by opposing thermodynamic forces. The equilibrium potential for the GABA_AR ($E_{\text{GABA}_A\text{R}}$) is largely determined by the receptors high permeability for Cl^- . These ions are subject to opposing forces. The transmembrane Cl^- gradient favours the inward flux due to the diffusion force. However, in the intracellular compartment, the electrostatic forces between Cl^- ions repel each other forcing some Cl^- ions to move outwards. The balance of these two forces sets the equilibrium potential for Cl^- (E_{Cl}), which closely resembles the $E_{\text{GABA}_A\text{R}}$.

The equilibrium potential for Cl^- (E_{Cl}) and HCO_3^- (E_{HCO_3}) through the GABA_AR can be calculated by the Nernst Equation. As the GABA_AR is permeable to both Cl^- and HCO_3^- , the $E_{\text{GABA}_A\text{R}}$ is determined by combining E_{Cl} and E_{HCO_3} through the Goldman-Hodgkin-Katz (GHK) equation ($E_{\text{GABA}_A\text{R}}$, **Equation 1.8**), which includes the Universal gas constant (R), the Faraday constant (F) and absolute temperature (T). The GHK equation is derived from the Nernst equation and can accommodate the different ions the channel is permeable to. As the permeability for Cl^- is four times greater than HCO_3^- , the $E_{\text{GABA}_A\text{R}}$ is considered mostly equivalent to the E_{Cl} .

$$E_{\text{GABA}_A\text{R}} = -\frac{RT}{F} \ln \left(\frac{[\text{Cl}^-]_e + 0.25[\text{HCO}_3^-]_e}{[\text{Cl}^-]_i + 0.25[\text{HCO}_3^-]_i} \right) \quad (1.8)$$

1.2.2 The neuronal transmembrane chloride gradient is set by membrane co-transporters

The $E_{\text{GABA}_A\text{R}}$ is a key parameter that sets the operating mode of the GABA_AR. As explained in **Equation 1.7**, the $E_{\text{GABA}_A\text{R}}$ is intrinsically linked to the state of the transmembrane Cl^- gradient. Therefore the systems that regulate intracellular Cl^- are directly responsible for setting the $E_{\text{GABA}_A\text{R}}$ and, by extension, the manner in which the

GABA_AR deploys its effects. Cl⁻ homeostasis is maintained through interacting active and passive processes occurring at the neuronal membrane. These include both transporters and channels that operate in concert to transport Cl⁻ in and out of the intracellular space (**Figure 1.5**). In doing so, these mechanisms establish the transmembrane Cl⁻ gradient, which is responsible for setting the E_{GABA_AR}.

Of the different cation-chloride co-transporters (CCCs) active on the neuronal membrane, two have a standout role in setting the transmembrane Cl⁻ (Blaesse et al., 2009; Doyon, Vinay, Prescott and De Koninck, 2016; Kaila et al., 2014). The first is sodium-potassium-chloride transporter (NKCC1), which transports Cl⁻ into the intracellular space. The main opposition to this Cl⁻ influx is the potassium-chloride co-transporter (KCC2), which extrudes Cl⁻ back into the extracellular compartment. Together, the relative activity of NKCC1 and KCC2 can either increase or decrease intracellular Cl⁻ and, therefore, move the E_{GABA_AR} in either direction.

The relative activity of NKCC1 and KCC2 can vary over the lifetime of a neuron (Ben-Ari, 2002; Blaesse et al., 2009; Kaila et al., 2014). Early in brain development, high NKCC1 leads cortical neurons to operate with high intracellular Cl⁻ (Ben-Ari et al., 1989; LoTurco et al., 1995; Yamada et al., 2004). This causes GABAergic signalling to be depolarising, which has an important function in forming synaptic connections (Akerman and Cline, 2006; Leinekugel et al., 1997; Pfeffer et al., 2009; Wang and Kriegstein, 2011). However, as the brain matures, NKCC1 is downregulated and KCC2 expression increases to shift the neurons into a lower Cl⁻ state leading E_{GABA_AR} to become more hyperpolarised (Rivera et al., 1999). In this way, KCC2 becomes the principal Cl⁻ extrusion mechanism and therefore sets the driving force (Blaesse et al., 2009; Doyon, Vinay, Prescott and De Koninck, 2016; Kaila et al., 2014).

As a secondary transporter, KCC2 utilises the cell's energy 'indirectly' in the form of transmembrane gradients that are established by other mechanisms and membrane proteins. Specifically, KCC2 relies on the co-transport of potassium (K⁺) to remove Cl⁻ from the intracellular space. In this way KCC2 is dependent on the transmembrane K⁺ gradient (Viitanen et al., 2010). The sodium-potassium ATPase (Na⁺/K⁺ ATPase) is the central mechanism that sets the K⁺ gradient by actively transporting K⁺ ions into the intracellular space. As the majority of neuronal energy expenditure is dedicated

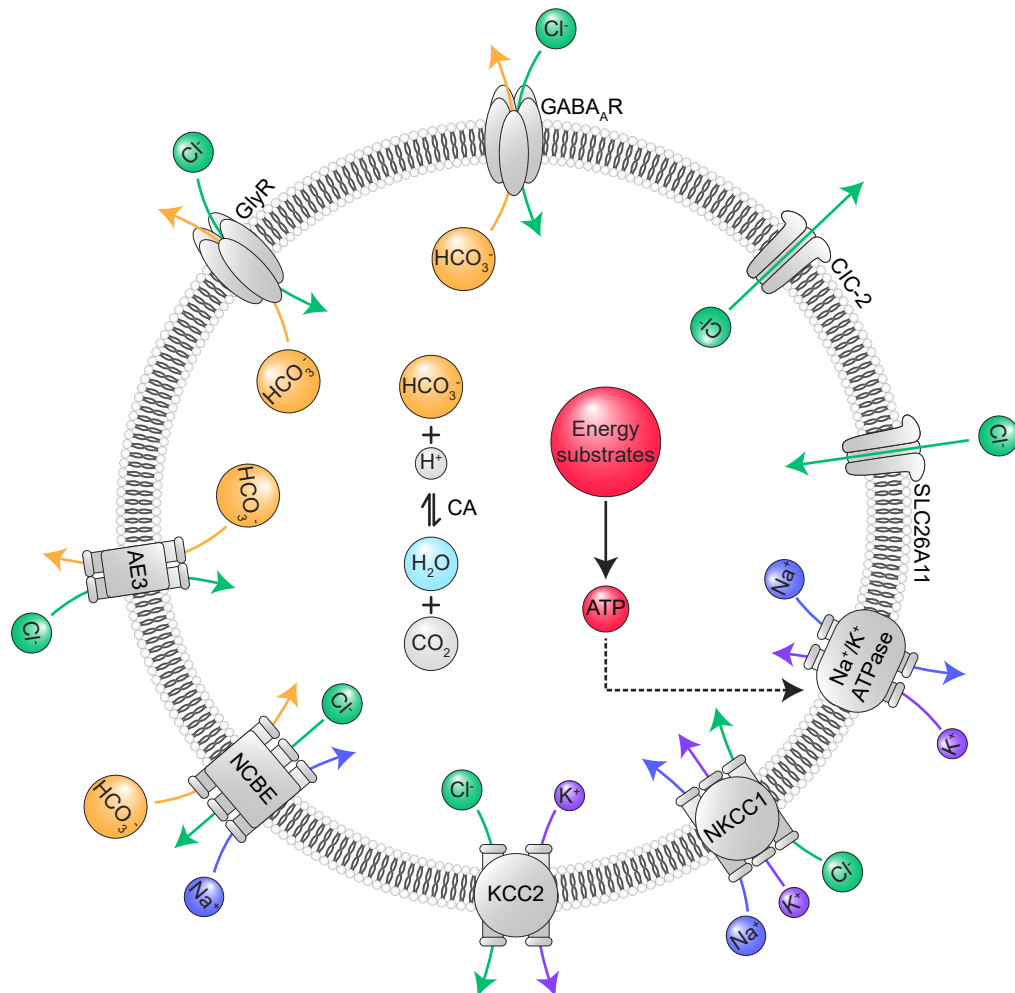


Figure 1.5: Neuronal chloride homeostasis is maintained by multiple membrane transporters and channels. Cation-chloride co-transporters (CCCs) use the gradients of other ions to transport Cl⁻ in opposing directions. Cl⁻ is extruded through the potassium-chloride co-transporter (KCC2) and the sodium-chloride-bicarbonate exchanger (NCBE). The sodium-potassium cotransporter (NKCC1) and anion exchanger (AE3) transport Cl⁻ into the intracellular space. The GABA-A receptor (GABA_AR) and glycine receptor (GlyR) are ligand-gated channels that permit Cl⁻ to flow when the receptors are activated by their respective neurotransmitters, GABA and glycine. The voltage-gated CIC-2 and SLC26A11 channels also permit Cl⁻ flux. The sodium-potassium ATPase (Na⁺/K⁺ ATPase) consumes significant amounts of energy to establish Na⁺ and K⁺ gradients that are utilised by the CCCs. Select transporters (i.e. NCBE, AE3) and channels (i.e. GABA_AR, GlyR) transport bicarbonate ions (HCO₃⁻) and therefore have a function in modulating neuronal pH. This is mainly achieved through the buffering of intracellular HCO₃⁻ by the carbonic anhydrase (CA). Figure is based on illustrations from Doyon and colleagues (2016), Rahmati and colleagues (2018) and Kilb (2021).

towards the Na^+/K^+ ATPase, by association, maintaining the function of KCC2 requires significant energy consumption (Doyon, Vinay, Prescott and De Koninck, 2016; Harris et al., 2012).

In addition to the CCCs, it has been proposed that large charged molecules (e.g. proteins) on either side of the neuronal membrane are responsible for setting the transmembrane Cl^- gradient (Delpire and Staley, 2014; Glykys et al., 2014; Rahmati et al., 2021). This is due to these impermeant anions exerting an electrostatic force by causing unequal distribution of negative charge across cellular compartments by establishing a Gibbs-Donnan effect (Donnan, 1995). Whilst it has been suggested that impermeant anions are involved in volume control and implicated in setting the distribution of Cl^- across the membrane (Glykys et al., 2014; Rahmati et al., 2021), the driving force of Cl^- through the GABA_AR is exclusively controlled by the function of the CCCs (Düsterwald et al., 2018; Hille, 2001b; Otsu et al., 2020; Rivera et al., 1999; Voipio et al., 2014).

1.2.3 The equilibrium potential of the GABA-A receptor determines the strength of synaptic inhibition

The polarity of $\text{DF}_{\text{GABA}_A\text{R}}$ determines the direction in which Cl^- ions will flow through an open GABA_AR . An influx or efflux of negative charge can affect V_m in different ways (**Figure 1.6A**). When $E_{\text{GABA}_A\text{R}}$ is more negative than E_{Leak} , $\text{DF}_{\text{GABA}_A\text{R}}$ is positive and therefore represents a driving force that promotes Cl^- influx and membrane hyperpolarisation. The opposite is true in that if $E_{\text{GABA}_A\text{R}}$ is more positive than E_{Leak} , $\text{DF}_{\text{GABA}_A\text{R}}$ is negative and Cl^- will flow out of an open GABA_AR , with this efflux of negative charge depolarising the membrane. In the case where $E_{\text{GABA}_A\text{R}}$ is equal to E_{Leak} , $\text{DF}_{\text{GABA}_A\text{R}}$ will be zero and there will be no net electrochemical driving force to move Cl^- in either direction.

The effect of GABA_AR activation on the neuronal membrane is determined by the $\text{DF}_{\text{GABA}_A\text{R}}$. When $E_{\text{GABA}_A\text{R}}$ is hyperpolarised relative to V_m , inhibition is enacted by pushing V_m further away from the action potential (AP) threshold (Eccles, 1961; Farrant and Kaila, 2007; Kaila, 1994; Rivera et al., 1999). Another form of preventing action potential generation is by reducing the local R_m , so that the effects of incoming excitatory currents are diminished and summation is prevented. This “shunting”

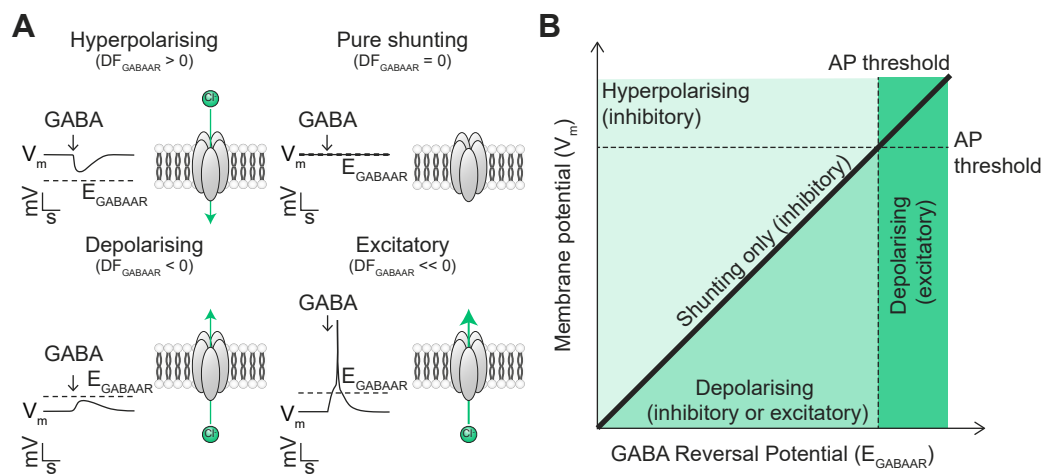


Figure 1.6: The the equilibrium potential of the GABA-A receptor determines the strength and nature of synaptic inhibition. **A**, The relationship between the reversal potential (E_{GABAAR}) and resting membrane potential (V_m) determines the driving force (DF_{GABAAR}) and in doing so sets the direction that chloride ions (Cl^-) will flow through the $GABA_A R$. When E_{GABAAR} is more negative than V_m , $GABA_A R$ activation permits Cl^- influx, which hyperpolarises the V_m , pushing the neuron further away from the action potential (AP) threshold. If E_{GABAAR} equals V_m , opening $GABA_A R$ decreases the local membrane resistance but does not permit Cl^- flux. When E_{GABAAR} is above V_m but below the AP threshold, activation of the $GABA_A R$ will depolarise the membrane. When this occurs, $GABA_A R$ activation can either repress or facilitate incoming synaptic inputs through shunting or summation. If E_{GABAAR} goes beyond the AP threshold, $GABA_A R$ action will trigger APs, and so can be considered to be excitatory. **B**, A plot of the relationship between E_{GABAAR} and V_m showing where the different responses described are likely to occur. Part A is adapted from Burman and colleagues (2022).

inhibition occurs whenever the GABA_AR is opened, however as $DF_{\text{GABA}_A\text{R}}$ approaches zero, it becomes the predominant function of the GABA_AR. When $E_{\text{GABA}_A\text{R}}$ is above V_m , it depolarises the membrane. The effect of GABA_AR-mediated depolarisation varies depending on the timing of incoming excitatory synaptic inputs, as well as their location on the neuron (Alger and Nicoll, 1979; Andersen et al., 1980; Gulledge and Stuart, 2003; Jean-Xavier et al., 2007; Kilb, 2021; Staley and Mody, 1992). For example, at the dendrite, depolarising GABA_AR responses are exclusively excitatory and can facilitate action potential generation (Gulledge and Stuart, 2003). However, at the soma, if the depolarising GABA_AR response occurs at the same time as an excitatory input, it will have an inhibitory effect by shunting the incoming synaptic current (Gulledge and Stuart, 2003). By contrast, if the somatic GABA_AR response occurs before the excitatory input, it can have an excitatory effect (Gulledge and Stuart, 2003). In the most exaggerated situation, if $E_{\text{GABA}_A\text{R}}$ is allowed to equal or go beyond the AP threshold, subsequent activation of the GABA_AR becomes exclusively excitatory as it is able to elicit action potentials regardless of its temporal or spatial proximity to other incoming excitatory synaptic inputs (Staley et al., 1995). In this way, GABA_AR function can be plotted by relating $E_{\text{GABA}_A\text{R}}$ to V_m (**Figure 1.6B**).

1.2.4 Different types of synaptic inhibition have distinct computational properties

Hyperpolarising and shunting inhibition have different effects on neural computation. This is largely due to the temporal kinetics with which these two forms of synaptic inhibition exert their effects (**Figure 1.7**). Hyperpolarising inhibition has a longer time course as the recovery of the membrane potential is set by the decay constant of the membrane, which is a feature of the neuron's cable properties (Alvarez-Leefmans and Delpire, 2009; Eccles, 1961). In this way, the voltage effect will last beyond the closure of the GABA_AR (Pavlov et al., 2011). By contrast, as shunting operates via conductance effects, this type of inhibition is more temporally-locked to when the GABA_AR is open (Staley and Mody, 1992).

Both hyperpolarising and shunting inhibition have been shown to affect synaptic integration and, in doing so, modify coincidence detection. Hyperpolarising inhibition has a long tail of effect that prohibits summation of incoming depolarising currents

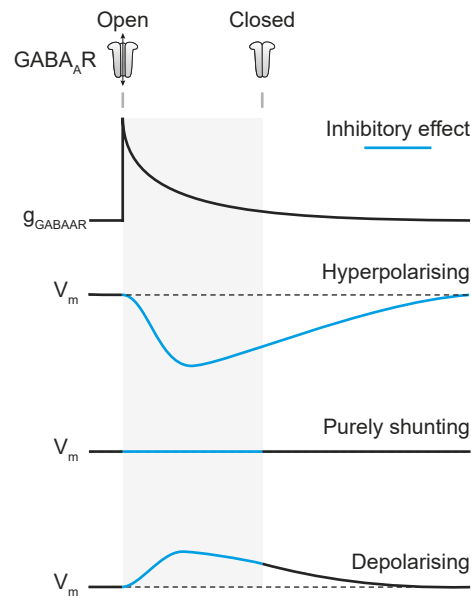


Figure 1.7: Hyperpolarising and shunting inhibition differ in their duration of effect on the membrane potential. Hyperpolarising inhibition has a prolonged effect on the membrane potential (V_m) as the inhibitory effect (cyan line) outlasts the duration of the GABA_AR conductance (g_{GABAAR}), which is set when the GABA_AR is in an open or closed state. By contrast, shunting inhibition has a narrower duration of inhibitory effect as it is only active when the GABA_AR is open. Even when GABA_AR activation is depolarising, it can have an inhibitory effect through shunting incoming excitatory inputs. Figure is adapted from (Gonzalez-Burgos et al., 2011).

(Pavlov et al., 2011). However, shunting inhibition has a much shorter duration of effect, such that only the excitatory synaptic inputs that occur at the same time as local GABA_AR activation are attenuated (Gulledge and Stuart, 2003). Furthermore, previous computational studies have linked shunting inhibition to key optimisations in network function. For example, shunting inhibition has been implicated in allowing neuronal networks to generate high-frequency gamma oscillations (Tikidji-Hamburyan and Canavier, 2020; Vida et al., 2006). However, these computational predictions have been based on features of networks of interneurons operating in the hippocampus. It is, therefore, unknown how different modes of inhibition may affect cortical networks *in vivo*.

1.2.5 A common view is that the GABA-A receptors provide hyperpolarising synaptic inhibition

Over the past three decades there have been numerous computational studies that have modelled synaptic inhibition. In most studies, the mode of inhibition is predefined by

setting a fixed value for E_{Leak} and equilibrium potential for synaptic inhibition ($E_{\text{Inhibition}}$), which can be considered equivalent to $E_{\text{GABA}_{\text{A}}\text{R}}$. A small scale review of this literature reveals that there is a clear tendency to define synaptic inhibition as having a hyperpolarising effect (**Figure 1.8** and **Table 1.1**), even though these parameters are largely based on observations made from *in vitro* studies.

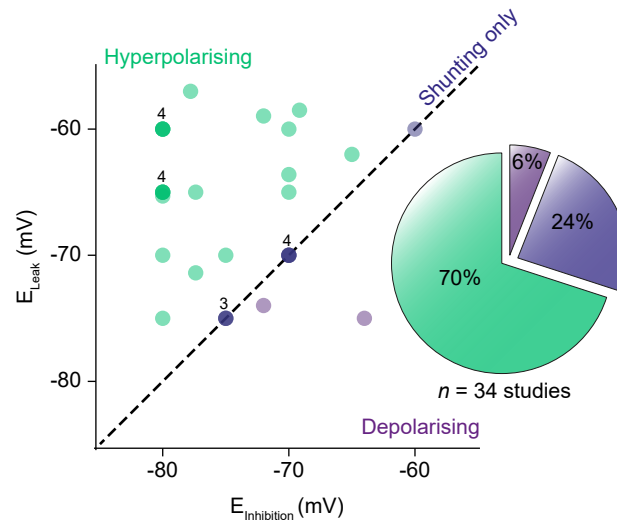


Figure 1.8: Current bias towards hyperpolarising inhibition in computational studies modelling synaptic inhibition. A small scale review was conducted of computational studies that explicitly state the parameters used to establish synaptic inhibition in their models ($n = 34$ studies). The equilibrium potential for inhibition ($E_{\text{Inhibition}}$, taken to be equivalent to $E_{\text{GABA}_{\text{A}}\text{R}}$) and the equilibrium potential for the leak conductances (E_{Leak}) were plotted to determine the mode of synaptic inhibition that was deployed. The line of equivalence (dashed line) reflects where $E_{\text{Inhibition}}$ is equal to E_{Leak} and therefore where inhibition would be purely shunting (blue points). '4' and '3' refer to the number of studies that used the same values. To the left of the line are studies that used hyperpolarising inhibition (green). Two studies used depolarising inhibitory conductances (purple). Overall, the majority of studies used hyperpolarising inhibition (inset). Figure plotted from data in **Table 1.1**.

Insights into GABA_AR synaptic signalling under conditions of undisturbed Cl⁻ gradients have been almost exclusively based upon *in vitro* studies performed in either acute or cultured brain slices. Whilst these experimental preparations provide excellent experimental access for studying synaptic signalling, recordings in brain slices are likely to distort synaptic inhibitory function (Doyon, Vinay, Prescott and De Koninck, 2016). The slicing process itself is thought to cause a traumatic insult that may alter intracellular Cl⁻ (Berglund et al., 2008; Dzhalala et al., 2012; Glykys and Staley, 2016). In addition, *in vitro* slices show significantly reduced levels of spontaneous synaptic activity and notably lower numbers of GABAergic synaptic inputs compared to that observed *in vivo* (Bindman et al., 1988; Destexhe et al., 2003; Steriade, 2001). As local

Author	Year	$E_{\text{Inhibition}}$	E_{Leak}	$DF_{\text{Inhibition}}$	Polarity
To et al	(2022)	-80	-65	15	Hyperpol.
Jin et al	(2021)	-70	-60	10	Hyperpol.
Currin et al	(2021)	-77.41	-65	12.41	Hyperpol.
Liou et al	(2020)	-77.84	-57	20.84	Hyperpol.
Currin et al	(2020)	-77.41	-71.35	6.06	Hyperpol.
Agnes et al	(2020)	-80	-65	15	Hyperpol.
Guet-Mcceight et al	(2019)	-70	-60	10	Hyperpol.
Latimer et al	(2019)	-80	-60	20	Hyperpol.
Bukoski et al	(2018)	-70	-63.56	6.44	Hyperpol.
Düsterwald	(2018)	-85.06	-72.6	12.49	Hyperpol.
Symmonds et al	(2018)	-70	-70	0	Shunt.
Muthukum. et al	(2015)	-90	-70	20	Hyperpol.
Pouille et al	(2013)	-72	-74	-2	Depol.
Sidiropoulou et al	(2012)	-80	-65	15	Hyperpol.
Vogels et al	(2011)	-80	-60	20	Hyperpol.
Moran et al	(2011)	-90	-70	20	Hyperpol.
Akam et al	(2010)	-80	-60	20	Hyperpol.
Vattikuti et al	(2010)	-70	-60	10	Hyperpol.
Rothman et al	(2009)	-75	-75	0	Shunt.
Vogels et al	(2009)	-80	-60	20	Hyperpol.
Ayaz et al	(2009)	-80	-65.3	14.7	Hyperpol.
Morita et al	(2005)	-64	-75	11	Depol.
Mo et al	(2004)	-60	-60	0	Shunt.
Murphy et al	(2003)	-70	-70	0	Shunt.
Mitchell et al	(2003)	-75	-75	0	Shunt.
Prescott et al	(2003)	-70	-70	0	Shunt.
Chance et al	(2002)	-80	-65	15	Hyperpol.
Destexhe et al	(1999)	-80	-75	5	Hyperpol.
Wilson et al	(1999)	-75	-75	0	Shunt.
Destexhe et al	(1997)	-80	-70	10	Hyperpol.
Holt et al	(1997)	-70	-70	0	Shunt.
Wang et al	(1996)	-75	-70	5	Hyperpol.
Gabbiani et al	(1994)	-65	-62	3	Hyperpol.
Bernander et al	(1991)	-70	-65	5	Hyperpol.

Table 1.1: Parameters used in modelling synaptic inhibition. Data plotted in **Figure 1.7**. Studies were selected if they explicitly stated both the equilibrium potential for inhibition ($E_{\text{Inhibition}}$) and the equilibrium potential for membrane leak conductances (E_{Leak}). Only studies simulating pyramidal neurons were analysed. Polarity was defined by the value of the driving force for inhibition ($DF_{\text{Inhibition}}$). Hyperpolarising ('Hyperpol.') was defined as $DF > 0$, pure shunting ('Shunt.') as $DF = 0$, and depolarising ('Depol.') as $DF < 0$. 'Muthukum.', Muthukumaraswamy.

network activity has a profound impact on the levels of synaptic connectivity and activity, it has been predicted that the conductance of cortical neurons in the *in vivo* brain will have important implications for how GABA_AR-mediated signalling operates (Doyon et al., 2011; Doyon, Vinay, Prescott and De Koninck, 2016). For example, a low membrane conductance is representative of low levels of coincident synaptic excitation and inhibition (as illustrated in **Figure 1.2**). Here, a cortical neuron will receive few inhibitory synaptic inputs, resulting in relatively low Cl⁻ influx through GABA_ARs, that is adequately managed by KCC2 extrusion (**Figure 1.9A**). In this situation, the [Cl⁻]_i will be kept low and result in a hyperpolarising DF_{GABA_AR}. As the level of synaptic activity increases, a neuron will receive a greater number of inhibitory synaptic inputs that will lead to increased activation of the GABA_AR. Now the amount of Cl⁻ influx can overwhelm the immediate extrusion capacity of KCC2 leading to an increased [Cl⁻]_i and a depolarising DF_{GABA_AR} (**Figure 1.9B**). These theoretical predictions of the impact of high GABA_AR activity on intracellular Cl⁻ have been supported by *in vitro* experiments (Yelhekar et al., 2017), but have not been explored in the context of the intact brain.

1.2.6 Short-term ionic plasticity at GABA-A receptors

The strength of GABA_AR-mediated synaptic inhibition can be regulated in multiple ways in order to accommodate high levels of synaptic activity. These include changes to the number of GABA_ARs (Goodkin, 2005; Kapur and Coulter, 1995), their location (Feng et al., 2008; Goodkin et al., 2008; Terunuma et al., 2008), and the receptor's sensitivity to activation by GABA (Frosch et al., 1992; Jones and Westbrook, 1995; McCarren and Alger, 1985; Overstreet et al., 2000). Synaptic activity can also affect the transmembrane Cl⁻ gradient that shapes the function of GABA_ARs (Doyon, Vinay, Prescott and De Koninck, 2016; Kaila et al., 2014; Raimondo et al., 2017; Wright et al., 2011). These activity-driven alterations to the transmembrane Cl⁻ gradient can be long-lasting and due to changes to CCC function (Kaila et al., 2014; Rivera et al., 2004). Alternatively, these changes to the transmembrane Cl⁻ gradient can also be transient and caused by intense activation of the GABA_AR, a process referred to as short-term ionic plasticity (Raimondo, Markram and Akerman, 2012; Wright et al., 2011).

Transient changes in intracellular Cl⁻, lasting milliseconds to minutes, can occur as a result of strong GABA_AR activity (**Figure 1.10A**). This was first noticed in early electrical

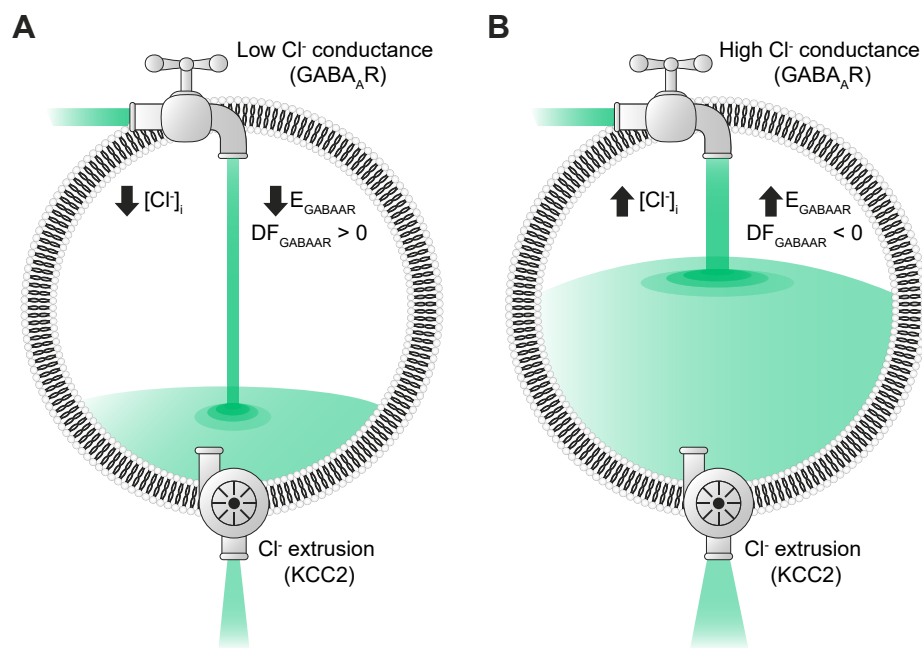


Figure 1.9: High GABA-A receptor conductance is predicted to depolarise the equilibrium potential of the GABA-A receptor. **A**, During periods of low GABA-A receptor (GABA_AR) conductance, there is limited entry of chloride (Cl⁻) into the cell. The potassium-chloride co-transporter (KCC2) is able to effectively manage the load and the intracellular concentration of Cl⁻ ([Cl⁻]_i) remains low. This sets a hyperpolarised equilibrium potential for the GABA_AR (E_{GABAAR}) and a positive driving force for the GABA_AR (DF_{GABAAR}). **B**, During states of high GABA_AR activity, Cl⁻ influx via the GABA_AR is increased. Under such conditions, KCC2's capacity to deal with the large amounts of Cl⁻ entering the cell can become overwhelmed and therefore intracellular Cl⁻ starts to accumulate. The elevated [Cl⁻]_i leads to a more depolarised E_{GABAAR} and a negative DF_{GABAAR} . Figure is based on a review by Doyon et al (2016).

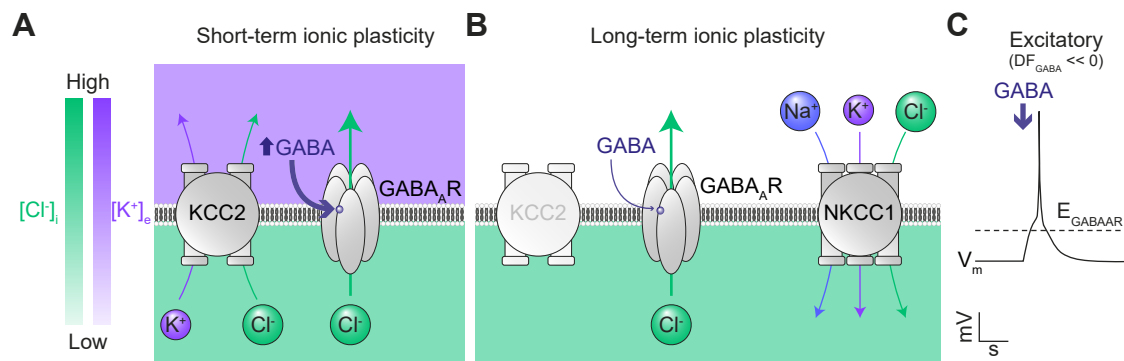


Figure 1.10: Activity-dependent mechanisms of ionic plasticity at GABA-A receptors. **A**, Short-term ionic plasticity is caused by strong activation of GABA_ARs, which causes a large influx of Cl⁻. This initiates increased K⁺ extrusion via KCC2, which elevates [K⁺]_e, thereby reducing KCC2's ability to deal with the large Cl⁻ load. **B**, Long-term ionic plasticity is mediated by decreased function of KCC2 (e.g. via phosphorylation), increased contribution of NKCC1 or a combination of both. Together this creates a higher [Cl⁻]_i. **C**, Ionic plasticity at different time scales can cause an excitatory shift in GABAergic signalling.

stimulation studies, which observed that repetitive activation of the GABA_AR can erode the receptor's ability to generate hyperpolarising potentials, and can even lead to GABAergic currents flipping polarity to become depolarising (Alger and Nicoll, 1979; Andersen et al., 1980; Thompson and Gahwiler, 1989). This shift in GABA_AR polarity is mediated by a rapid influx of Cl⁻ that exceeds the immediate extrusion capacity of KCC2 (Isomura et al., 2003; Kaila et al., 1997; Staley and Proctor, 1999; Staley et al., 1995). Indeed, in trying to compensate for the increased Cl⁻ load, the elevated KCC2 activity can cause a greater efflux of K⁺, which further reduces the capacity of the transporter to transport Cl⁻ (Viitanen et al., 2010). Together, the collapse of the transmembrane Cl⁻ and K⁺ gradients causes E_{GABAAR} to depolarise to an extent that it may even go beyond the AP threshold and become excitatory (Staley et al., 1995). This short-term ionic plasticity resolves soon after GABA_AR activation is discontinued and the intracellular Cl⁻ returns to its resting level (Lamsa and Taira, 2003; Staley and Proctor, 1999). This activity-driven excitatory shift in GABAergic signalling is most exaggerated during the hypersynchronous hyperexcitability associated with seizures. In this context, excitatory GABAergic signalling has been implicated in both facilitating the propagation of this pathological state, and making neuronal networks resistant to GABA_AR-targeting antiseizure medications such as benzodiazepines (Burman et al., 2020, 2022, 2019; Dubanet et al., 2021; Ellender et al., 2014; Fujiwara-Tsukamoto et al., 2010; Magloire et al., 2018).

1.2.7 Neuronal activity determines the setpoint for chloride homeostatic mechanisms

Activity-dependent plasticity at GABAergic synapses can be sustained and, consequently, completely reset the neuronal Cl^- setpoint (**Figure 1.10B**). This can have either a physiological or pathological effect depending on the state of network activity in which it occurs. For example, in the strengthening of synaptic connections during development or learning processes in the brain, repeated activation of GABAergic synapses can cause Cl^- extrusion via KCC2 to be reduced at feed-forward inhibitory synapses (Fiumelli and Woodin, 2007; Lamsa et al., 2010; Ormond and Woodin, 2011, 2009; Woodin et al., 2003). As a consequence, the $[\text{Cl}^-]_i$ will increase and depolarise $E_{\text{GABA}_A\text{R}}$. This positive shift in $E_{\text{GABA}_A\text{R}}$ will reduce the hyperpolarising effect of GABA_AR -mediated inhibition and in effect weaken postsynaptic inhibition. It is thought that this form of inhibitory synaptic plasticity is necessary to 'disinhibit' postsynaptic pyramidal cells, which further potentiate glutamatergic synaptic inputs and lead to a sustained increase in pyramidal cell firing (Ormond and Woodin, 2011, 2009).

Similarly, neurons that have participated in prolonged or recurrent seizure activity have been shown to have significantly lower levels of KCC2 expression and function in both animal and human studies (Burman et al., 2019; Huberfeld et al., 2007; Lee et al., 2010; Rivera et al., 2004; Silayeva et al., 2015). Concurrently, seizure activity has also been shown to increase NKCC1 expression (Barmashenko et al., 2011; Li et al., 2008). Collectively, these changes to the CCC revert the neuron's Cl^- homeostasis back to its development state, where GABAergic signalling is excitatory (Ben-Ari, 2002). During seizures, this excitatory shift in GABA_AR -mediated synaptic signalling may further contribute to the initiation and propagation of seizure activity (Alfonsa et al., 2015; Burman et al., 2019; Kelley et al., 2016).

In both the context of synaptic plasticity and seizures, these enduring changes in Cl^- homeostasis appear to be mediated by secondary messenger pathways (Doyon, Vinay, Prescott and De Koninck, 2016). The initiation of this process occurs as a result of calcium influx through co-incident activation of glutamatergic N-methyl-D-aspartate (NMDA) receptors (Lee et al., 2011) and L-type calcium (Ca^{2+}) channels (Ormond and Woodin, 2009). Alternatively, activation of neuromodulatory systems can also affect Cl^-

homeostasis (Doyon, Vinay, Prescott and De Koninck, 2016; Kaila et al., 2014; Mahadevan and Woodin, 2016; Rivera et al., 2004). These effects can be mediated through G-protein coupled receptors (GPCRs), such as serotonergic (Bos et al., 2013) and adenosine receptors (Ford et al., 2015; Ilie et al., 2012), and tyrosine kinase receptors, such as the brain-derived neurotrophic factor (BDNF) (Coull et al., 2005). In addition, GABA_BRs, which are also GPCRs, have also been implicated in modulating KCC2 function and influencing the driving force for GABA_ARs (Wright et al., 2017).

1.2.8 Cell-type specific differences in inhibitory signalling and chloride regulation

Most of our understanding on GABAergic signalling has come from *in vitro* studies in excitatory pyramidal neurons. However, there is limited evidence suggesting that somatic GABA_AR-mediated inhibition can differ in interneurons. Across brain regions, $E_{\text{GABA}_A\text{R}}$ in fast-spiking interneurons has been found to be more depolarised compared to regular spiking pyramidal neurons (Banke and McBain, 2006; Gulácsi et al., 2003; Martina et al., 2001; Otsu et al., 2020; Vida et al., 2006). The higher interneuron somatic $E_{\text{GABA}_A\text{R}}$, particularly in parvalbumin (PV) basket cells, facilitates a preference for shunting inhibition. Computational data suggests that these interneurons require this mode of synaptic inhibition in order to facilitate network connectivity through generating high-frequency oscillations (Vida et al., 2006).

The underlying mechanisms for these cell-type-specific differences in GABAergic signalling are unclear as there is conflicting evidence on the role of CCCs. Early work by Martina and colleagues (2001) had shown through pharmacological studies that somatic Cl⁻ regulation in interneurons is mediated by increased NKCC1 function, whereas KCC2 function predominates in pyramidal neurons. However, another more recent *in vitro* study by Otsu and colleagues (2020) has suggested that somatic KCC2-mediated Cl⁻ extrusion is more efficient in interneurons. Despite this discrepancy in the underlying mechanisms, the finding that intracellular Cl⁻ between pyramidal cells and PV cells is different suggests that Cl⁻ homeostasis is likely to be heterogeneous across neuronal cell types.

1.3 Current approaches to probe GABA-A receptor synaptic signalling

In the previous section, I presented current knowledge on how intracellular Cl^- is regulated and its effects upon GABA_AR-mediated synaptic signalling. These insights are largely based on experimental investigations that have used different electrical and optical methodologies to study GABA_AR function *in vitro* and *in vivo*. Here I will elaborate on the approaches used to study GABAergic signalling, whilst highlighting important technical caveats that need to be considered.

1.3.1 Electrophysiological and optical methods for measuring GABA-A receptor function

In order to accurately study the function of the GABA_AR, it is imperative to use an approach that causes minimal disruption to the $[\text{Cl}^-]_i$. Over time, different electrophysiological and optical strategies have been adopted to address this challenge by either studying GABA_AR function directly, or by inferring GABA_AR function by monitoring intracellular Cl^- . Each method varies in its spatial and temporal resolution, the different/particular parameters that are measured, as well as in the amount of disruption that the method causes to intracellular Cl^- .

Optical methods have included using Cl^- sensitive dyes or genetically-encoded biosensors to directly measure Cl^- levels across intracellular compartments (Arosio and Ratto, 2014; Bregestovski et al., 2009; Lodovichi et al., 2022). A major advantage of this approach is that it can measure intracellular Cl^- across a large number of cells both *in vitro* and *in vivo*, whilst preserving their native ionic state (Boffi et al., 2018; Glykys et al., 2014; Grimley et al., 2013; Kuner and Augustine, 2000; Pracucci et al., 2021; Rahmati et al., 2021; Sulis Sato et al., 2017). Cl^- imaging can be used to study long acting changes in intracellular Cl^- state (over minutes and longer) such as during seizure activity (Burman et al., 2019; Raimondo et al., 2013; Sulis Sato et al., 2017) and diurnal changes (Klett and Allen, 2017; Klett et al., 2022; Pracucci et al., 2021).

A limitation of Cl^- imaging is that it does not reveal the state of $\text{DF}_{\text{GABA}_A\text{R}}$, which is arguably the most important metric when investigating GABA_AR-mediated synaptic

transmission. Furthermore, Cl^- reporters have a relatively low signal-to-noise ratio, which reflects the lower driving force for Cl^- compared other ions such as Ca^{2+} (Zajac et al., 2020). This makes it very hard to detect synaptic Cl^- fluxes and therefore most Cl^- imaging studies either report resting intracellular Cl^- (Arosio et al., 2010; Boffi et al., 2018; Pracucci et al., 2021) or the result of intense periods of Cl^- influxes, such as during seizure-like activity (Burman et al., 2019; Dzhala et al., 2010; Lillis et al., 2012; Sulis Sato et al., 2017).

Electrophysiological approaches are able to overcome the limitations of Cl^- imaging approaches by directly probing synaptic GABA_AR function and can monitor how the receptor changes over much shorter time scales (down to the millisecond range). Different electrophysiology strategies have included cell-attached recordings to isolate GABA_AR channels (Tyzio et al., 2006, 2008) and extracellular recordings whilst activating GABA_AR conductances (Dubanet et al., 2021). However, the gold standard is considered to be gramicidin perforated patch-clamp recordings, which are able to provide access to intracellular physiology and GABA_AR dynamics without disrupting the transmembrane Cl^- gradient (Abe et al., 1994; Ebihara et al., 1995; Kyrozis and Reichling, 1995). Gramicidin perforated patch-clamp recordings have been used to study physiological and pathological changes in GABA_AR , including during synaptic plasticity (Woodin et al., 2003), over the sleep-wake cycle (Alfonsa et al., 2021) and during seizures (Burman et al., 2019; Ellender et al., 2014; Ilie et al., 2012). In addition, this technique can also study near instantaneous changes in GABA_AR driving force as a result of preceding synaptic activity (Raimondo, Kay, Ellender and Akerman, 2012).

Despite its unrivalled ability to measure GABA_AR -mediated synaptic inhibition, perforated patch-clamp recordings have been limited almost exclusively to *in vitro* preparations. This is because of the technical challenges associated with achieving and maintaining the perforation with cation-selective ionophores such as the antibiotic gramicidin, which does not disrupt transmembrane Cl^- gradients. There have been attempts at performing perforated recordings *in vivo* using amphotericin, an antifungal, but this perforating agent creates larger pores that do not preserve the integrity of the Cl^- gradient (Wang et al., 2016). Therefore, there remains an unmet need to be able to perform gramicidin perforated patch-clamp recordings in the living brain in order to

study GABA_AR function.

1.3.2 Different strategies of activating neuronal chloride flux

A key component of studying intracellular Cl⁻ dynamics is being able to assess the driving force for Cl⁻. In order for this to be achieved, Cl⁻-permeable channels, like the GABA_AR, need to be activated in order to permit the flux of Cl⁻ ions. Postsynaptic GABA_AR conductances can be activated in different ways (**Figure 1.11**). These include focal application of an exogenous GABA or a GABA_AR-selective agonist such as muscimol. This can be performed *in vitro* (Alger and Nicoll, 1979; Ellender et al., 2014; Ilie et al., 2012; Yelhekar et al., 2017) or *in vivo* (Kirmse et al., 2015). However, this exogenous modulation does not represent physiological GABAergic signalling. This is because the large amounts of GABA released can quickly saturate the GABA_AR, and it can be difficult to contain the locality of the effect.

An alternate approach is to stimulate endogenous presynaptic GABA release by activating local interneuron populations. Traditionally, this was done by electrical stimulation of presynaptic GABA-releasing axons and pharmacologically isolating the post-synaptic GABA response (Staley and Mody, 1992). The modern approach to do this is to use optogenetics, which involves expressing light-activated actuators in GABAergic interneurons (Kätzel et al., 2011; Zhao et al., 2011). A major advantage of this optogenetic approach is that it can be applied to different interneuron subpopulations, such as PV and somatostatin (SST)-expressing interneurons, and thereby make it possible to stratify the effects of GABA_AR-mediated synaptic inhibition across neuronal compartments (Pan-Vazquez et al., 2020; Udakis et al., 2020). In addition, this optogenetic approach can be used to probe synaptic inhibition *in vivo* (Dubanet et al., 2021; Valeeva et al., 2016). However, this approach has two major caveats. First, the optogenetic approach complicates how one can study GABA_AR signalling in different cell types as it relies on initiating GABA release from local interneurons. Second, changes in the presynaptic release of GABA can affect the postsynaptic measurements (Kullmann et al., 2005).

With the arrival of optogenetic technologies (Boyden et al., 2005; Nagel et al., 2002, 2003; Zemelman et al., 2002), new opportunities are being afforded by the development of

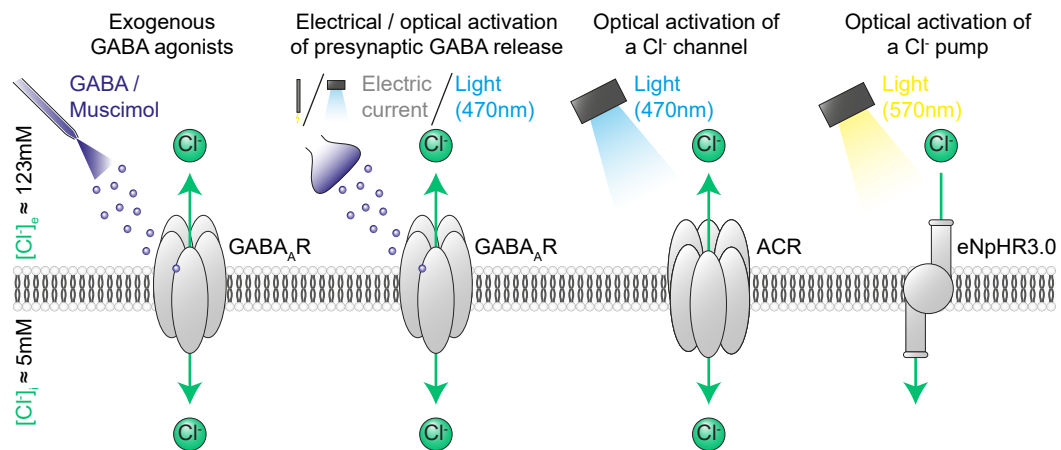


Figure 1.11: Different approaches for activating neuronal chloride fluxes. Exogenous GABA-A receptor (GABA_AR) agonists can be delivered to the neuron. Optogenetic or electrical stimulation of presynaptic GABAergic interneurons can evoke endogenous GABA release. An anion channelrhodopsin (ACR) is a light-activated chloride (Cl^-) channel and, therefore, similar to the GABA_AR in its dependence on the state of the transmembrane Cl^- gradient. This means that upon activation, Cl^- flux can occur in either direction. Halorhodopsin (eNpHR3.0) is a light-activated Cl^- pump that moves Cl^- into the intracellular space in a manner that is independent of the state of the transmembrane Cl^- gradient. As a result, activation of halorhodopsin will only permit Cl^- influx.

different optogenetic tools that offer a more direct way of activating Cl^- fluxes in neurons. Optogenetic silencing strategies often rely on Cl^- -mediated mechanisms (Wiegert et al., 2017). Halorhodopsin, was among the first tools used to inhibit specific neurons by causing Cl^- -mediated membrane hyperpolarisation (Gradinaru et al., 2010; Zhang et al., 2007). However, as this light-activated Cl^- transporter works independently of the transmembrane Cl^- gradient, it can shift the $[\text{Cl}^-]_i$ and, in doing so, can distort measurements of the Cl^- driving force (Alfonsa et al., 2015; Raimondo, Kay, Ellender and Akerman, 2012). A more viable optogenetic approach is to use a light-activated Cl^- channel, such as an anion channel rhodopsins (ACR), as the function of this channel would reflect the state of the transmembrane Cl^- gradient (Berndt et al., 2016; Govorunova et al., 2015; Mahn et al., 2018; Wietek et al., 2015, 2014). This optogenetic tool offers the potential to study Cl^- driving force directly and can be expressed in genetically-defined neuron populations.

1.4 Aims and Objectives

The overall aim of this thesis was to explore how GABA_AR-mediated synaptic signalling operates across different states of cortical network activity and neuronal cell types. In pursuing this aim, I addressed the aforementioned gaps in current knowledge in order to advance our understanding of GABAergic signalling in the intact mammalian cortex. To achieve this aim, I laid out the following experimental objectives:

The first objective was to establish a method of measuring the biophysical parameters that set GABAergic signalling *in vivo*. In **Chapter 3**, I present a new approach to performing gramicidin perforated patch-clamp recordings along with the first *in vivo* measurements of $E_{\text{GABA}_A\text{R}}$.

The second objective was to use this novel electrophysiological method to study how GABAergic signalling operates in different induced and physiological cortical states. I address this in **Chapter 4** where I compare differences in local network activity and GABA_AR-mediated synaptic signalling in the anaesthetised and awake cortex. Here I also explored how local network activity affects GABAergic signalling by monitoring changes in GABA_AR function after excitatory signalling had been shut down.

The third objective was to further explore the relationship between local network activity and GABA_AR function in the awake cortex. In **Chapter 5**, I investigate how an animal's sleep-wake history affects cortical activity and GABAergic signalling. I further expand on the contribution of local network activity in setting the biophysical parameters of GABA_AR function.

The fourth objective was to establish a direct method for studying neuronal Cl⁻ dynamics which does not require the activation of GABA_ARs. In **Chapter 6**, I present a novel optogenetic approach that can be used to determine the resting and dynamic [Cl⁻]_e. I further highlight the utility of this novel approach by measuring Cl⁻ dynamics in the intact rodent brain, as well as within different genetically-defined neuronal cell types.

Chapter 2

Methodology

2.1 Experimental model

For experiments investigating synaptic GABAergic signalling, mice with homozygous expression of Gad2 fused to Cre recombinase ($Gad2^{tm2(Cre)Zjh/J}$, Gad2-IRES-Cre) were crossed with mice with homozygous expression for the light-activated Na^+ channel, channelrhodopsin 2 (B6;129S-Gt(ROSA)^{26Sortm32}(CAG-COP4*H134R/EYFP)Hze/J, Ai32 line). This produced a heterozygous colony expressing channelrhodopsin 2 (ChR2, ChR2(H134R)-YFP) in Gad2-positive neurons, which includes the main subclasses of GABAergic interneurons (Abe et al., 1994; Kätzel et al., 2011; Ledri et al., 2014; Valeeva et al., 2016). For experiments investigating Cl^- dynamics across excitatory and inhibitory neuronal subtypes, mice homozygous for CaMKII α -Cre ($Tg(Camk2a-Cre)^{T29-1Stl}$) or PV-Cre (B6; 129P2-Pvalb^{tm1(Cre)Arbr}) were used, which express Cre recombinase in either pyramidal neurons (Tsien et al., 1996) or parvalbumin interneurons (Hippenmeyer et al., 2005), respectively. All transgenic mice were purchased from Jackson Laboratory (Maine, USA). For in utero electroporation experiments, C57 black 6 (C57/BL6) wild-type mice were used (purchased from Charles River Laboratories). Husbandry was provided to all mouse colonies by the Oxford Biomedical Services Unit. In all experiments, both male and female mice were used at specific ages, as required. Mice were maintained under a 12-h:12-h light-dark cycle and fed *ad libitum*. All mice were bred, housed and used in accordance with the United Kingdom Animals (Scientific Procedures) Act (1986).

2.2 Experimental preparations

2.2.1 Anaesthetised recordings

The preparation for anaesthetised recordings was adapted from previously published protocols (Jordan, 2021; Jouhanneau and Poulet, 2019; Margrie et al., 2002; Wang et al., 2016). Mice (6-8 weeks, P36-P48) were anaesthetised with an intraperitoneal (IP) injection of 25 % urethane (1g/kg, diluted in sterile PBS). To counteract adverse events caused by urethane, a bolus of the anticholinergic agent, Glycopyrronium Bromide (0.01 mg/kg) was administered subcutaneously (SC). Local anaesthetic (Marcain 2 mg/kg) was applied intradermally in the scalp and topically in the ears prior to mounting the mouse into the head holding apparatus (Narishige) under a surgical stereoscope (Olympus). The mouse's body temperature was maintained at 37°C using a heating mat and rectal probe. The animal's head was shaved and eye-protecting ointment (Viscotears) was applied to both eyes. The scalp was then removed using surgical scissors and the area expanded with blunt dissection to expose the skull. The site of the craniotomy over primary visual cortex was marked. Tissue adhesive (Vetbond) was applied to fix the surrounding scalp to the skull and to secure cranial sutures. Multiple layers of dental cement (Simplex Rapid) were applied to create a chamber on top of the skull. A 0.5 mm craniotomy was drilled over the marked region using a dental drill (Foredom). The craniotomy was submerged in cortex buffer (containing, in mM: 125 NaCl, 5 KCl, 10 HEPES, 2 MgSO₄·7H₂O, 2 CaCl₂·2H₂O, 10 Glucose). The bone flap and dura were removed. The animal was then relocated onto the recording apparatus. Recording sessions would last a maximum of six hours, after which the animal was culled.

2.2.2 Awake recordings

The preparation for awake recordings consisted of three phases based on published protocols (Jordan, 2021; Margrie et al., 2002). The first phase was the fixation of the head plate. Mice (4 weeks, P28) were anaesthetised with isoflurane (Zoetis) and mounted into a stereotaxic frame (Kopf). Subcutaneous analgesia (meloxicam 5mg/kg and buprenorphine 0.1mg/kg) was administered along with intradermal local analgesic (marcain 2mg/kg) into the scalp. The scalp was shaved (Wahl) and cleaned (Hibiscrub).

Eye-protecting ointment (Viscotears) was applied. The scalp was then removed and the site was washed with sterile cortex buffer (containing, in mM: 125 NaCl, 5 KCl, 10 HEPES, 2 MgSO₄·7H₂O, 2 CaCl₂·2H₂O, 10 Glucose). After drying the skull with adsorbent swabs (Haag-Streit), the periosteum was removed using a micro curette (Fine Science Tools). Tissue adhesive (Vetbond) was applied to secure cranial sutures and to fix surround scalp onto the underlying bone. A custom-designed aluminium headplate with a 7 mm well was bonded to the skull first with adhesive glue (Loctite) followed by serial layers of dental cement (Super-Bond). The well was then covered with silicone sealant (Kwik-Cast). The animal was then singly housed and allowed to recover. From day three after head plate fixation, the animal was habituated to head-fixation for increasing time intervals up to 60 minutes, over a period of 5-7 days. On the day of recording, the animal was first anaesthetised with isoflurane and mounted onto a stereotaxic frame. The silicone sealant was removed and the area washed with sterile cortex buffer. A 0.5 mm craniotomy was created using a dental drill (Foredom) and the bone flap removed. A durectomy was performed and the site was covered with a soft dressing soaked in cortex buffer. To minimise the effects of anaesthesia, this procedure was limited to 20 minutes. The animal was then remounted onto the head-fixation setup and placed onto the recording apparatus. The mouse was allowed to fully recover for at least 30 minutes before recording was commenced. Recording sessions would last a maximum of 3 hours, after which the animal was culled.

2.2.3 Postnatal intracerebral viral injection

To selectively express the light-activated stGtACR2 Cl⁻ channel in excitatory or inhibitory neurons in cortex, the floxed construct hSyn1-stGtACR2-FusionRed packaged in an adeno-associated virus (AAV1, [#105677-AAV1](#), Addgene) was injected into the cortex of mice homozygous for either CaMKII α -Cre (to target pyramidal neurons) or PV-Cre (to target PV interneurons). Briefly, mice (4 weeks, P28) were anaesthetised with isoflurane (Zoetis) and placed in a stereotaxic frame (Kopf). Analgesic management included intravenous meloxicam (5 mg/kg) and buprenorphine (0.1 mg/kg) as well as intradermal marcain (2 mg/kg) injected into the scalp. The cornea were protected with eye-protecting ointment (Viscotears) and the animal's temperature was monitored throughout the procedure. The scalp was shaved (Wahl) and cleaned with a

chlorhexidine-based solution (Hibiscrub). A midline incision was made that extended from the interpupillary line to the interaural line. Careful dissection was performed to expose the underlying cranium. The location of the craniotomy was positioned over the left primary somatosensory cortex (coordinates in relation to bregma: 3 mm lateral, 1.2 mm posterior). A craniotomy (diameter less than 1 mm) was performed with a dental drill (Foredom). A bevelled glass micropipette (Blaubrand IntraMARK) preloaded with undiluted virus was inserted 300 μm below the surface of the cortex. A total volume of 500 nL was injected at a rate of 33 nL/min. After every 100 nL, the micropipette was raised in 50 μm increments. Following completion of the injection, the micropipette was left in situ for an additional 5 minutes before it was removed. The scalp was sutured (6-0 Vicryl, Ethicon) and the animal recovered before being returned to its home cage. Following injections, mice were maintained for at least 2 weeks to allow for adequate expression of the stGtACR2 in the respective Cre-expressing cell types.

2.2.4 *In utero* electroporation

Breeding pairs from the C57/B6 colony were setup and females checked daily. The day on which plugging was observed was defined as embryonic day 0.5 (E0.5). In utero electroporation was performed at E15.5 to maximise targeting of cortical pyramidal neurons in layer 2/3 (Bitzenhofer et al., 2017; Briz et al., 2017; Langevin et al., 2007; Tabata and Nakajima, 2001). On the day of the procedure, pregnant dams were anaesthetised with isoflurane (Zoetis) and given subcutaneous analgesia (5 mg/kg meloxicam and 0.1 mg/kg buprenorphine). A midline laparotomy was performed and the uterine horns were exposed. DNA plasmids were loaded into a micropipette made from borosilicate glass capillaries (Harvard Apparatus) using a horizontal puller (Sutter). The DNA plasmids consisted of: Cre recombinase under the Chicken β -actin (CAG) promoter ([#13775](#), Addgene), floxed stGtACR2 under the hSyn1 promoter ([#105677](#), Addgene) and the reporter tandem tomato (Tdtomato) under the CAG promoter ([#83029](#), Addgene). The plasmids were combined in the following amounts: 7 μL of CAG-Cre (3 $\mu\text{g}/\mu\text{L}$), 7 μL of stGtACR2 (2.6 $\mu\text{g}/\mu\text{L}$) and 5 μL of CAG-TdTomato (3.7 $\mu\text{g}/\mu\text{L}$). 1 μL of 0.03 % fast green dye (Sigma) was added to make a total volume of 20 μmL . For each embryo, 1.5 μL of the plasmid-containing mixture was injected intraventricularly. The anode of a 5 mm Platinum Tweezertrode (BTX) was then

positioned over the dorsal telencephalon external to the uterine muscle and five pulses of 36 V (each pulse 50 ms separated by a 950 ms interval) was delivered using a pulse generator (BTX). After all embryos had been electroporated, the uterine horn was returned to the abdominal cavity which was filled with warm sterile saline solution. The abdominal wall was sutured closed with Vicryl (Ethicon) and the skin closed with Prolene sutures (Ethicon). Dams were closely monitored during the recovery period. Pups were screened for successful electroporation on the third postnatal day (P3), by illuminating the head with a custom-built light-emitting diode (LED, 525 nm, 30 W) to detect TdTomato florescence. The pups were weaned at three weeks of age (P21) and maintained until 6-8 weeks for experiments.

2.2.5 Acute slice preparation

In vitro electrophysiological experiments were performed in left primary somatosensory cortex (S1) of adult mice (6-8 weeks postnatal), 2-3 weeks after intracerebral viral injection (Section 2.2.3). Mice were first anaesthetised with isoflurane (Zoetis) and decapitated. The brain was then sliced into 350 μ m coronal sections using a microtome (HM650 V, Thermo Scientific) in carbonated high-sucrose solution maintained at 4°C and consisting of: 185 mM sucrose, 1.2 mM NaH₂PO₄, 25 mM NaHCO₃, 2.5 mM KCl, 25 mM glucose, 2 mM CaCl₂ and 2 mM MgCl₂ (all purchased from Merck). The pH was adjusted to 7.3-7.4 (S20, Mettler Toledo) and osmolarity to 300-310 mOsm (5520 Vapro, Wescor). Brain slices were recovered in an incubation chamber maintained at 37°C and containing artificial cerebrospinal fluid (ACSF) consisting of: 120 mM NaCl, 3 mM KCl, 1.2 mM NaH₂PO₄, 23 mM NaHCO₃, 11 mM D-Glucose, 2 mM CaCl₂ and 2 mM MgCl₂ (pH 7.3-7.4, mOsm 300-310 mOsm). Slices were recovered for at least 60 minutes before starting electrophysiological recordings.

2.3 Immunohistochemistry

2.3.1 Preparation of fixed brain slices

For all whole brain histology, mouse brains were fixed via transcardial perfusion of phosphate buffered solution (PBS, 0.1 M, Sigma) and 4 % paraformaldehyde solution (PFA, Sigma). Brains were stored for 24 hours at 4°C in 4 % PFA and then washed and

stored in PBS containing 0.05 % sodium-azide (Merck). Within a week of perfusion, brains were washed in PBS and mounted onto a microtome (HM650V, Thermo Scientific) before being sectioned into 100 μ m thick coronal slices whilst bathed in PBS.

2.3.2 Antibody staining

Thereafter, slices were washed with fresh phosphate buffered saline (PBS) before being washed with PBS containing 0.3 % triton (Sigma; 0.3 % PBST). Slices were then blocked in a solution containing 20 % normal goat serum (NGS) in 0.3 % PBST for 2 hours. The slices were washed with 0.3 % PBST after blocking and then primary antibody incubation setup in a solution containing 10 % NGS, 0.3 % PBST plus the relevant primary antibodies (Table 2.1). For biocytin labelling, only the primary antibody incubation was performed with an anti-streptavidin antibody linked to fluorophore.

Antibody	Host	Conc.	Supplier	Code
Gad67	Mouse	1:100	Merck Millipore	MAB5406
Cux1	Rabbit	1:300	Santa Cruz	SC-13024
PV	Guinea pig	1:1000	Synaptic Systems	195004
Streptavidin-Cy3	-	1:1000	Thermo Fisher	438315

Table 2.1: Primary antibodies. 'Conc.', concentration of antibody used.

Primary incubation was completed at 4°C. After 24 hours, slices were again washed in 0.3 % PBST before incubation with the secondary antibody in a solution containing 5 % NGS, 0.3 % PBST and a fluorophore-linked secondary antibody specific for the relevant primary antibody (Table 2.2). Secondary antibody incubations were completed at room temperature (25°C) for 2 hours before slices were washed in fresh PBS. Slices were mounted on glass cover slips with VectaShield (Vectorlabs).

Antibody	Alexa Fluor	Host	Conc.	Supplier	Code
Anti-mouse	594	Goat	1:1000	Thermo Fisher	A-21135
Anti-rabbit	635	Rabbit	1:300	Thermo Fisher	A-31577
Anti-guinea pig	488	Goat	1:1000	Thermo Fisher	A-11073

Table 2.2: Secondary antibodies. 'Conc.', concentration of antibody used.

2.3.3 Imaging

Confocal imaging was performed using a LSM 880 microscope (Zeiss) equipped with 488 nm, 561 nm and 633 nm lasers. All images were captured using a 20x

water-immersion objective (W Plan-Apochromat NA 1.0) through the ZEN software (Zeiss). Image processing was performed in ImageJ software (NIH).

2.4 Electrophysiology

All electrophysiological recordings were performed using an Axopatch 700A amplifier with data acquired using Clampex software (Molecular Devices). A HumBug noise eliminator (Digitimer) was used to remove 50 Hz noise.

2.4.1 Whole-cell patch-clamp recordings

For *in vivo* experiments, micropipettes were prepared from borosilicate glass capillaries (Harvard Apparatus) using a vertical puller (Narishige) to obtain a long shaft and tip size of 5-8 M Ω (Jouhanneau and Poulet, 2019). Pipettes were filled with K⁺ methanesulfonate internal solution (containing in mM: 110 KMeSO₃, 6 NaOH, 3 MgCl₂·6H₂O, 0.02 CaCl₂, 40 HEPES, 0.05 EGTA, 2 Na₂ATP, 0.5 NaGTP, 2 MgATP, 10 Biocytin), mounted into an 'Optopatcher' pipette holder 7 (A-M Systems) on a micro-manipulator (Sutter) and lowered onto the brain surface. A silver ground electrode (Multichannel Systems) was lowered onto the surface of the nearby skull. The recording site was submerged in cortex buffer. Positive pressure was applied to the pipette and the pipette was then inserted into the brain at a 45 degree angle from lateral to medial, to a depth of 200-300 μ m from the surface of the brain. Blind patching of cortical neurons was performed as described previously (Jordan, 2021; Margrie et al., 2002; Wang et al., 2016). Briefly, neurons were identified by consistent changes in electrode resistance, at which point positive pressure was removed to allow for the formation of gigaseal and a holding potential of -70 mV was applied to the pipette. Whole-cell configuration was achieved by applying brief and gentle suction pulses.

For *in vitro* experiments, micropipettes (2-5 M Ω) with a short shaft were prepared from borosilicate glass capillaries (Harvard Apparatus) using a horizontal puller (Sutter) and filled with a K⁺ gluconate based internal solution, which contained (in mM): 126 K-Gluconate, 4 KCl, 4 Na₂ATP, 0.3 NaGTP, 10 Na₂-PhosphoCreatine, 10 HEPES, 0.05 EGTA. For experiments in which intracellular Cl⁻ was varied (20 mM and 70 mM), this was achieved by adjusting the amount of K⁺ gluconate and K⁺ chloride. For the 20 mM

Cl⁻ internal solution, 110 mM of K-Gluconate and 20 mM KCl were used. For the 70 mM Cl⁻ internal solution, 60 mM K-Glu and 70 KCl were used. All other reagents were maintained the same. For all internal solutions, pH was adjusted to 7.3 using KOH (Mettler Toledo) and osmolarity was adjusted to 287-293 mOsm (Wescor). Neurons were visualised using Dodt contrast and a 60x objective (LUMPlanFI/IR, NA 0.90, Olympus).

2.4.2 Perforated patch-clamp recordings

To perform perforated patch-clamp recordings, the internal pipette solution was prepared immediately prior to recording by combining a high chloride (150 mM) solution (in mM: 141 KCl, 9 NaCl, 10 HEPES) heated to 37°C with a stock solution of gramicidin A (4 mg/ml - dissolved in dimethyl sulfoxide, DMSO, Merck) to achieve a final concentration of 80 µg/mL gramicidin (Kyrozis and Reichling, 1995). The solution was then vortexed (40 seconds) and sonicated (20 seconds). The pipette was then back-filled with the gramicidin solution before being lowered onto the brain surface and blind patching commenced, as described above. Once the gigaseal had formed, perforation was then monitored by observing changes in series resistance. Recording protocols were started once the series resistance had stabilised at <50 MΩ for *in vitro* recordings and <100 MΩ for *in vivo* recordings. Rupture or break through of the perforation into the whole-cell configuration was detected by sudden and persistent depolarisation of the measured E_{GABA}, consistent with dialysis of the neuron with the high Cl⁻ pipette solution. The same procedure was followed for *in vitro* perforated patch-clamp recordings, except that neurons were visualised using dot contrast and a 60x objective (LUMPlanFI/IR, NA 0.90, Olympus).

2.4.3 Two-photon guided patch-clamp recordings

In vivo targeted patch-clamp recordings were performed in mice (6-8 weeks) following the protocol described by Jouhanneau and Poulet (Jouhanneau and Poulet, 2019). Briefly, mice were anaesthetised and the cortex exposed as described in **Section 2.2.1**. Patch pipettes were filled with same internal solution described for either whole-cell (**Section 2.4.1**) or perforated (**Section 2.4.2**) patch-clamp recordings, with the addition of 15 mM Alexa Fluor 594 dye (Thermo Fisher). Pipettes with positive pressure (200-300 mBar) were lowered onto the brain surface under 4x magnification (Plan N, NA 0.10,

Olympus). The pipette was then visualised under 40x magnification (LUMPlanFI/IR NA 0.80, Olympus), inserted into the brain (200-300 μm from the surface), and the positive pressure lowered to 20-30 mBar. The pipette was then located using a custom-built two-photon microscope, which consisted of a modified confocal scan unit (Olympus FV300) coupled to a Ti:Sapphire laser (Newport Spectra-Physics Mai Tai HP; wavelength 820 nm, power 50 mW). The positive pressure released the Alexa dye into the extracellular space, revealing the 'shadows' of neuronal cell bodies (Kitamura et al., 2008). A neuron was approached and, when consistent changes in electrode resistance appeared, the positive pressure was released and the holding potential clamped at -70 mV in voltage-clamp mode to assist gigaseal formation. Targeted patch-clamp recordings of labelled neurons were performed in mice that had undergone IUE (Section 2.2.4). In these recordings the craniotomy was placed over the area of maximum tdTomato expression detected using a custom-built LED (525 nm, 30 W). Before pipettes were inserted into the cortex, neurons were first visualised using the two-photon microscope (930 nm) and images were acquired using FluoView software (Olympus).

2.5 Manipulations

2.5.1 Activation of synaptic GABA signalling

To probe synaptic GABA responses *in vivo*, presynaptic GABA release was evoked by activating channelrhodopsin-expressing Gad2⁺ interneurons via a 50 μm fibre (Thorlabs) embedded in the Optopatcher pipette holder (A-M Systems) and connected to a 473 nm laser (MBL-FN-473-150 mW, CNI Laser). For *in vitro* studies, focal GABA (100 μM , Torcis Biosciences) was applied in the vicinity of the soma via a micropipette attached to a picospritzer (5 psi, 10 ms, General Valve).

2.5.2 Blocking intraneuronal chloride extrusion

KCC2 function was blocked using the selective antagonist, VU0463271 (Delpire et al., 2012). First, a 0.5 mM stock solution was made by dissolving VU0463271 (Torcis) in DMSO. For *in vivo* experiments, a working solution of 100 μM VU was used by diluting the VU stock in cortex buffer and this solution was injected directly into the cortex using

a micropipette at a rate of 33 nL/min to a total volume of 200 nL. For *in vitro* experiments, VU was added to the circulating ACSF solution to achieve a final concentration of 10 μ M.

2.5.3 Altering extracellular anions

Negatively charged extracellular glycosaminoglycans were lysed using the proteolytic enzyme chondroitinase ABC (ChABC, Merck) following the same protocol outlined by Glykys and colleagues (2014). ChABC was first reconstituted in 0.01 % bovine serum albumin (BSA) aqueous solution. On the day of the experiment, the reconstituted ChABC was diluted in a buffer (50 mM Tris, 60 mM sodium acetate and 0.02 % BSA, pH 8.0) to create a working solution of 2 U/mL before being applied to the cortex. ChABC experiments were performed after adding a glutamatergic receptor blocker (see **Section 2.5.4**) to eliminate the potential for ChABC to affect the recorded neuron indirectly via changes to local network activity (Glykys et al., 2014).

2.5.4 Shutting down cortical network activity

To reduce network activity, the α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor blocker, 2,3-dihydroxy-6-nitro-7-sulfamoylbenzo (F) quinoxaline (NBQX) was used (Sheardown et al., 1990). A stock solution of 1 mM was created by dissolving NBQX (Tocris) in DMSO. On the day of recording, a working solution of 100 μ M was made by diluting the stock solution in ACSF. This solution was then injected directly into the cortex (250 nL total at a rate of 33 nL/min).

2.5.5 Acute sleep deprivation

Acute sleep deprivation was performed following the same protocol described by Alonsa and colleagues (2021). During habituation, animals were exposed to novel objects to encourage them to engage in exploratory behaviour. On the day of recording, animals were sleep deprived for three hours starting from the onset of the light cycle. Animals were kept awake by presenting novel objects whilst being under continuous observation. As soon as the animal appeared to lose interest in the object, it was replaced with an alternative object. Immediately following the three hour sleep deprivation, a craniotomy and dural removal was performed prior to starting the patch-clamp recordings (as described in **Section 2.2.2**).

2.5.6 Blocking GABA-B receptor activation

During *in vitro* experiments, focal application of exogenous GABA non-selectively activates both GABA_ARs and GABA_BRs. Therefore, to isolate GABA_AR function, GABA_BRs were blocked with the selective antagonist, CGP 55845 (Cunningham and Enna, 1996). A stock solution of 100 mM was made by dissolving CGP 55845 hydrochloride (Torcis) in DMSO. On the day of recording, stock solution was added to circulating ACSF to give a working concentration of 10 μ M.

2.5.7 Activation of stGtACR2

During *in vitro* experiments, stGtACR2 was activated via a 1000 μ m fibre connected to a 405 nm laser (MDL-III-405-300 mW, CNI Laser). Light was delivered to brain slices through the 60x objective (LUMPlanFI/IR, NA 0.90, Olympus). For *in vivo* experiments, stGtACR2 was activated using a 50 μ m fibre (Thorlabs) embedded in the Optopatcher pipette holder (A-M Systems) and connected to a 473 nm laser (MBL-FN-473-150 mW, CNI Laser)

2.6 Recording protocols and data analysis

Recording protocols were configured on Clampex (Molecular Devices). Data was analysed offline using custom-made Python scripts, unless otherwise stated. All raw data is securely stored on the Akerman laboratory internal server managed by the University of Oxford Medical Science Division IT support staff.

2.6.1 Offline corrections

Whole-cell patch-clamp recording measurements were corrected for liquid junction potential (Barry and Lynch, 1991; Marty and Neher, 1995). The liquid junction potential (LJP) was calculated using the stationary Nernst–Planck equation (Marino et al., 2014) using the open-source [LJPcalc](#) software package. For the K⁺ methanesulfonate internal solution the LJP was -8.6 mV and for the K⁺ gluconate internal solution the LJP was -13.5 mV. For perforated patch-clamp recordings, the effect of the liquid junction potential is believed to be negligible. This is because the lack of anion permeability means that the negative charge is balanced on either side of the neuronal membrane

(Kyrozis and Reichling, 1995).

Furthermore, the concentration of K^+ , which is the main permeant cation, eventually reaches an equilibrium which leads to a balance of charge between the intracellular and pipette compartments. For both whole-cell and perforated recordings, the pipette offset (mV) was measured at the end of each recording and subtracted from the current-clamp recordings (Marty and Neher, 1995).

During perforated patch-clamp experiments, online series resistance compensation was not used. The high amount of activity in the *in vivo* brain would cause large fluctuations in required input current, which is known to increase the rate of perforation rupture (Lundbæk et al., 1997; Staley and Proctor, 1999). However, to correct for series resistance effects, offline correction was performed (Traynelis, 1998). The voltage drop (V_D) caused by the series resistance (R_s) was calculated by multiplying the measured current response (I) with 90 % of the R_s . The V_D is then subtracted from the command voltage (V_C) to calculate the estimated voltage (V_E) of the neuronal membrane (see **Equation 2.1** and **Equation 2.2**).

$$V_D = I \times 0.9 \times R_s \quad (2.1)$$

$$V_E = V_C - V_D \quad (2.2)$$

2.6.2 Measurements of membrane properties

Input resistance (R_i) was calculated from measuring the steady-state change in current in response to a -10 mV step during voltage-clamp recordings (**Equation 2.3** and **Equation 2.4**).

$$\Delta I = I_{\text{After -10mV step}} - I_{\text{Before -10mV step}} \quad (2.3)$$

$$R_i = \frac{-10mV}{\Delta I} \quad (2.4)$$

To calculate series resistance (R_s ; also known as access resistance), a combination of two methods were used. First, the peak current in response to a -10mV voltage step was used to calculate the R_s (peak method, **Equation 2.5**).

$$R_{sPeak} = \frac{V_{Step}}{I_{Peak\ measured}} \quad (2.5)$$

Second, an exponential was fitted to the decay of the transient current in response to a -10mV step to estimate the peak (exponential method, **Equation 2.6** and **Equation 2.7**).

$$I_{Peak\ estimated} = I_{Peak\ measured} \times \exp\left(\frac{-t}{\tau_{Decay}}\right) - \Delta I \quad (2.6)$$

$$R_{sExponential} = -1 \times \frac{V_{Step}}{I_{Peak\ estimated}} \quad (2.7)$$

Where the t describes the time required for the current to return to baseline and the decay time constant (τ_{Decay}) is measured from the peak of the transient current to the resting current. These two methods gave very similar values and so the numerical average of both methods was used as a final estimate of R_s (**Equation 2.8**).

$$R_s = \frac{R_{sPeak} + R_{sExponential}}{2} \quad (2.8)$$

To isolate the R_m , R_s was subtracted from the measured R_i (**Equation 2.9**).

$$R_m = R_i - R_s \quad (2.9)$$

2.6.3 Estimating the intracellular concentration of chloride

To estimate $[Cl^-]_i$ from $E_{GABA_{AR}}$, the GHK equation (Equation 2.10, based on Equation 1.7 from Section 1.2.1) was used:

$$[Cl^-]_i = \exp\left(\frac{E_{GABA_{AR}} \times F}{RT}\right) \times ([Cl^-]_e + 0.25[HCO_3^-]_e) - 0.25[HCO_3^-]_i \quad (2.10)$$

Where the extracellular concentration of Cl^- ($[Cl^-]_e$) was 137 mM (based on the cortex buffer recipe for *in vivo* recordings, see Section 2.2.1, and adjusted to 131 mM for the ACSF used during *in vitro* recordings, see Section 2.2.5). The extracellular concentration for bicarbonate ($[HCO_3^-]_e$) was 15 mM and intracellular concentration for bicarbonate ($[HCO_3^-]_i$) as 24 mM (derived from typical extra- and intracellular pH values presented by Raimondo and colleagues (2015) based on pH measurements by Casper and colleagues (1972) and Raimondo and colleagues (2012)). Constants used were the universal gas constant ($R = 8.3144598$), the Faraday constant ($F = 96.485$ C/mol) and absolute temperature ($T = 310.55$ K).

2.6.4 Membrane potential measurements from current-clamp recordings

To measure the resting membrane potential from current-clamp recordings, spontaneous activity was recorded for five minutes in current-clamp mode. During analysis, membrane potential values greater than -40 mV were removed to avoid distortions caused by action potentials. Average distribution plots were created by concatenating all membrane potential values across all neurons in each group, to which a Gaussian kernel-density estimate was then fitted.

2.6.5 Background synaptic activity

The level of background subthreshold synaptic activity was determined by measuring the rate of change in the membrane potential over time, after excluding action potentials and transitions between states. The rate of change in the membrane potential was calculated by taking the first derivative (V_m dV/dt in mV/ms). To remove distortions

caused by large fluctuations in membrane potential (i.e. during the transition between cortical up and down states), the derivative value was first winsorised before calculating the mean of the modulus of the $V_m \, dV/dt$ for each neuron.

2.6.6 Polarity of GABAergic responses

GABA responses were evoked with a 10 ms light stimulation, presented during one second sweeps. The average of 15 sweeps was then calculated and normalised to mean voltage across the 100 ms preceding the light stimulation. The effect of the GABA response was determined by calculating the mean normalised membrane potential for 100 ms from the start of the light stimulation. If the mean value was greater than zero, responses were classified as depolarising, whereas values below zero were classified as hyperpolarising. To test for differences in the polarity of responses, proportions were compared between groups using a Fisher-exact test with an alpha value of 0.05 (i.e. the threshold for statistical significance was defined as $p < 0.05$).

2.6.7 GABA-A receptor equilibrium potential

As described in **Section 1.2.1**, the $E_{GABA_A R}$ is the voltage across the neuronal membrane at which point there is no net ion flux through the $GABA_A R$. To measure $E_{GABA_A R}$, the voltage where the current through the activated receptor is equal to zero was measured using two types of voltage-clamp protocol: a ramp protocol and a step protocol. The ramp protocol was used most often to calculate $E_{GABA_A R}$ and involved clamping the neuron at -70mV, and then imposing two consecutive voltage ramps, each lasting 150 ms, which extended from 60 mV below the holding voltage to 40 mV above the holding voltage (i.e. from -130 mV to -30 mV, at a rate of 0.7 mV/ms). The first ramp ('baseline ramp') served as a control for the neuron's intrinsic membrane currents and the second ramp ('light ramp') included a GABA conductance that was initiated 30 ms prior to the start of the ramp, to ensure the evoked GABA current was at its peak during the ramp.

During analysis, the first and last 15 ms of each ramp were excluded to avoid large transient currents caused by the discharge of pipette capacitance. The currents from both ramps were then superimposed on a current-voltage (IV) plot using the series corrected voltage (see **Section 2.6.1**) and to avoid action potentials and capacitance

transients distorting measurements, the current responses were cropped to only include regions that were clear of these sources of contamination. A straight line was fitted to both currents. The current from the baseline ramp was used to infer the reversal potential of the leak current, defined as the membrane potential at which the fitted line was equal to zero. The point at which the fitted lines for the two ramps intersected is $E_{\text{GABA}_{\text{AR}}}$. This can also be calculated by subtracting the current response of the baseline ramp from the light ramp. The voltage at which a fitted line to this subtracted current is equal to zero, is equivalent to $E_{\text{GABA}_{\text{AR}}}$. The driving force can then be calculated by subtracting the measured $E_{\text{GABA}_{\text{AR}}}$ from the E_{Leak} which is reflective of the resting membrane potential (RMP) (**Equation 1.6**). In addition, the slope of the subtracted current is equal to the GABA conductance.

The step protocol involved estimating $E_{\text{GABA}_{\text{AR}}}$ from a voltage-clamp protocol in which the neuron was exposed to a series of voltage steps, whilst eliciting a GABA response at each step. The voltage was stepped in 10 mV increments from -130 mV to -30 mV. Each step lasted 500 ms, with 10 s between steps. The GABA conductance was activated 100 ms after the start of the step, using a 100 ms pulse of blue light to optically release presynaptic GABA. Current measurements were made before and 30 ms after activation of the GABA conductance. The two currents were then analysed in a similar way to that described for the ramp protocol. In experiments on stGtACR2-expressing neurons, the reversal potential for stGtACR2 (E_{stGtACR2}) was calculated using the same voltage ramp and step protocols, except that the Cl^- flux was generated by activating stGtACR2.

2.6.8 KCC2-mediated chloride extrusion kinetics

To measure Cl^- extrusion kinetics, voltage-clamp recordings were performed in stGtACR2-expressing neurons, where Cl^- fluxes could be activated optically. During the baseline period, the holding current was measured by performing a ramp protocol (as described in **Section 2.6.7**). Thereafter, neurons were loaded with Cl^- by prolonged (1 s) activation of stGtACR2, whilst concurrently increasing the Cl^- driving force by clamping the membrane potential at +20 mV. Immediately following this 1 s loading period, sequential measurements of E_{stGtACR2} were made by performing voltage ramps whilst activating stGtACR2 (starting 100 ms after loading and then at 5 s intervals). The changes in E_{stGtACR2} were then plotted over time and a single exponential curve was

then fitted to the data to calculate the one phase decay constant for E_{stGtACR2} (τ_{stGtACR2}) using **Equation 2.11**.

$$\tau_{\text{stGtACR2}} = -\frac{1}{K} \times \ln\left(\frac{E_{\text{stGtACR2}(\infty)}}{E_{\text{stGtACR2}(t1)} - E_{\text{stGtACR2}(\infty)}}\right) \quad (2.11)$$

Where $E_{\text{stGtACR2}(t1)}$ is the E_{stGtACR2} measured immediately after the loading period, $E_{\text{stGtACR2}(\infty)}$ is the E_{stGtACR2} during steady state, and K is the rate constant (expressed as the inverse second, s^{-1}). The time constant, tau (τ_{stGtACR2}), is the reciprocal of the rate constant (reported in s) and reflects the time taken for the E_{stGtACR2} to reach a steady state. The τ_{stGtACR2} therefore captures how quickly the neuron is able to extrude the Cl^- introduced during the loading period.

2.6.9 Biostatistics

The majority of the statistical analyses was performed using the Python SciPy library. The only exception was the analysis of time constants which was performed using the one phase decay analysis tool in the GraphPad Prism software package (v8.0.0, Dotmatics). In all analyses, an alpha value (p-value, p) of < 0.05 was considered significant. All data is reported as mean $\pm$ standard error of the mean (SEM). For comparative statistics, the distribution of the continuous data was first established using the Shapiro-Wilk test for normality, which guided the subsequent use of appropriate parametric and non-parametric tests.

Chapter 3

Investigating GABA-A receptor signalling *in vivo*

3.1 Introduction

Fast synaptic inhibition is principally mediated by Cl^- permeable $\text{GABA}_\text{A}\text{R}$. In order to accurately study the function of these receptors, we require methods that preserve the transmembrane Cl^- gradient, whilst also affording the opportunity to activate synaptic $\text{GABA}_\text{A}\text{Rs}$. Gramicidin perforated patch-clamp recordings remain the gold standard for studying $\text{GABA}_\text{A}\text{R}$ dynamics, as they afford the ability to study the driving force on the receptor under conditions where the transmembrane Cl^- gradient is left intact (Abe et al., 1994; Ebihara et al., 1995; Kyrozis and Reichling, 1995). This method is technically challenging due to the long recording times and requirement for pipette stability, and for these reasons have been performed using *in vitro* preparations such as dissociated neuronal cultures (Abe et al., 1994; Ebihara et al., 1995; Kyrozis and Reichling, 1995), organotypic brain slice cultures (Mohajerani and Cherubini, 2005) and acute brain slice preparations (Tanaka et al., 1997). However, while *in vitro* studies offer excellent access for studying inhibitory synaptic signalling, they are limited in that they are not representative of the activity and connectivity of the intact brain (Bindman et al., 1988). Imaging with genetically-encoded fluorescent reporters has offered an alternative approach to estimate intracellular Cl^- *in vivo* (Boffi et al., 2018; Glykys et al., 2014; Pracucci et al., 2021; Rahmati et al., 2021; Sulis Sato et al., 2017). However, these

reporters do not provide information on a cell's membrane potential and lack the sensitivity to reveal synaptic Cl^- fluxes, and therefore cannot capture the electrochemical driving force for Cl^- at GABAergic synapses. For this reason, there remains a compelling case for developing a method that preserves native transmembrane Cl^- gradients in the intact brain and is also able to provide information on both the membrane potential and ion flux through synaptic GABA_A Rs.

The first objective of this Chapter was to establish a method for selectively evoking synaptic GABAergic signalling *in vivo*. This involved replicating previous work in which an optogenetic approach was used to elicit presynaptic GABA release in order to activate postsynaptic GABA signalling (Valeeva et al., 2016). The second objective was to measure the driving force for GABA_A R currents *in vivo*. To achieve this, I modified established methods for performing *in vivo* whole-cell patch-clamp recordings (Margrie et al., 2002), in such a way that the transmembrane Cl^- gradient remains unperturbed. Having established a method of recording the driving force for GABA_A R currents, I then focussed on the mechanisms that establish the driving force for this receptor *in vivo*. There is strong *in vitro* evidence demonstrating the importance of the Cl^- cotransporter, KCC2, in setting the Cl^- driving force (Blaesse et al., 2009; Rivera et al., 1999), but this has not been demonstrated in the intact brain. Therefore, the third objective was to test whether blocking KCC2 modulates $\text{DF}_{\text{GABA}_A\text{R}}$ *in vivo*. Finally, previous controversial work has proposed that impermeant anions are responsible for setting the transmembrane Cl^- gradient in neurons (Glykys et al., 2014; Luhmann et al., 2014; Voipio et al., 2014). Subsequent computational and *in vitro* experimental data have suggested that this mechanism cannot account for the driving force for GABA_A R currents (Düsterwald et al., 2018), although it remains unknown how impermeant anions operate in the intact brain. The final objective of this Chapter was therefore to investigate how manipulating extracellular impermeant anions affect GABA_A R signalling *in vivo*.

3.2 Optical activation of interneurons elicits postsynaptic GABA_A receptor responses *in vivo*

As described in **Section 1.3.2**, there are different methods for activating GABA_A Rs in order to study the receptor's equilibrium potential. Traditionally this has been

performed by focal application of exogenous GABA. However, as this approach requires positioning a micropipette in close proximity to the recorded neuron, it is not feasible *in vivo*, especially when the patch-clamp recordings are performed blind. Furthermore, exogenous GABA is likely to activate extrasynaptic receptors on the recorded neuron, rather than those that are restricted to GABAergic synapses. To overcome these limitations, an optogenetic approach was adopted here. This involved producing a transgenic mouse in which the light-activated cation channel, Channelrhodopsin-2 (ChR2), was expressed in all Glutamate Decarboxylase (Gad) expressing GABA-releasing interneurons (Kätzel et al., 2011). When these interneurons are exposed to blue light, ChR2 is activated, allowing cation influx and suprathreshold neuronal excitation (Boyden et al., 2005). This results in the release of presynaptic GABA and activation of postsynaptic GABA_ARs. This approach is amenable to *in vivo* recordings as it does not require the insertion of an additional pipette or cell targeting. In addition, the blue light can be delivered via a customised pipette holder, an 'Optopatcher', which delivers the light close to the tip of the recording pipette (Katz et al., 2013).

The first experiment focused on reproducing this optogenetic approach to study synaptic GABA_AR signalling *in vivo*. A transgenic colony was generated by crossing homozygous Gad2-Cre mice with homozygous ChR2-EYFP mice (as explained in **Section 2.1**), to produce heterozygous Gad2-Cre-ChR2-EYFP mice. To confirm that light pulses could indeed evoke post-synaptic inhibitory potentials in this mouse, I performed *in vivo* whole-cell patch-clamp recordings in juvenile mice (postnatal age 4-8 weeks) anaesthetised with urethane (**Figure 3.1A**). Using a blind approach in which neurons are identified by monitoring current deflections (Margrie et al., 2002), I performed current-clamp recordings from either pyramidal neurons or ChR2-expressing interneurons (**Figure 3.1B**). Upon activation of the ChR2 with blue laser light (470nm), the membrane potential of the pyramidal neuron exhibited a hyperpolarising deflection, indicative of post-synaptic inhibition (**Figure 3.1C**). Meanwhile, blue light caused the rapid firing of presynaptic action potentials in a ChR2-expressing interneuron (**Figure 3.1C**).

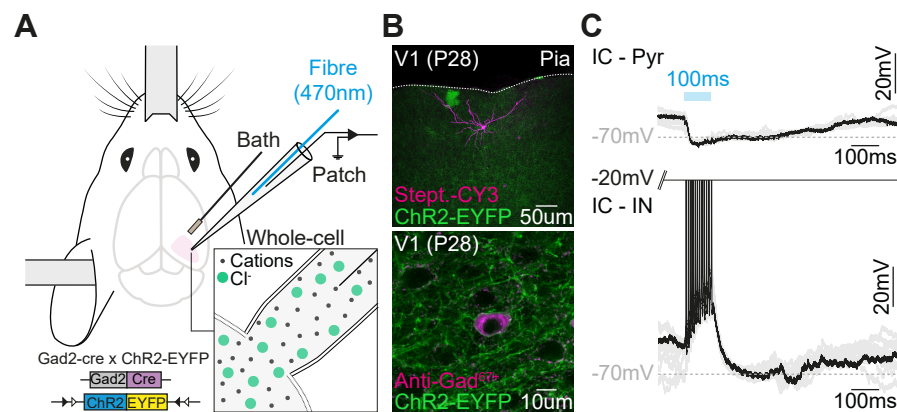


Figure 3.1: Optical activation of Gad2-positive interneurons evokes post-synaptic GABA receptor responses in cortical pyramidal neurons *in vivo*. **A**, Schematic of whole-cell patch-clamp recording in visual area 1 (V1) of a transgenic mouse expressing channelrhodopsin-2 fused to enhanced yellow fluorescent protein (ChR2-EYFP) in Gad2⁺ interneurons. The Gad2⁺ interneurons express Cre-recombinase and are therefore able to remove loxP sites controlling ChR2-YFP expression. Using this system, it is possible to selectively express ChR2-YFP only in Gad⁺ GABA-releasing interneurons. To activate ChR2, blue laser light pulses were delivered through a 50 μ m optical fibre that is positioned close to the pipette tip. This allows for the activation of ChR2-expressing pre-synaptic neurons that synapse onto the recorded neuron. Inset shows the whole-cell recording configuration, where the concentration of chloride (Cl⁻) in the pipette solution is able to dialyse and influence the intracellular Cl⁻ level. **B**, Confocal images of a biocytin-filled layer 2/3 pyramidal neuron stained with streptavidin-fluorophore conjugate, streptavidin-CY3 (top) and a layer 2/3 Gad2⁺ interneuron stained with a Gad67 antibody (bottom). **C**, Current-clamp recordings ('IC') of a light-evoked response (100ms blue light illumination) from a pyramidal neuron (top) and a Gad2⁺ interneuron (bottom).

3.3 Gramicidin perforated patch-clamp recordings *in vivo*

Having successfully reproduced an optical method of eliciting post-synaptic GABA receptor responses *in vivo*, I next focussed on establishing perforated patch-clamp recordings *in vivo*. A major limitation of the whole-cell recording technique is that the contents of the pipette dialyse the neuronal cytosol and thereby affect transmembrane ion gradients (Marty and Neher, 1983). This can be avoided by creating ion-specific pores in the membrane that provide electrical access to the neuron, thereby avoiding the need to create a large, non-specific hole in the membrane. The antibiotic gramicidin creates cation-specific pores in the membrane that only permits Na^+ and K^+ ionic flux (Hladky and Haydon, 1984; Myers and Haydon, 1972). This allows for the electrical activity of the neuron to be measured without disrupting the transmembrane Cl^- gradient, thereby preserving the native function of GABA_ARs (Abe et al., 1994; Ebihara et al., 1995; Kyrozis and Reichling, 1995).

As with the whole-cell recordings described in **Section 3.2**, layer 2/3 cortical pyramidal neurons in V1 were targeted using the same blind patching approach, but now with a pipette containing gramicidin (**Figure 3.2A**). Once a gigaseal had been obtained (defined as a total resistance $> 1 \text{ G}\Omega$), membrane perforation was observed by monitoring changes in the current deflection following a 10 mV step, also known as a ‘seal’ or ‘membrane’ test (**Figure 3.2B**). As the gramicidin pores became embedded in the neuronal membrane, changes in the total resistance were observed. Total resistance is the sum total of the membrane resistance (R_m) and the access or series resistance (R_s , defined as the resistance to electric charge at the junction between the tip of the micropipette and neuronal membrane). After 10 minutes from when the gigaseal had formed, I observed a 20-fold change in the R_s (mean $R_{s-0\text{min}}$: $1126.9 \pm 35.8 \text{ M}\Omega$ vs $R_{s-10\text{min}}$: $50.2 \pm 3.8 \text{ M}\Omega$), indicating that the membrane patch had been perforated. In this state, I could record spontaneous neural activity and optically-evoked synaptic GABA responses (**Figure 3.2C and D**).

The R_s limits the flow of charge between the neuronal membrane and the silver electrode within the micropipette. This can compromise the ability to control the neuron’s membrane potential when using voltage-clamp recordings, resulting in a distortion between the voltages that are set by the amplifier (the holding potential) and

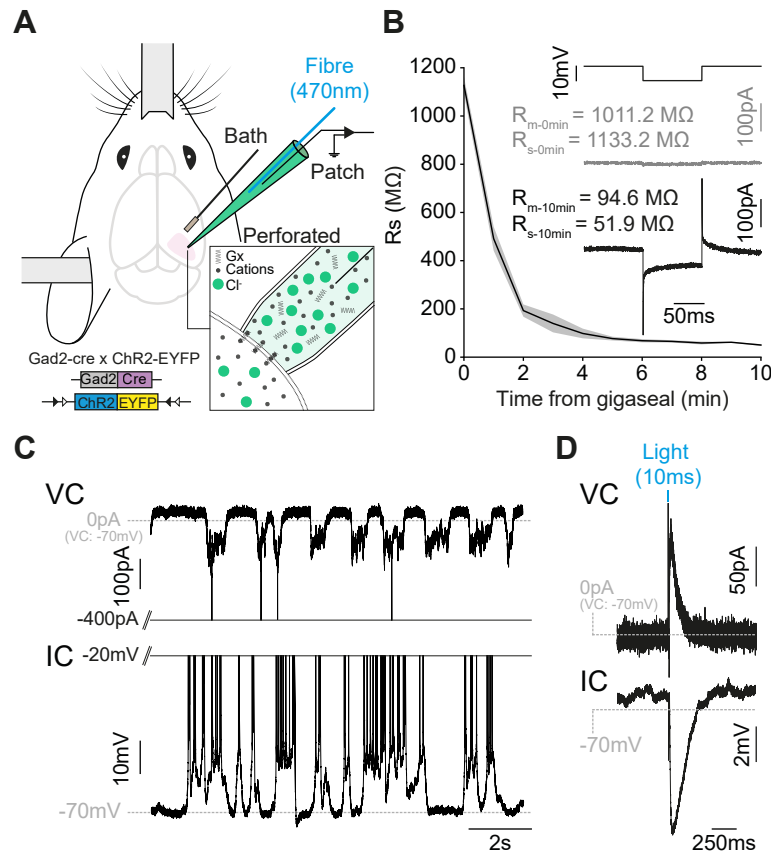


Figure 3.2: *In vivo* gramicidin perforated patch-clamp recordings. **A**, Schematic of gramicidin perforated patch-clamp recordings in primary visual cortex (V1). The inset shows how gramicidin creates cation-selective pores on the neuronal membrane that avoid disruption of intracellular chloride (Cl^-) levels. **B**, A summary plot of series resistance (R_s) over time (black line indicates mean, grey shading indicates standard error of the mean, $n = 10$ neurons, 7 mice). Inset traces are from a voltage-clamp (VC) recording from a layer 2/3 pyramidal neuron showing current responses to a 10 mV hyperpolarising pulse at gigaseal (0 min) and after perforation (10 min). **C**, VC and current-clamp (IC) recordings of spontaneous pyramidal neuron activity. **D**, Light-evoked post-synaptic GABA currents (in VC) and potentials (in IC) recorded from a representative layer 2/3 pyramidal neuron. ' R_m ', membrane resistance; ' R_s ', series resistance.

the actual voltage reached by the neuronal membrane. For this reason, I recorded the R_s throughout all recordings and used this to estimate the actual voltage of the neuron, by correcting for the voltage that is lost, or 'dropped', at the tip of the microelectrode (as described in **Section 2.6.1**).

To validate the integrity of the perforation created by gramicidin, I conducted perforated patch-clamp recordings under two-photon guidance (**Figure 3.3A**). By including an AlexaFluor fluorescent dye in the pipette solution, I was able to visualise the pipette in the cortex using two-photon microscopy (Margrie et al., 2003). Under positive pressure, the dye is released into the extracellular space revealing the 'shadow' of cortical neurons, which do not take up the dye (Kitamura et al., 2008). Furthermore,

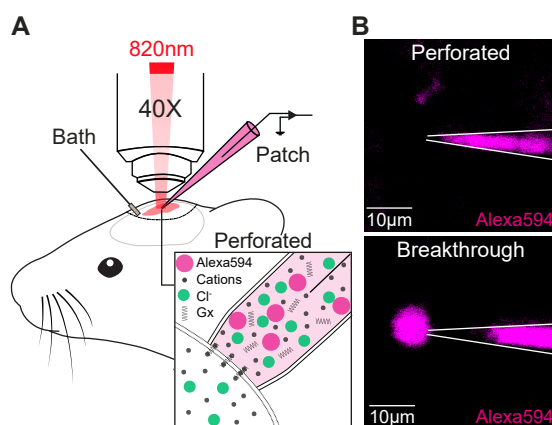


Figure 3.3: Two-photon imaging confirms gramicidin perforated patch-clamp recording *in vivo*. **A**, Schematic of experimental setup. A micropipette filled with gramicidin and AlexaFluor 594 (Alexa594) was visualised using two-photon microscopy at a wavelength of 820 nm. Inset shows that in the perforated configuration, Alexa594 cannot pass through the gramicidin pores and therefore cannot enter the intracellular space of the recorded neuron. **B**, Two-photon images of a gramicidin perforated patch-clamp recording showing Alexa594 retained within the pipette during the perforated stage (top) and then after breakthrough into the whole-cell configuration when the neuron filled with Alexa594 (bottom).

as the AlexaFluor dye is a large molecule, it would not be able to pass through the cation-selective pores created by the gramicidin. I confirmed this by observing that when the perforation was intact, the dye remained in the pipette (**Figure 3.3B**). However, when I broke through into the whole-cell configuration, the neuron filled with the dye (**Figure 3.3B**).

3.4 The first *in vivo* measurements of the GABA-A receptor equilibrium potential

To measure the equilibrium potential for light-evoked synaptic GABA responses, I performed voltage-clamp recordings. This allowed the voltage of the neuron to be controlled, whilst the current required to change the neuron's membrane potential was recorded. As described in **Section 1.2.1**, the equilibrium potential for GABA is the voltage at which there is no net ion flux, or charge, through the GABA receptors, and therefore the measured current will be zero. In line with previous studies (Garduno et al., 2002; Ilie et al., 2012; Jin et al., 2005; Owens et al., 1996; Yelhekar et al., 2016), I used a voltage ramp protocol in which the holding potential was varied from -130 mV to -30 mV (60 mV below the holding potential of -70 mV to 40 mV above the holding potential, **Figure 3.4A**). The protocol consisted of two ramps, each 150 ms in duration with a

change in the voltage (dV/dt) of ~ 0.7 mV/ms, and 500 ms between the two ramps. The first ramp served as a control and the second ramp was timed to occur during the light-evoked synaptic GABA conductance (started 30 ms before the start of the ramp). The resulting ramp currents could then be superimposed on a current-voltage (IV) plot (**Figure 3.4B**). The voltage at which the control ramp current equals zero can be considered the RMP. The RMP can then be used to infer the equilibrium potential for the neuron's leak conductance (E_{Leak}). Meanwhile, the difference between the two ramp currents represents the current-voltage relationship of the activated GABA conductance, and the voltage at which the first and second ramp currents are equal provides an estimate of E_{GABA} . This can be more clearly seen by plotting the result of subtracting the first ramp current from the second ramp current (**Figure 3.4C**).

To confirm the integrity of the perforation, the gramicidin pipette solution contained a high concentration of Cl^- (~ 141 mM). The rationale was that if this solution were to dialyse with the intracellular cytosol, there would be a significant increase in $[Cl^-]_i$ and a pronounced depolarizing shift in E_{GABA} (Howard et al., 2007; Raimondo, Kay, Ellender and Akerman, 2012; Staley, 1994). To illustrate this, equivalent data is shown during perforation and after forcing breakthrough into a whole-cell mode (**Figure 3.4A-C**). Finally, to confirm the stability of the perforated configuration, the RMP, E_{GABA} , and DF_{GABA} were monitored over a 30-minute period in a subset of neurons (**Figure 3.4D**). I repeated the ramp protocols to measure E_{GABA} every one to three minutes. These cellular parameters showed minimal fluctuation over a 30-minute recording, with the driving force remaining consistently hyperpolarising before the forced breakthrough was initiated (E_{GABA} post-breakthrough: -35.22 ± 3.7 mV, $n = 6$ from 6 mice, data not shown in the figure).

To confirm that the ramp protocol provided an accurate estimate of $E_{GABA,R}$, the results were compared to measurements made with a voltage step protocol (**Figure 3.5A-E**). This involved stepping the voltage in 10 mV intervals, each step lasting 500 ms, during which a light-evoked post-synaptic GABA conductance was elicited. This approach makes it possible to visually identify E_{GABA} , but the protocol takes much longer to complete as each voltage step is performed individually and with sufficiently long intervals to avoid altering $[Cl^-]_i$. However, one advantage of this step protocol is that it

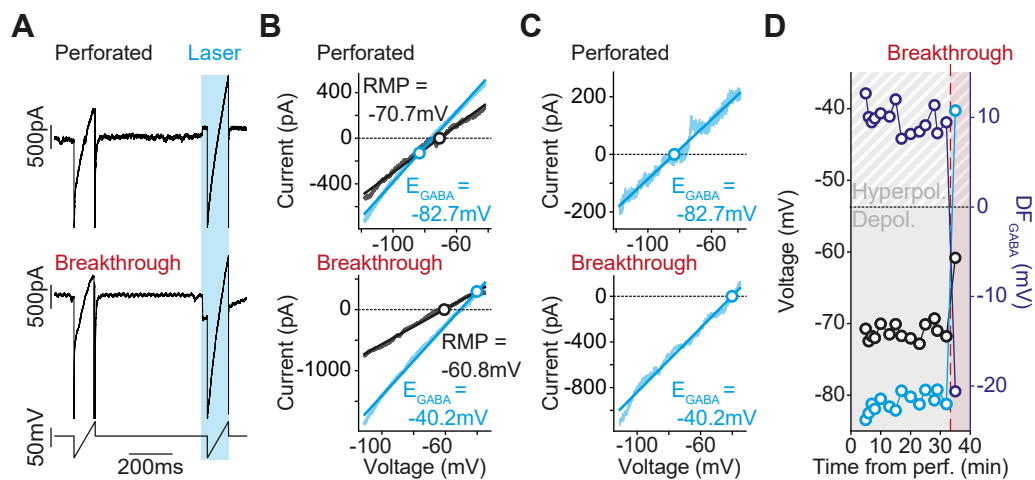


Figure 3.4: Measuring the GABA equilibrium potential *in vivo*. **A**, Ramp protocol performed in the perforated recording configuration (top) and then following breakthrough into the whole-cell configuration (middle) in the same neuron. The voltage protocol delivered to the neuron from the amplifier (i.e. before series resistance correction) is also shown (bottom). The protocol consisted of two ramps. The first ramp served as a control ('baseline ramp') and the second ramp was timed to occur during the light-evoked synaptic GABA conductance ('light ramp'). **B**, Current-voltage (IV) plots for the baseline (black) and light (cyan) ramps performed under perforated (top) and breakthrough (bottom) conditions. The resting membrane potential (RMP) and GABA equilibrium potential (E_{GABA}) are indicated with black and cyan circles, respectively. **C**, IV plot of the subtracted current (light ramp current minus the baseline ramp current). **D**, Changes in E_{GABA} (cyan), RMP (black) and driving force ($DF_{GABA} = RMP - E_{GABA}$, dark blue) over time for an individual recording. The DF_{GABA} remains stable during perforated recording and shows a sharp depolarising shift after forced breakthrough (red shaded area).

allows for a closer assessment of the contribution of the GABA_ARs and the GABA_BR. Previous *in vitro* work by Davies and colleagues (1990) has shown that the early current response to GABA activation predominantly reflects GABA_AR activation (peak at ~29 ms from the start of the GABA current), whilst the later component predominantly reflects GABA_BR activation (peak at ~139 ms from the start of the GABA current). Therefore, to test whether the measurements of E_{GABA} from the ramp protocol are reflective of E_{GABA_A} , I compared the measurements of E_{GABA} from the ramp protocol with E_{GABA} estimated at two different time points during the step protocol (**Figure 3.5D**). E_{GABA} measurements from the ramp were significantly and strongly correlated ($R^2 = 0.92$, $p < 0.0001$) with the measurements of E_{GABA} made using the early phase (~20 ms) of the evoked GABA current during the step protocol. By contrast, there was no correlation between E_{GABA} from the ramp protocol, with E_{GABA} measurements made using the late phase (~220 ms) of the evoked GABA current. These data demonstrate that E_{GABA} measurements made using the ramp protocol reflect E_{GABA_A} .

Finally, to assess whether the amplitude of the evoked response introduced biases in the estimates of E_{GABA} , I inferred the amplitude of the light-evoked postsynaptic GABA conductance from the slope of ramp current (see detailed explanation in **Section 2.6.7**). Using predefined quality control inclusion criteria (see **Sections 2.4.2** and **2.6**, I found no correlation between the conductance of the evoked GABA current and E_{GABA} (**Figure 3.5E**). In addition, there was also no correlation between E_{GABA} and R_s (within defined quality control criteria, see detailed explanation in **Section 2.6.1**).

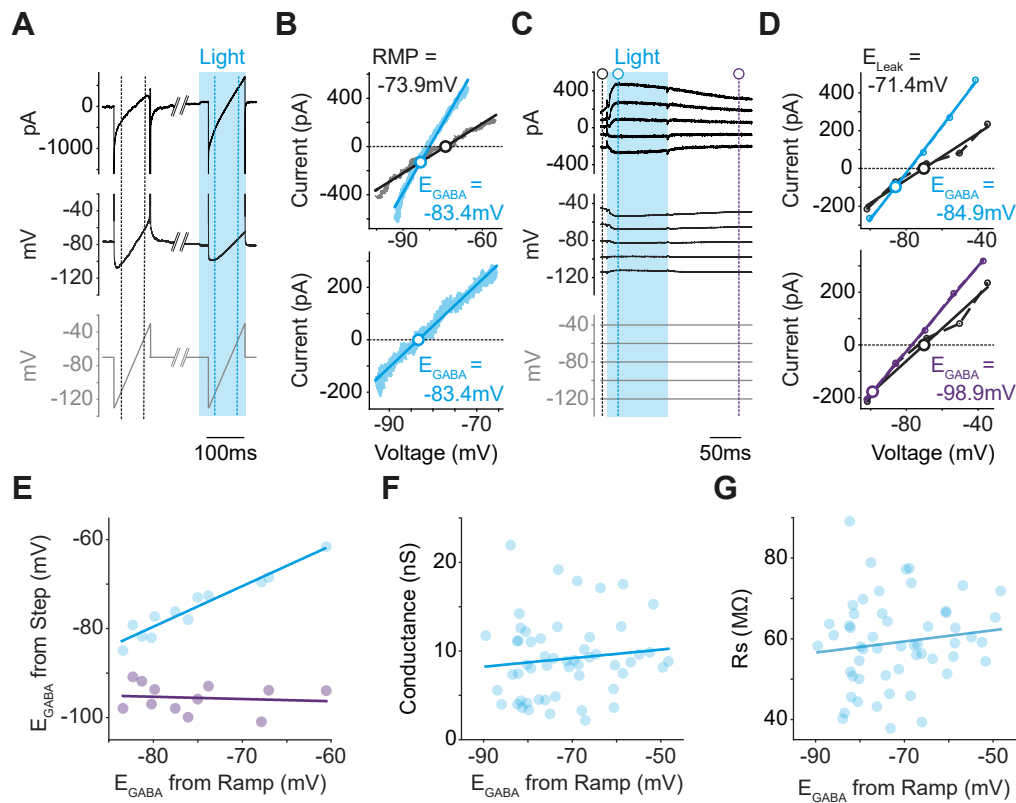


Figure 3.5: The measurements of the equilibrium potential of GABA using the voltage ramp protocol reflect the equilibrium potential of the GABA-A receptor. **A**, Raw trace of a ramp protocol showing the holding voltage (bottom, grey), the estimated voltage after applying 90% series resistance ($R_s = 52.1 \text{ M}\Omega$, middle, black), and the measured current response (top, black). During the second ramp, light-evoked post-synaptic GABA currents were evoked with blue light pulses ('Light'; cyan shaded area). Defined sections of the ramps were analysed (between two pairs of vertical dashed lines). **B**, Current-voltage (IV) plot from ramp protocols (top) where the baseline (black) and light (cyan) current responses are plotted. The intersection of the two fitted lines is E_{GABA} , while the point at which the baseline current response crosses zero is the resting membrane potential (RMP). Subtracting the baseline current response from the light current response reveals the GABA current (light ramp minus baseline ramp, bottom), which has a value of zero at the E_{GABA} . **C**, A voltage step protocol can also be used to estimate E_{GABA} in the same neuron as in 'A'. The voltage step protocol (bottom, grey), estimated voltage ($R_s = 50.8 \text{ M}\Omega$, middle, black) and current response (top, black) are shown. GABA currents were evoked using light pulses (470 nm, 100 ms, cyan shading). The black dashed line indicates where measurements were taken to calculate E_{Leak} . The dark blue dashed line indicates where measurements were made from the early phase of the GABA current ($\sim 20 \text{ ms}$ from light onset), whereas the purple dashed line indicates where measurements were made from the late phase of the GABA current ($\sim 220 \text{ ms}$ from the onset). **D**, IV plots (top panel) of baseline current (black) and light current during the early light-evoked GABA response (cyan). The bottom panel shows the IV plot of the baseline current and the total current using measurements taken later in the GABA response (purple). **E**, Scatter plot showing the correlation between E_{GABA} values calculated from the voltage step and ramp protocols for each neuron ($n = 12$ neurons, 6 mice). There is a significant and strong correlation between E_{GABA} measurements from the ramp protocol and the early GABA current in the step protocol (cyan, $R^2 = 0.92$, $p < 0.0001$) but not the late GABA current (purple, $R^2 = 0.01$, $p = 0.73$). **F**, No correlation between E_{GABA} and the conductance of the light-evoked GABA response (pooled mean $10.62 \pm 0.6 \text{ nS}$, $R^2 = 0.01$, $p = 0.41$). **G**, No correlation between E_{GABA} measurements and the R_s (pooled mean $59.13 \pm 1.5 \text{ M}\Omega$, $R^2 = 0.02$, $p = 0.33$).

3.5 KCC2 sets the driving force for GABA-A receptor signalling *in vivo*

Having acquired measurements of $E_{\text{GABA}_{\text{AR}}}$ *in vivo* using the ramp protocol, the next experiments tested whether these measurements were sensitive to changes in intracellular Cl^- . KCC2 is thought to be the primary Cl^- extrusion system in the adult brain (see **Section 1.2.1**). Evidence from *in vitro* studies have suggested that blocking KCC2 with antagonists (e.g. furosemide or VU0463271) results in a depolarising shift in $E_{\text{GABA}_{\text{AR}}}$, consistent with an increase in $[\text{Cl}^-]_{\text{i}}$ (Düsterwald et al., 2018; Jarolimek et al., 1999; Otsu et al., 2020; Thompson and Gahwiler, 1989). As the RMP is not affected by KCC2 blockade, the net result is a change in $\text{DF}_{\text{GABA}_{\text{AR}}}$. As the $\text{DF}_{\text{GABA}_{\text{AR}}}$ is calculated by using the RMP, this is a measurement of the resting $\text{DF}_{\text{GABA}_{\text{AR}}}$ for the recorded neuron, as per **Equation 1.7**. Whilst these observations have been well-established *in vitro*, they have not been demonstrated *in vivo*. To address this, *in vivo* gramicidin perforated patch-clamp recordings were performed from layer 2/3 pyramidal neurons before and after delivering a selective KCC2 antagonist. VU0463271 (VU) was chosen as it specifically targets KCC2 (Delpire et al., 2012), unlike furosemide which can affect both KCC2 and NKCC1. After the baseline, $E_{\text{GABA}_{\text{AR}}}$ was measured using the ramp protocol (see **Section 3.4**), VU was slowly injected (100 μM , total volume ~ 250 nL delivered at a rate of 66 nL/minute) into the area of cortex surrounding the recorded neuron (**Figure 3.6A**). At least 10 minutes after the injection was completed, $E_{\text{GABA}_{\text{AR}}}$ measurements were repeated in the same neuron and compared to baseline (**Figure 3.6B**).

In vivo application of VU resulted in a significant depolarising shift in $E_{\text{GABA}_{\text{AR}}}$ (**Figure 3.6C**), without affecting the RMP (**Figure 3.6D**). Consequently, there was a significant depolarising shift in $\text{DF}_{\text{GABA}_{\text{AR}}}$ (**Figure 3.6E**). Whereas $\text{DF}_{\text{GABA}_{\text{AR}}}$ was strongly hyperpolarising under baseline conditions ($p = 0.008$, *one-sample t-test*), after VU application, $\text{DF}_{\text{GABA}_{\text{AR}}}$ was close to zero ($p = 0.7$, *one-sample t-test*). Thus, consistent with *in vitro* evidence, KCC2 functions to establish a hyperpolarising driving force for GABA_{AR} signalling *in vivo*.

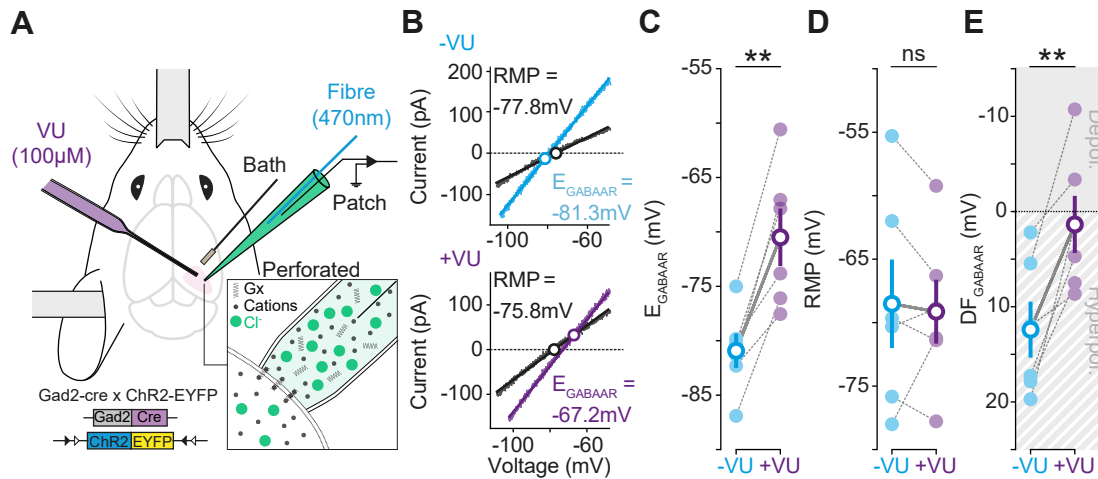


Figure 3.6: Blocking KCC2 causes a depolarising shift in the driving force of the GABA-A receptor. **A**, Experimental schematic in which VU0463271 (VU, 100 μ M) was injected into the area of cortex surrounding the recorded neuron. **B**, IV plots showing E_{GABAAR} and E_{Leak} under baseline conditions before VU (-VU, cyan, top) and after VU application (+VU, purple, bottom). **C**, Population data ($n = 6$ neurons, 6 mice) showing a significant depolarising shift in E_{GABAAR} following VU application (-VU: -80.9 ± 1.6 mV vs +VU: -70.9 ± 2.6 mV, $p = 0.002$, paired t -test). **D**, VU did not affect RMP (-VU: -68.5 ± 3.5 mV vs +VU: -69.1 ± 2.5 mV, $p = 0.73$, paired t -test). **E**, VU caused a significant depolarising shift in the DF_{GABAAR} , defined as $E_{Leak} - E_{GABAAR}$ (-VU: 12.4 ± 2.9 mV vs +VU: 1.4 ± 3.0 mV, $p = 0.006$, paired t -test). 'ns', non-significant ($p > 0.05$); **, $p < 0.01$.

3.6 Modulating extracellular anions does not affect the driving force of GABA-A receptor synaptic responses *in vivo*

Glykys and colleagues (2014) have previously claimed that altering extracellular impermeant anions can affect the polarity of local GABA_AR-mediated synaptic inhibition. This claim is based on *in vitro* experiments using Cl⁻ imaging, which showed that reducing the concentration of impermeant extracellular anions causes an increase in intracellular Cl⁻. The concentration of impermeant anions was modulated by enzymatic cleavage of the sulfated carbohydrate moieties of extracellular proteoglycans, using chondroitinase ABC (ChABC). Proteoglycans are sulfated glycosaminoglycans, which are known to be a key component of the extracellular neuronal membrane (Bandtlow and Zimmermann, 2000). According to the reasoning of Glykys and colleagues (2014), ChABC is predicted to cause the glycoproteins to release their constituent sulfate ions (SO₄⁻) and thereby reduce the extracellular concentration of local negatively charged ionic species, which would increase local [Cl⁻]_e and shift the equilibrium condition for transmembrane cation-chloride transport towards higher [Cl⁻]_i (see Figure 3.7A for a schematic interpretation). However, biophysical modelling work predicts that

modulating impermeant anions can affect the Cl^- levels on either side of the membrane, but only affects the Cl^- equilibrium potential when the charge on the impermeant anions is changed, and does not affect the $\text{DF}_{\text{GABA}_{\text{A}}\text{R}}$ due to concomitant effects on V_{m} . To test these ideas experimentally, *in vivo* perforated recordings were performed to measure the effects of ChABC upon RMP, $E_{\text{GABA}_{\text{A}}\text{R}}$ and the $\text{DF}_{\text{GABA}_{\text{A}}\text{R}}$ after the cortex was exposed to ChABC (**Figure 3.7B**).

ChABC was delivered to V1 and allowed to act for one hour before it was then removed by washing with ACSF (**Figure 3.7B**). This followed a similar procedure to the original *in vitro* experiment in which organotypic cultured slices were incubated with ChABC in the media (Glykys et al., 2014). After exposure to ChABC, $E_{\text{GABA}_{\text{A}}\text{R}}$ and RMP measurements were made using the IV plot (**Figure 3.7C** generated from the ramp protocol (as shown in **Figure 3.4A** and **Figure 3.5A**). R_{m} was calculated using a 10 mV voltage step (**Figure 3.7D**). As with VU, neurons exposed to ChABC exhibited a depolarising shift in $E_{\text{GABA}_{\text{A}}\text{R}}$ when compared to control neurons recorded under baseline conditions (**Figure 3.7E**, control vs VU: $p < 0.0001$, control vs ChABC: $p < 0.0001$, *one-way ANOVA with Bonferroni tests for multiple comparisons*). There was no detectable difference in $E_{\text{GABA}_{\text{A}}\text{R}}$ between the VU and ChABC groups ($p > 0.9999$, *one-way ANOVA with Bonferroni tests for multiple comparisons*). By contrast, VU and ChABC had different effects upon E_{Leak} of the neurons. Consistent with the results of **Figure 3.6D**, RMP in the VU was not different from control (control vs VU: $p > 0.05$, *one-way ANOVA with Bonferroni tests for multiple comparisons*). However, the ChABC group showed a significant depolarising shift in RMP, when compared to either the control condition or the VU condition (**Figure 3.7F**, control vs ChABC: $p = 0.0013$; VU vs ChABC: $p = 0.004$, *one-way ANOVA with Bonferroni tests for multiple comparisons*). As a consequence of the differential effects upon $E_{\text{GABA}_{\text{A}}\text{R}}$ and RMP, VU and ChABC had different effects upon the $\text{DF}_{\text{GABA}_{\text{A}}\text{R}}$. Whilst VU caused a significant decrease in $\text{DF}_{\text{GABA}_{\text{A}}\text{R}}$ (**Figure 3.7G**, control vs VU: $p = 0.001$; VU vs ChABC: $p = 0.009$, *Kruskal-Wallis test with Dunn's tests for multiple comparisons*), $\text{DF}_{\text{GABA}_{\text{A}}\text{R}}$ remained strongly hyperpolarising after ChABC treatment (control vs ChABC: $p > 0.9999$, *Kruskal-Wallis test with Dunn's tests for multiple comparisons*).

Finally, in addition to the differential effects upon RMP, the R_{m} was found to be

significantly lower in the ChABC group than in both the control ($p = 0.01$) and VU groups ($p = 0.004$, *Kruskal-Wallis test with Dunn's tests for multiple comparisons*, **Figure 3.7H**). VU did not affect R_m ($p > 0.9999$). Together, these findings suggest that lysing extracellular proteoglycans has broader effects on the neuron than modulating the equilibrium condition for cation-chloride transport. They support the idea that the modulation of impermeant anions could contribute to setting the absolute $E_{\text{GABA}_A\text{R}}$, but not the $DF_{\text{GABA}_A\text{R}}$ due to concomitant effects on the membrane potential.

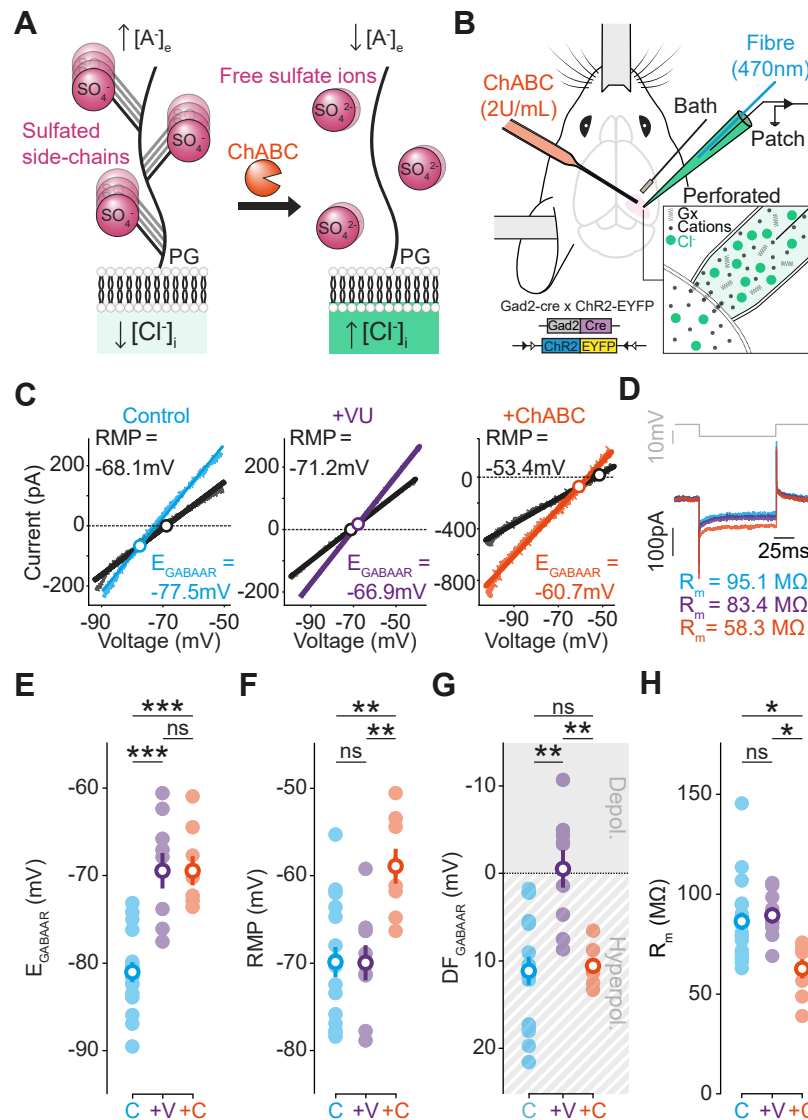


Figure 3.7: Modulating extracellular proteoglycans with chondroitinase ABC does not affect the driving force of GABA-A receptor-mediated synaptic responses. **A**, Schematic interpretation of Glykys and colleagues' (2014) prediction that, by lysing the sulfated side chains of extracellular membrane-bound proteoglycans, sulfate ions are released. This causes a decrease in the extracellular concentration of negatively-charged impermeant anions ($[A^-]_e$) which, according to the authors' reasoning, causes an increase in the intracellular concentration of Cl^- ($[Cl^-]_i$). **B**, Experimental schematic showing the delivery of chondroitinase ABC (ChABC, 2 U/mL) into the cortex, by injection and application to the cortical surface. **C**, Current-voltage plot following a ramp protocol (as shown in **Figure 3.3A**) with E_{GABAAR} and RMP marked for control (C, cyan, $n = 16$ neurons, 12 mice), VU (+VU, purple, $n = 9$ neurons, 7 mice) and ChABC (+ChABC, orange, $n = 8$ neurons, 5 mice) groups. **D**, Raw traces of the current response to -10 mV step to calculate the membrane resistance (R_m) for the control, VU and ChABC groups. **E**, E_{GABAAR} population data for control (C, -81.1 ± 1.2 mV), VU (+V, -69.7 ± 2.1 mV) and ChABC (+C, -68.5 ± 1.7 mV). **F**, RMP population data for control (-69.9 ± 1.7 mV), VU -70.1 ± 2.0 mV) and ChABC (-58.9 ± 1.9 mV). **G**, Driving force ($DF_{GABAAR} = RMP - E_{GABAAR}$) for each group (control: 11.1 ± 1.6 mV; VU: -0.5 ± 2.2 mV; ChABC: 10.5 ± 0.8 mV). Both control and ChABC groups exhibited DF_{GABAAR} that were greater than zero (one sample t -test compared to zero; control: $p < 0.0001$; VU: $p = 0.8$; ChABC: $p < 0.0001$). **H**, R_m was compared across the three groups (control: 86.5 ± 4.3 m Ω ; VU: 89.4 ± 4.0 m Ω ; ChABC: 62.6 ± 4.8 m Ω). 'ns', non-significant ($p > 0.05$); *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.0001$.

3.7 Discussion

This Chapter combined an optogenetic approach to probe postsynaptic GABA_AR signalling with gramicidin perforated patch-clamp recordings in order to provide the first *in vivo* estimates of $E_{\text{GABA}_A\text{R}}$. The measured $E_{\text{GABA}_A\text{R}}$ was reflective of the state of intracellular Cl^- as antagonising KCC2 function caused a depolarising shift in the driving force of $E_{\text{GABA}_A\text{R}}$ currents. By contrast, manipulating extracellular anions with ChABC caused a depolarising shift in both $E_{\text{GABA}_A\text{R}}$ and RMP, leading to no net effect in $\text{DF}_{\text{GABA}_A\text{R}}$.

Previous work by Valeeva and colleagues (2016) demonstrated that synaptic GABA receptor responses could be investigated *in vivo* using the optical activation of presynaptic interneurons. These authors examined the frequency of synaptic GABA receptor-mediated currents during light activation (lasting 500 ms) while clamping the neuron at the equilibrium potential for glutamatergic signalling (~ 0 mV). The results of this Chapter extend this work by showing that the same optogenetic strategy can be used to study post-synaptic GABA responses using brief (10 ms) light activation of presynaptic GABAergic interneurons. By isolating a single GABA response, it was possible to study the key biophysical parameters that determine the mode of GABA_AR signalling *in vivo*.

A limitation of this optogenetic strategy is that it is not specific to the activation of GABA_ARs. The fast postsynaptic response following light activation is consistent with the major conductance being mediated via GABA_ARs, although there is the potential that slower metabotropic GABA_BRs could also be activated (Bettler et al., 2004). The use of fast ramp protocols made it possible to isolate the early component of the postsynaptic response, which is associated with the GABA_AR and was supported by the observations that the estimates of $E_{\text{GABA}_A\text{R}}$ were close to those expected for E_{Cl} and were sensitive to the manipulation of KCC2. By contrast, the slower GABA_BR has been shown to exhibit an equilibrium potential close to the reversal for potassium (E_K , ~ -100 mV) and would not be affected by a decrease in KCC2 function (Khalilov et al., 2017). *In vitro* studies routinely isolate GABA_AR responses by focal application of a GABA_AR agonist or by widespread delivery of GABA_BR antagonists (Nissbrandt and Engberg, 1996). However, the use of GABA_AR agonists fails to isolate synaptic GABA_ARs and the

use of GABA_BR antagonists is expected to alter *in vivo* network activity via their complex pre- and postsynaptic actions (Bettler et al., 2004).

To further support the measurements made with the voltage ramp protocol, I was able to show that E_{GABA} estimates from the ramp protocol were similar to E_{GABA} estimates made in the early phase of the GABA current during the voltage step protocol when the contribution of GABA_ARs predominates (Davies et al., 1990). An important similarity in the experimental design between the original *in vitro* work of Davies and colleagues (1990) and my *in vivo* findings, is that both use endogenous synaptic GABA release to measure the equilibrium potential of GABA currents, evoked via either electrical or optical stimulation. By contrast, Yelhekar and colleagues (2016) have previously shown in an *in vitro* study, using amphotericin perforated patch-clamp recordings and a similar voltage ramp protocol, that E_{GABA} becomes increasingly depolarising in the late phase of an evoked GABA response. However, in their study, they apply a high concentration (1 mM) of exogenous GABA whilst holding neurons at sustained voltages for longer periods of time (>10 seconds). Under these conditions, the artificially evoked GABA currents are likely to induce Cl⁻ loading within the neurons and thereby depolarise E_{GABA} .

In vivo perforated patch-clamp recordings were first demonstrated by Wang and colleagues (2016), who used amphotericin as a perforating agent. Amphotericin is weakly permeable to Cl⁻ and, over time, results in intracellular Cl⁻ reaching an equilibrium between the intracellular and pipette solutions (Kleinberg and Finkelstein, 1984; Kyrozis and Reichling, 1995). For this reason, gramicidin is better suited to study GABAergic signalling as it is not permeable to Cl⁻ and therefore maintains the transmembrane gradient. Interestingly, the time taken to perforate the neuronal membrane *in vivo* (defined as the time taken to reach a stable access resistance of <100 MΩ), was relatively fast for both amphotericin (Wang and colleagues (2016) report ~5 minutes) and the gramicidin recordings that I present here (~10 minutes). *In vitro* studies often report longer gramicidin perforation periods, which likely reflects the time required for the dimeric cation-selective channels to form and embed themselves in the neuronal membrane (Ebihara et al., 1995; Hladky and Haydon, 1984; Kyrozis and Reichling, 1995). The relatively fast perforation observed *in vivo* may suggest that this

process occurs more quickly in the environment of the intact brain and at physiological temperatures.

By using gramicidin perforated patch-clamp recordings, it was possible to generate direct measurements of the $E_{\text{GABA}_{\text{A}}\text{R}}$ *in vivo*. In layer 2/3 pyramidal neurons within the visual cortex of adult anaesthetised mice, the mean $E_{\text{GABA}_{\text{A}}\text{R}}$ using a voltage ramp protocol was found to be approximately ~ -80 mV. This would translate to an $[\text{Cl}^-]_{\text{i}}$ of ~ 3 mM (using the modified GHK equation presented in **Section 2.4.1**). *In vivo* Cl^- imaging measurements from layer 2/3 pyramidal neurons in anaesthetised mice have reported $[\text{Cl}^-]_{\text{i}}$ values of ~ 5 -10 mM (Boffi et al., 2018; Sulis Sato et al., 2017), which would translate to an $E_{\text{GABA}_{\text{A}}\text{R}}$ of approximately -70 mV (using **Equation 1.8** with the same parameters described in **Section 2.4.1**). This discrepancy between electrophysiological and imaging estimates may partly reflect the challenges associated with performing quantitative imaging with different wavelengths *in vivo* (Sulis Sato et al., 2017), the impact of pH upon Cl^- reporters (Boffi et al., 2018), or genuine differences in the somatic Cl^- concentration compared to local transmembrane gradients at $\text{GABA}_{\text{A}}\text{R}$ -containing synapses.

A major limitation with gramicidin perforated patch-clamp recordings is that relatively high series resistances can significantly impede the ability to clamp the membrane potential. This can be overcome to a certain extent by performing series resistance correction in which the actual neuronal voltage can be estimated. However, complete correction is not possible and therefore the measurements in voltage-clamp recordings are likely to be affected, to some degree, by the error caused by an uncorrected voltage drop across the membrane. Interestingly, Yelhekar and colleagues (2016) reported differences in $E_{\text{GABA}_{\text{A}}\text{R}}$ measured using different protocols. The authors show that $E_{\text{GABA}_{\text{A}}\text{R}}$ measurements made from the interpolation of current amplitudes measured during voltage step protocols can be less accurate than measurements from voltage ramp protocols (Yelhekar et al., 2016). The data presented in this Chapter suggests that estimates of $E_{\text{GABA}_{\text{A}}\text{R}}$ *in vivo* are equivalent when inferred from voltage step protocols and ramp protocols. This may reflect the relatively small synaptic GABA conductances that were elicited *in vivo* and the fact that the protocols were designed to allow Cl^- homeostasis to be fully re-established between the GABA conductances.

Measurements of $E_{\text{GABA}_{\text{AR}}}$ are strongly related to intracellular Cl^- homeostasis. This was confirmed *in vivo* by blocking KCC2, which led to an expected depolarising shift in the driving force for evoked GABA currents. While previous *in vivo* imaging experiments have implicated KCC2 in Cl^- homeostasis (Boffi et al., 2018), the data in this Chapter is the first demonstration that KCC2 determines the driving force for Cl^- -mediated synaptic signalling *in vivo* and confirms previous *in vitro* data (Banke and McBain, 2006; D sterwald et al., 2018; Hewitt et al., 2009; Jarolimek et al., 1999; Otsu et al., 2020; Rivera et al., 1999; Thompson and Gahwiler, 1989). Recently, the role of CCCs in setting the Cl^- driving force has been brought into question, based on evidence that blocking NKCC1 and KCC2 does not cause a measurable change in $[\text{Cl}^-]_i$ when imaging Cl^- in cultured organotypic brain slices (Glykys et al., 2014). From these findings, the authors concluded that CCCs do not set the driving force for Cl^- -mediated signalling. Subsequent theoretical commentaries have tried to explain these controversial findings by highlighting issues associated with measuring CCC function under conditions of low Cl^- conductances and the effects of hyperpolarised E_{Leak} found in brain slices (Doyon, Vinay, Prescott and De Koninck, 2016; Johansson et al., 2016). The results in the current Chapter provide direct experimental evidence that emphatically implicates KCC2 in setting $E_{\text{GABA}_{\text{AR}}}$ and the driving force for Cl^- -mediated GABAergic signalling *in vivo*.

Impermeant anions have been implicated in creating a Gibbs-Donnan equilibrium between charged species on either side of the neuronal membrane, and to establish the set point for the transmembrane Cl^- gradient (Delpire and Staley, 2014; Glykys et al., 2014, 2017; Rahmati et al., 2021). Previous *in vitro* data support that altering intracellular impermeant anions can indeed shift $E_{\text{GABA}_{\text{AR}}}$, but does so without affecting the driving force (D sterwald et al., 2018). The findings presented in this Chapter support these predictions by showing that *in vivo*, altering extracellular impermeant anions can indeed shift $E_{\text{GABA}_{\text{AR}}}$ with a negligible effect on driving force. This *in vivo* experiment was inspired by previous *in vitro* observations, which showed that lowering impermeant anions in organotypic brain slices causes a significant increase in the intracellular Cl^- measured using Cl^- imaging (Glykys et al., 2014). Whilst impermeant anions may have a role in setting the transmembrane Cl^- gradient, a series of unresolved parameters must be considered. First, it is unknown how ChABC cleaves the sulfonated side chains on the extracellular proteoglycans, nor how quickly the realised negatively-charged sulfate

ions diffuse away. Without this knowledge, it is difficult to postulate how changes in extracellular anions can affect the interactions between ionic species in the local extracellular space. Second, ChABC affects other membrane properties, as seen by an increase in membrane conductance and a depolarising shift in E_{Leak} . Whilst *in vitro* data suggests that ChABC does not induce neuronal death through apoptosis (Glykys et al., 2014), it remains unclear how altering the extracellular matrix affects membrane structural integrity. Third, one of the claims made by Delpire and Staley (2014) in support of the ChABC-induced effects on $[Cl^-]_i$ is that when extracellular impermeant anions are removed, they are replaced by Cl^- diffusing into the local extracellular space. This increase in $[Cl^-]_e$ is then thought to be able to shift the Cl^- driving force. However, this theory has not been quantified experimentally, and how $[Cl^-]_e$ changes in response to alterations in extracellular impermeant anions. The findings in **Figure 3.7G** do not support this as ChABC appeared to not affect on $DF_{GABA_A R}$.

In summary, this Chapter has introduced an electrophysiological approach to studying synaptic $GABA_A R$ signalling in the intact anaesthetised brain whilst preserving the integrity of the transmembrane Cl^- gradient. Moreover, this novel *in vivo* method has confirmed the importance of KCC2 in setting the driving force for $GABA_A R$ -mediated synaptic signalling.

Chapter 4

Cortical states set the mode of GABA-A receptor signalling

4.1 Introduction

The cortex is a highly dynamic system that can transition between different modes or 'states'. Each cortical state is defined by a distinct pattern of network activity, which is the product of co-occurring excitatory and inhibitory synaptic conductances (see **Section 1.1.3**). Together these synaptic inputs can have a profound impact on the membrane properties of the constituent neurons (Destexhe et al., 2003; Ferguson and Cardin, 2020; Haider and McCormick, 2009; Harris and Thiele, 2011). Whilst excitation and inhibition in the cortex closely follow one another, during states of increased network activity, as seen in the awake brain, inhibition has a stronger influence on shaping cortical processing (Anderson, Carandini and Ferster, 2000; Borg-Graham et al., 1998; Galarreta and Hestrin, 1998; Haider et al., 2013; Rudolph et al., 2007; Wilent and Contreras, 2005). This predominance of synaptic inhibition in the awake cortex involves a sustained activation of GABAergic synaptic conductances, which would in turn lead to a greater influx of Cl^- (Doyon, Vinay, Prescott and De Koninck, 2016; Johansson et al., 2016). In this high Cl^- conductance state (**Figure 1.9, Section 1.2.5**), the elevated intracellular Cl^- would be predicted to shift $E_{\text{GABA}_A\text{R}}$ and in doing so, affect the manner in which GABA_ARs exert their function (**Figure 1.6, Section 1.2.4**). For this reason, one can hypothesise that GABAergic signalling in the high conductance state of the awake

brain is likely to differ from GABAergic signalling in the low conductance state of *in vitro* brain slices, where most of our understanding of GABAergic signalling originates. Having established gramicidin perforated patch-clamp recordings *in vivo* (**Chapter 3**), I was now able to use this technique to test this hypothesis experimentally by examining GABAergic signalling during different states of cortical activity in the intact brain.

The first objective of this Chapter was to explore whether there are differences in cortical GABAergic signalling in the anaesthetised and awake *in vivo* state. Here I use gramicidin perforated patch-clamp recordings in layer 2/3 cortical pyramidal neurons to compare the membrane properties in these two states. After that, I use the same optogenetic method for evoking GABA-mediated synaptic inhibition presented in **Chapter 3** to study the driving force of GABAergic signalling in the anaesthetised and awake cortex. To confirm whether the state of the local network can influence GABAergic signalling, the second objective was to determine the contribution of local network activity to set $E_{\text{GABA}_{\text{AR}}}$ in the awake brain. Here I modulate network activity and measure the subsequent changes in the $DF_{\text{GABA}_{\text{AR}}}$.

4.2 The awake brain is characterised by high levels of cortical network activity

Previous work using either sharp intracellular electrodes in cats (Destexhe and Paré, 1999; Steriade et al., 2001) or whole-cell patch-clamp recordings in rodents (Constantinople and Bruno, 2011; Poulet and Petersen, 2008) has demonstrated that the awake cortex is highly active due to a near-constant bombardment of desynchronised synaptic inputs. By contrast, in the urethane-anaesthetised cortex, network activity is synchronised, and consists of alternating phases of high and low activity referred to as up and down states (González-Rueda et al., 2018; Steriade, Nunez and Amzica, 1993). These differences in activity patterns between the anaesthetised and awake brains can have opposing effects on the membrane properties of cortical neurons (**Section 1.1.3**). For this reason, neurons in the awake brain operate in a high-conductance state due to a large amount of synaptic activity, whereas in the anaesthetised brain, when averaging across the cortical up and down states, neurons operate in a lower conductance state (Destexhe et al., 2003). To confirm whether this phenomenon is valid in my *in vivo*

preparation, I used *in vivo* perforated patch-clamp recordings (introduced in **Chapter 3**) to characterise the differences in synaptic activity and membrane conductance in head-fixed urethane-anaesthetised and awake juvenile mice (**Figure 4.1A**, see detailed procedures in **Sections 2.2.1** and **2.2.2**). As some of this data is re-used in subsequent analyses, appropriate statistical tests to correct for multiple comparisons were performed.

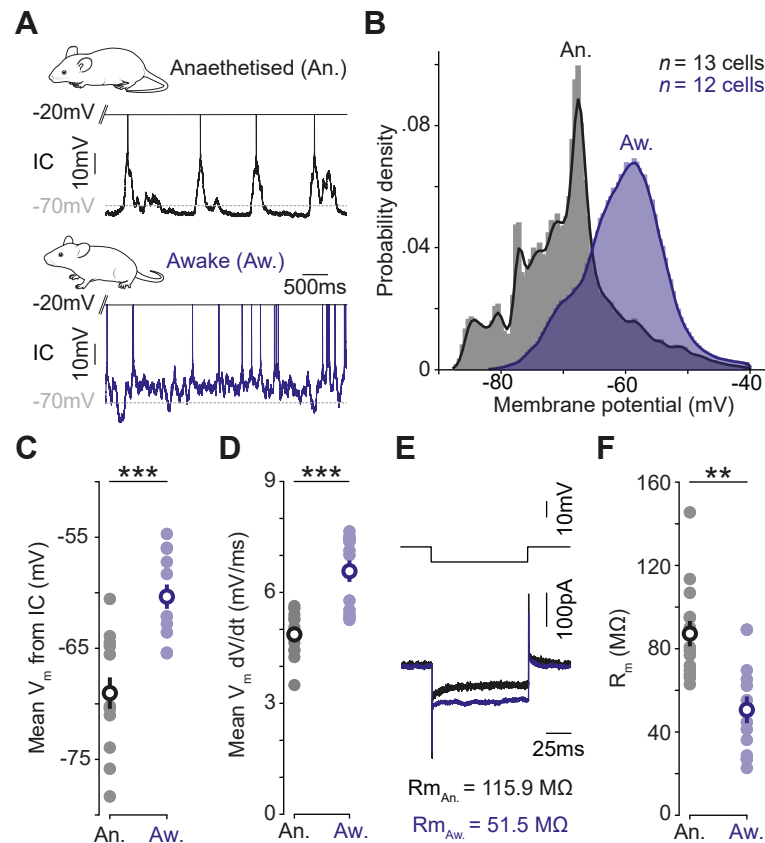


Figure 4.1: The awake brain is characterised by high levels of network activity. **A**, Example current-clamp (IC) traces of spontaneous neuronal activity in a layer 2/3 pyramidal neuron in visual area 1 (V1) of an anaesthetised (An., black, top) and awake (Aw., blue, bottom) mouse. **B**, Probability density function over the distribution of membrane potentials (V_m) for pyramidal neurons in the anaesthetised ($n = 13$ neurons, 7 mice) and awake state ($n = 12$ neurons, 10 mice). **C**, The mean V_m from IC recordings of spontaneous recordings was significantly more depolarised in the awake brain (An.: -69.1 ± 1.4 mV vs Aw.: -60.3 ± 1.1 mV, $p < 0.0001$, one-way ANOVA with Bonferroni tests for multiple comparisons). **D**, Mean instantaneous change in V_m was significantly greater in the awake brain (An.: 4.9 ± 0.2 mV vs Aw.: 6.6 ± 0.3 mV/ms, $p = 0.0004$, one-way ANOVA with Bonferroni tests for multiple comparisons). **E**, Raw trace of the current response to a 10 mV hyperpolarising step (An.: black; Aw.: blue). **F**, Significant decrease in membrane resistance (R_m) in the awake brain (An.: 87.2 ± 6.1 M Ω , $n = 14$ neurons, 8 mice vs Aw.: 50.6 ± 6.4 M Ω , $n = 13$ neurons, 10 mice, $p = 0.009$, one-way ANOVA with Bonferroni tests for multiple comparisons). **, $p < 0.01$; ***, $p < 0.001$.

Current-clamp recordings of spontaneous activity in layer 2/3 pyramidal neurons in the visual cortex of adult mice showed that the distribution (**Figure 4.1B**) and mean

membrane potential (**Figure 4.1C**) of the awake brain are more depolarised compared to the urethane-anaesthetised brain. Furthermore, to explore the amount of background subthreshold synaptic activity an individual cortical neuron receives in each brain state, the fluctuation in membrane potential over time was measured by calculating the first-order derivative (explained in **Section 2.6.5**). In the awake cortex, the larger fluctuations in membrane potential are consistent with a greater amount of background synaptic activity (**Figure 4.1D**).

To measure the membrane conductance of the cortical neurons, voltage-clamp recordings were used to deliver a 10 mV hyperpolarising step (**Figure 4.1E**). The current response was then used to calculate the R_m , which reflects the conductance state of the membrane (see **Section 1.1.3** for further explanation and **Section 2.6.2** for details on the analysis of membrane properties). Using this protocol, I found that the R_m in the awake cortex was significantly lower than in the urethane-anaesthetised brain (**Figure 4.1F**). Together, the findings of this first experiment largely confirm the observations made in previous studies and demonstrate that cortical neurons in the awake brain are indeed characterised by a high level of background synaptic activity and operate in a high conductance state.

4.3 Shunting inhibition predominates in the awake cortex

Having confirmed differences between the activity profiles in the anaesthetised and awake cortex, I next used the optogenetic approach introduced in **Section 3.2** to probe GABA-mediated synaptic responses (**Figure 4.2A**, top panel). The polarity of the light-evoked potentials was measured in current-clamp by calculating the average V_m over a 100 ms period following light stimulation (as described in **Section 2.6.6**). In the anaesthetised brain, the GABAergic responses were exclusively hyperpolarising, whereas in the awake brain, there was a mixture of both depolarising and hyperpolarising responses (**Figure 4.2A**, bottom panel).

As the polarity of GABA_AR-mediated signalling is related to the $DF_{GABA_A R}$, the increase in depolarising responses seen in the awake brain suggests differences in $E_{GABA_A R}$ between the two brain states. To test whether this is the case, I used the same voltage ramps that were introduced in **Section 3.4** to measure $E_{GABA_A R}$ of cortical neurons in the urethane-anaesthetised and awake brain (**Figure 4.2B**). Overall, $E_{GABA_A R}$ in the awake

cortex was significantly more depolarised (**Figure 4.2C**). Moreover, while the E_{Leak} was also more depolarised in the awake cortex (**Figure 4.2D**), the net effect on the $DF_{\text{GABA}_A\text{R}}$ was also depolarising (**Figure 4.2E**). The $DF_{\text{GABA}_A\text{R}}$ in the awake cortex was not significantly different from zero, indicating that GABA_AR -mediated synaptic signalling has a shunting effect in the awake state. Furthermore, between the anaesthetised and awake states, the conductance of the evoked GABA currents were not significantly different (anaesthetised: 11.1 ± 1.8 nS vs awake: 11.4 ± 1.5 nS, $p = 0.87$, *one-way ANOVA with Bonferroni tests for multiple comparisons*, data not shown in the figure).

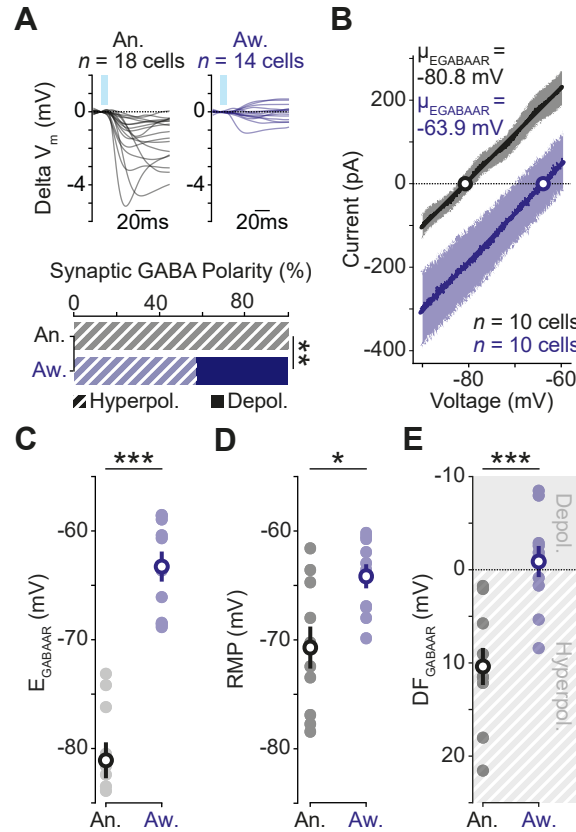


Figure 4.2: The equilibrium potential for the GABA-A receptor is shunting in the awake cortex. **A**, Overlaid averaged and normalised traces of light-evoked postsynaptic GABA responses in the urethane-anaesthetised (top panel, black, $n = 18$ neurons, 10 mice) and awake (blue, $n = 14$ neurons, 11 mice) states. There was a greater proportion of light-evoked currents causing depolarising shifts in V_m (Depol.) compared to hyperpolarising shifts (Hyperpol.) in the awake brain (bottom panel, An.: Depol. 0/18 Hyperpol. 18/18 vs Aw.: Depol. 6/14 Hyperpol. 8/14, $p = 0.003$, Fisher-Exact test). **B**, Averaged subtracted GABA currents for pyramidal neurons in anaesthetised ($n = 10$ neurons, 6 mice) and awake ($n = 10$ neurons, 9 mice) mice with mean E_{GABAAR} marked ($\mu_{EGABAAR}$). **C**, Population data showing depolarising shift in E_{GABAAR} in the awake brain (An.: -81.1 ± 1.7 mV vs Aw.: -63.3 ± 1.4 mV, $p < 0.0004$, one-way ANOVA with Bonferroni tests for multiple comparisons). **D**, RMP was significantly more depolarised in the awake cortex (An.: -70.7 ± 1.9 mV vs Aw.: -64.2 ± 1.1 mV, $p = 0.02$, one-way ANOVA with Bonferroni tests for multiple comparisons). **E**, The GABA_AR driving force ($DF_{GABAAR} = RMP - E_{GABAAR}$) was significantly more depolarised in the awake brain (An.: 10.4 ± 2.0 mV vs Aw.: -0.9 ± 1.7 mV, $p = 0.0004$, one-way ANOVA with Bonferroni tests for multiple comparisons). The DF_{GABAAR} in the awake brain was not significantly different to zero, which is consistent with synaptic GABA_AR signalling principally exerting a shunting effect (An.: $p = 0.0006$, one-sample t -test; Aw.: $p = 0.61$, one-sample t -test). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

4.4 The awake network affects neuronal membrane properties

The findings from the first experiment confirmed that the awake cortex is highly active and has a preference for shunting GABA_AR-mediated synaptic responses. To determine whether the depolarising shift in $E_{\text{GABA}_A\text{R}}$ in the awake brain is caused by the high levels of local network activity, the second experiment performed the same measurements after glutamatergic signalling had been antagonised with a local injection of the AMPA receptor antagonist, NBQX (**Figure 4.3A**, see **Section 2.5.4** for details on NBQX).

After NBQX injection, the distribution (**Figure 4.3B**) and mean (**Figure 4.3C**) membrane potential of cortical neurons was hyperpolarised. In addition, membrane potential fluctuations were also reduced, consistent with the idea that background synaptic activity had been suppressed (**Figure 4.3D**). This change in local cortical activity was reflected in the membrane conductance of the neuron, which decreased after NBQX, as shown by voltage-clamp measurements of membrane resistance (**Figure 4.3E and F**). These findings establish that shutting down glutamatergic signalling with NBQX in the awake cortex significantly reduces local cortical network activity, which lowers the conductance state of the neuronal membrane.

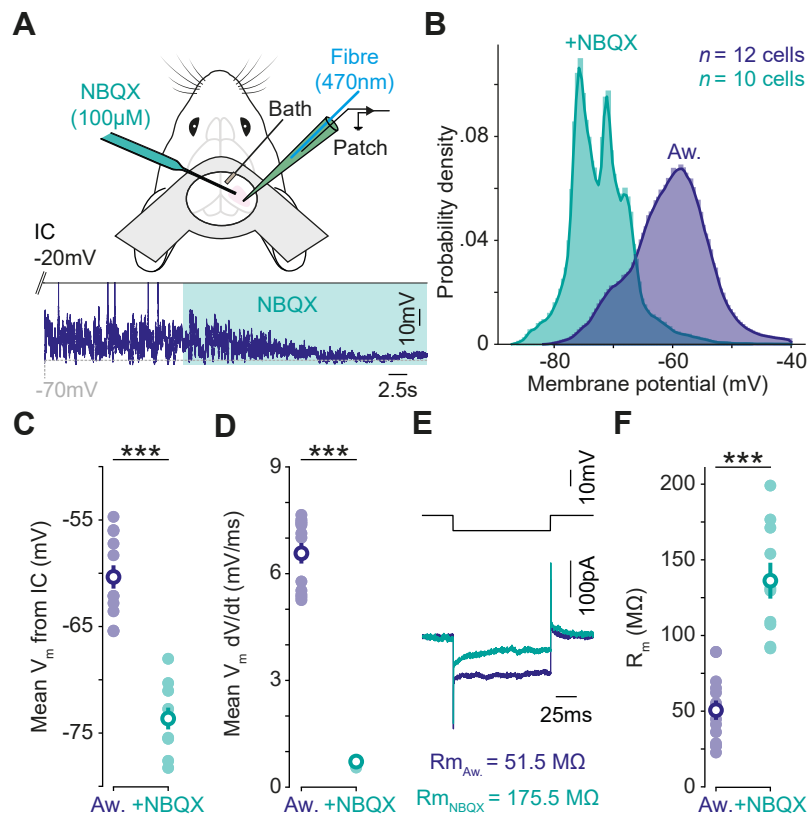


Figure 4.3: Blocking glutamatergic signalling shuts down cortical network activity in the awake brain. **A**, Current-clamp (IC) recording showing the change in neuronal activity after glutamate-receptor blocker, NBQX (100 μ M), was injected locally in the awake brain. **B**, Distribution and probability density estimate of membrane potentials (V_m) in the awake brain with and without NBQX (awake without NBQX, Aw., blue: $n = 12$ neurons, 10 mice vs awake with NBQX, +NBQX, turquoise: $n = 10$ neurons, 7 mice). Data in the awake group was re-used from Figure 4.1. **C**, NBQX hyperpolarises mean V_m measured from IC recordings (Aw.: -60.3 ± 1.1 mV vs +NBQX: -72.6 ± 1.0 mV, $p < 0.0001$, one-way ANOVA with Bonferroni tests for multiple comparisons). **D**, The fluctuations in membrane potential are significantly reduced after NBQX application (Aw.: 6.6 ± 0.3 mV/ms vs +NBQX: 0.7 ± 0.1 mV/ms, $p < 0.0001$, one-way ANOVA with Bonferroni tests for multiple comparisons). **E**, Raw trace of the current response to 10 mV hyperpolarising step (Aw.: blue; +NBQX: turquoise). **F**, NBQX causes significant decrease in membrane resistance (R_m , Aw.: $50.6 \pm 6.4 \text{ M}\Omega$, $n = 13$ neurons from 10 mice vs +NBQX: $136.2 \pm 11.9 \text{ M}\Omega$, $n = 10$ neurons from 7 mice, $p < 0.0001$, one-way ANOVA with Bonferroni tests for multiple comparisons). ***, $p < 0.001$.

4.5 Network activity determines GABAergic signalling in the awake state

To test whether levels of local network activity in the awake cortex affect GABAergic signalling, the polarity of GABA_AR response was measured with and without NBQX. The light-evoked potentials became exclusively hyperpolarised in the quietened network state following NBQX injection (**Figure 4.4A and B**). Consistent with this finding, NBQX caused a hyperpolarising shift in $E_{\text{GABA}_A\text{R}}$ in cortical neurons (**Figure 4.4B and C**). When combined with the negative shift in E_{Leak} (**Figure 4.4D**), the net effect on the $DF_{\text{GABA}_A\text{R}}$ was hyperpolarising (**Figure 4.4E**). Furthermore, NBQX did not appear to significantly affect the light-evoked GABA conductance (awake without NBQX: 11.4 ± 1.5 nS vs awake with NBQX: 10.4 ± 0.7 nS, $p = 0.52$, *one-way ANOVA with Bonferroni tests for multiple comparisons*). These findings, when combined with the results in **Section 4.5**, demonstrate that the more depolarised $E_{\text{GABA}_A\text{R}}$ values in the awake cortex are caused by the high level of background synaptic activity.

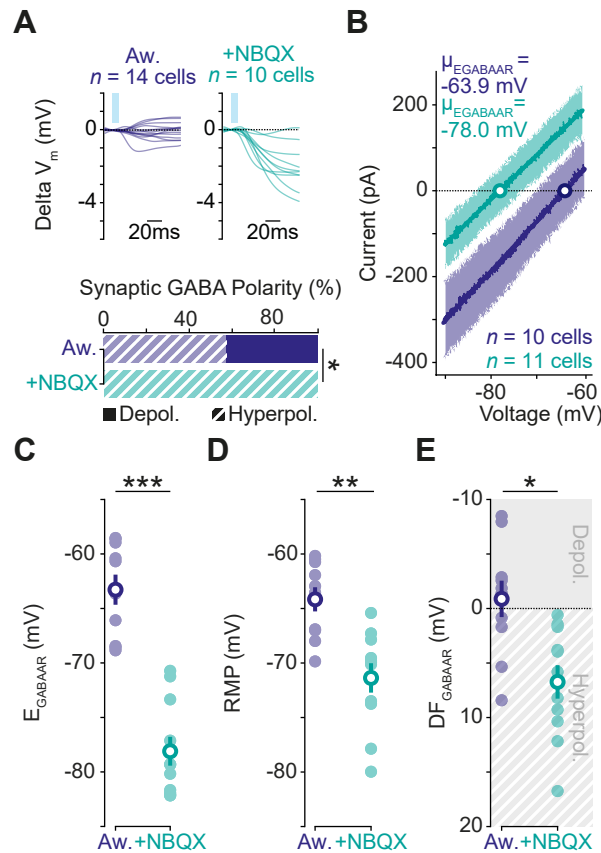


Figure 4.4: Network activity establishes a shunting equilibrium potential for the GABA-A receptor in the awake cortex. **A**, Overlaid averaged and normalised traces of light-evoked post-synaptic GABA currents in the awake brain with and without NBQX (top panel, awake without NBQX, Aw., blue: $n = 14$ neurons from 11 mice vs awake with NBQX, + NBQX, turquoise: $n = 10$ neurons from 7 mice). NBQX causes a hyperpolarising shift in the polarity of light-evoked GABA currents (bottom panel, Aw.: Depol. 6/14 Hyperpol. 8/14 vs +NBQX: Depol. 0/10 Hyperpol. 10/10, $p = 0.03$, Fisher-Exact test). Data in the awake group was re-used from Figure 4.1. **B**, Averaged current-voltage (IV) plot of subtracted light-evoked current (light-evoked current minus baseline current) showing a hyperpolarising shift in E_{GABAAR} after NBQX application (Aw.: $n = 10$ neurons from 9 mice vs +NBQX: $n = 11$ neurons from 7 mice). Mean E_{GABAAR} is indicated ($\mu_{EGABAAR}$). **C**, Population data of mean E_{GABAAR} values (Aw.: -63.3 ± 1.4 mV vs +NBQX: -78.1 ± 1.3 mV, $p < 0.0001$, one-way ANOVA with Bonferroni tests for multiple comparisons). **D**, NBQX causes a hyperpolarising shift in the RMP (Aw.: -64.2 ± 1.1 mV vs +NBQX: -71.4 ± 1.4 mV, $p = 0.009$, one-way ANOVA with Bonferroni tests for multiple comparisons). **E**, NBQX causes a hyperpolarising shift in the driving force for the GABA_AR response ($DF_{GABAAR} = RMP - E_{GABAAR}$, Aw.: -0.9 ± 1.7 mV vs +NBQX: 6.7 ± 1.5 mV, $p = 0.03$, one-way ANOVA with Bonferroni tests for multiple comparisons). After NBQX application, the DF_{GABAAR} in the awake brain became statistically significantly different from zero, which represents a complete shunting effect (Aw.: $p = 0.61$, one-sample t -test vs +NBQX: $p = 0.001$, one-sample t -test). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

4.6 Discussion

In this Chapter, I provide the first *in vivo* evidence that GABAergic synaptic signalling varies across different cortical states. This is evident in how the high levels of network activity seen in the awake brain are linked to GABA_AR-mediated synaptic responses being predominantly shunting. The effect of evoked GABA_AR-mediated signalling can be modulated by shutting down local cortical network activity, which causes GABAergic synaptic signalling to become hyperpolarising. These results demonstrate a relationship between how local network activity contributes to setting the mode of GABA_AR-mediated synaptic signalling in the cortex.

Each cortical state appears to be characterised by distinct neuronal membrane properties. From my recordings in the awake brain, cortical neurons appeared to receive a greater amount of subthreshold synaptic input. This is evidenced by the neurons in this cortical state having a more depolarised (**Figure 4.1C**) and fluctuating (**Figure 4.1D**) V_m compared to neurons in a urethane-anaesthetised brain. In addition, I measured a lower membrane resistance in the awake brain which, as explained in **Section 1.1.3**, indicates that the membrane conductance is high (**Figure 4.1E and F**). Consistent with the idea that these features reflect synaptic activity levels, each feature was shifted when excitatory synaptic inputs were antagonised by NBQX (**Figure 4.3**). Collectively, these measures of neuronal membrane properties appear to be linked to one another, as they all shift across each of the cortical states. Furthermore, these findings validate what has been proposed previously by Destexhe and colleagues (2003), as they support the idea that the awake brain operates in a high conductance state. Other studies that support this finding include intracellular sharp electrode recordings in the awake cortex of rodents and cats (Baranyi et al., 1993; Destexhe and Paré, 1999; Matsumura et al., 1988; Steriade et al., 2001; Woody and Gruen, 1978) as well as whole-cell patch-clamp recordings in rodent cortex (Fernandez et al., 2018; Yamashita et al., 2013).

Previous work has reported opposing findings on the membrane properties of cortical neurons between anaesthetised and awake states. For example, Constantinople and colleagues (2011) performed whole-cell patch-clamp recordings in mouse cortex and reported that the R_i increases as the network transitions from being under isoflurane anaesthesia into the awake state. Moreover, the anaesthetised brain is often considered

to reflect a sleep state, with this being evident in how urethane can replicate features of both NREM and REM sleep phases (Clement et al., 2008; Mondino et al., 2022; Steriade, Nunez and Amzica, 1993). In this context, Steriade and colleagues (2001) reported from intracellular sharp electrode recordings in the cat cortex that R_i also increases when transitioning from sleep into wakefulness. However, these observations need to be interpreted with caution. First, comparing findings across different anaesthetic preparations may not be helpful as these agents can have pleiotropic and contrasting effects on network activity (Bharieke et al., 2022). Furthermore, the anaesthetised brain cannot be considered entirely synonymous with natural sleep as it is an artificially-induced state in which there is pharmacological manipulation of the cortical network (Franks, 2008). The more compelling evidence supporting the relationship between the amounts of synaptic input a neuron receives and its membrane resistance comes from the experiments performed within the awake state. By blocking excitatory synaptic activity with NBQX, I observed that the membrane resistance increased. This observation is consistent with previous whole-cell experiments in which the sodium-channel antagonist tetrodotoxin was used to reduce network activity (Fernandez et al., 2018).

The more depolarised E_{GABA_A} in awake cortex indicates that intracellular Cl^- is higher than that observed in the urethane-anaesthetised brain (**Figure 4.2B and C**). Specifically, in the awake cortex, the mean E_{GABA_A} was -63 mV, which predicts a $[Cl^-]_i$ of ~10 mM (using the GHK equation **Section 2.6.3**). By contrast, an E_{GABA_A} of -81 mV in the urethane-anaesthetised brain, predicts a $[Cl^-]_i$ of 3.1 mM. These differences in intracellular Cl^- are likely caused by the different levels of Cl^- conductances in each of these states, which reflect the different levels of synaptic inhibition (Doyon et al., 2011; Doyon, Vinay, Prescott and De Koninck, 2016). This is strongly suggested by the significant differences in membrane potential fluctuations (**Figure 4.1D**), driven by incoming synaptic inputs, as well as in the R_m (**Figure 4.1F**) - a proxy measure for total membrane conductance. As explained in **Section 1.1.3**, $GABA_A$ inhibitory conductances predominate in highly active neuronal networks, such as seen in the awake brain. When coupled with a depolarised membrane potential (**Figure 4.1B and C**), this creates a cortical state that generates large net Cl^- influxes, resulting in higher levels of intracellular Cl^- and a more depolarised E_{GABA_A} (**Figure 4.2B and C**). This

finding is consistent with previous computational (Doyon et al., 2011) and *in vitro* studies (Yelhekar et al., 2017), which have demonstrated how resting Cl^- conductance, which is closely linked to the level of background synaptic activity, is a critical component in setting intracellular Cl^- homeostasis.

Theoretically, these state-dependent differences in intracellular Cl^- could reflect changes in Cl^- co-transporters that underlie $E_{\text{GABA}_A\text{R}}$. However, this is unlikely as shutting down local network activity with NBQX (**Figure 4.3A**) was able to hyperpolarise $E_{\text{GABA}_A\text{R}}$ in the awake state to -78 mV (estimated $[\text{Cl}^-]_i$ of ~ 4 mM), which is close to the value measured in the urethane-anaesthetised state (-81 mV, ~ 3 mM). In addition, theoretical evidence suggests that the efficiency of KCC2-mediated Cl^- extrusion is tightly coupled to Cl^- conductance (Doyon, Vinay, Prescott and De Koninck, 2016; Johansson et al., 2016). This model predicts that during periods of heightened network activity, KCC2 would become more efficient at managing the Cl^- load and serve to prevent a rise in intracellular Cl^- . However, the findings from this Chapter suggest that KCC2 extrusion does not fully compensate for the greater influxes of Cl^- conductance, but rather helps to establish a new equilibrium where $[\text{Cl}^-]_i$ is relatively high and $E_{\text{GABA}_A\text{R}}$ is relatively depolarised so that it favours shunting inhibition.

As described in **Section 1.1.2**, different cortical states are defined by varying levels of local network activity. In this Chapter, these activity patterns were defined by taking average measurements across each of the states (**Figure 4.1** and **Figure 4.3**). However, this approach does not account for the fact that within each cortical state, networks make transitions between different phases of low and high network activity. These are referred to as cortical up and down states in the urethane-anaesthetised brain (González-Rueda et al., 2018; Haider et al., 2006; Mondino et al., 2022; Steriade, Nunez and Amzica, 1993), while in the awake brain the cortex transitions between transient periods of arousal and quiescence (Crochet and Petersen, 2006; Poulet and Crochet, 2019; Poulet and Petersen, 2008; Tan et al., 2014; Yamashita et al., 2013). Furthermore, there are also similarities between phases across cortical states. This is most evident in how the depolarised up state is similar to the pattern of activity that occurs in the aroused awake brain (Constantinople and Bruno, 2011). Therefore, it is plausible that there are further fluctuations in the GABA_AR -mediated responses across the different

phases operating within a single cortical state.

In summary, the data presented in this Chapter demonstrates that the mode in which GABAergic signalling operates in the cortex is closely linked to the state of local network activity. This supports the hypothesis that the amount of background inhibitory synaptic input a neuron receives has a direct influence on the $DF_{\text{GABA}_{\text{AR}}}$. However, certain physiological brain states, such as sleep, are thought to affect both the level of synaptic activity and the function of Cl^- co-transporters (Alfonsa et al., 2021). In **Chapter 5**, I explore the interaction between these two mechanisms by testing how sleep history affects GABAergic signalling in the awake cortex.

Chapter 5

Sleep-wake history modulates GABAergic receptor signalling

5.1 Introduction

In Chapter 4, I showed how the levels of network activity influence the mode of GABAergic signalling in cortex. In the awake cortex, the level of network activity can vary considerably as it is influenced by co-occurring physiological processes across different temporal scales. One parameter that can directly influence the activity of the cortex is sleep-wake history, which reflects an animal's sleep pressure or 'need to sleep' (as explained in **Section 1.1.2**). This has been demonstrated through acute sleep deprivation studies, which have reported transient increases in cortical excitability in rodents that experience a prolonged period of wakefulness (Dash et al., 2009; Vyazovskiy et al., 2011, 2009). In other words, sleep deprivation is associated with short-term changes in the level of local network activity in the awake cortex.

Recent data from Alfonsa and colleagues (2021) has shown that sleep-wake history can also modulate synaptic GABA_AR signalling. Specifically, the authors report that in animals which have undergone acute sleep deprivation, $E_{\text{GABA}_A\text{R}}$ is more depolarised compared to animals that have had adequate sleep and are well-rested. Underlying this depolarising shift in $E_{\text{GABA}_A\text{R}}$ appears to be an upregulation of the NKCC1 transporter, which would lead to an elevation of intracellular Cl^- . However, as this study used *in vitro* recordings from acute brain slices to reveal the effect of sleep-wake history, it is not

clear whether the effects upon $E_{\text{GABA}_{\text{A}}\text{R}}$ are evident *in vivo*, or how they relate to state-dependent changes in local network activity. To explore how sleep deprivation affects *in vivo* $\text{GABA}_{\text{A}}\text{R}$ dynamics, in this Chapter I use the perforated patch-clamp technique to compare GABAergic signalling in mice with different sleep-wake histories. The first objective was to confirm if acute sleep deprivation can affect local network activity in the awake cortex. Thereafter, the second objective was to determine whether sleep deprivation could affect the mode in which GABAergic signalling operates in the cortex. In the previous Chapter, I used the AMPA-antagonised NBQX to shut down local activity in the cortex so that its contribution to $E_{\text{GABA}_{\text{A}}\text{R}}$ could be measured. Using this same approach, the third objective was to explore the contribution of local network activity to setting $\text{GABA}_{\text{A}}\text{R}$ function in the sleep-deprived cortex.

5.2 Acute sleep deprivation affects cortical network activity

Mice spend the majority of their time awake during the dark period and the majority of their time sleeping during the light period. *In vivo* perforated patch-clamp recordings were started three hours after light onset (i.e. Zeitgeber time 3, ZT3) and performed in awake mice that had either been allowed to sleep naturally during the preceding three hours (referred to as ‘rested’ animals; data from **Chapter 4**, see **Section 4.2**) or had experienced a period of prolonged wakefulness as a result of a sleep deprivation protocol during the preceding 3 hours (referred to as ‘sleep-deprived’ animals; see detailed protocol in **Section 2.5.5**). The sleep deprivation protocol involved exposing the animals to novel objects, and controlled for circadian phase and light exposure, so that the principle difference between the rested and sleep-deprived mice was their sleep-wake history and associated sleep pressure (Alfonsa et al., 2021). The first experiment compared the activity profile of layer 2/3 pyramidal neurons in V1 of rested and sleep-deprived mice. Spontaneous activity was found to differ between the two cortical states (**Figure 5.1A**), with sleep deprivation resulting in a more depolarised membrane potential (**Figure 5.1B** and **C**) and greater fluctuations in the membrane potential (**Figure 5.1D**), but no significant effect on the membrane resistance when compared to neurons in rested cortex (**Figure 5.1E**).

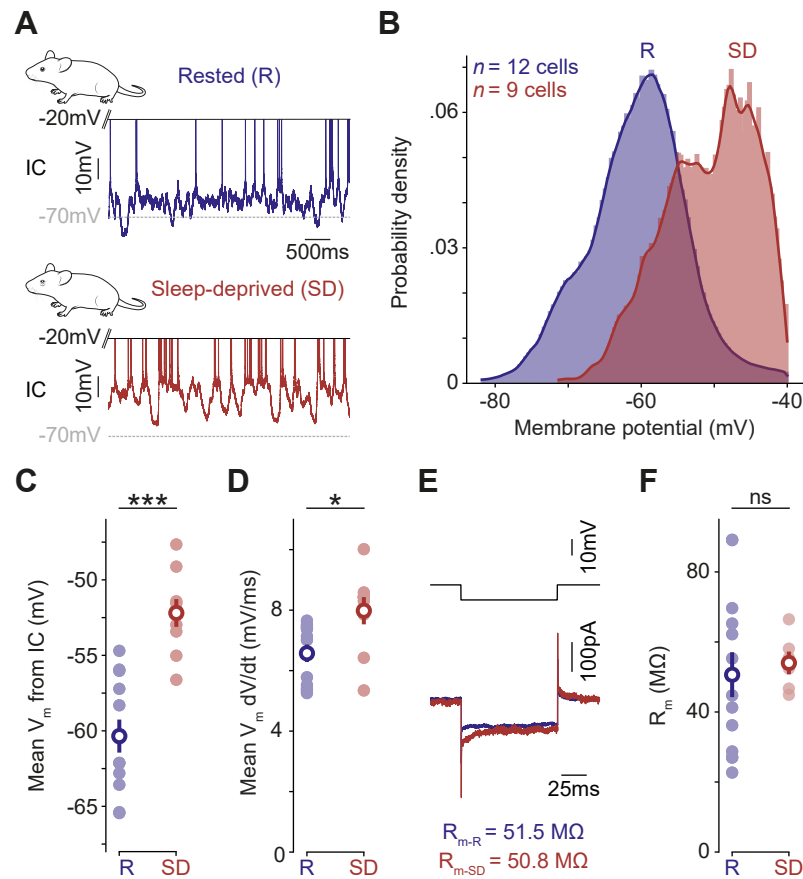


Figure 5.1: Acute sleep deprivation increases cortical network activity. **A**, Example of perforated current-clamp (IC) recordings of spontaneous activity from pyramidal neurons in visual area 1 of rested mice (blue, top) and in mice which were acutely sleep deprived for three hours (red, bottom). **B**, Distribution of membrane potential (V_m) values shown as a probability density function in the rested ($n = 12$ neurons from 10 mice) and sleep-deprived cortex ($n = 9$ neurons from 9 mice). Data for the rested group is re-used from the 'awake' group in Figure 4.1. **C**, Population data of mean V_m from IC recordings of spontaneous activity (R: -60.3 ± 1.1 mV vs SD: -52.2 ± 0.9 mV, $p = 0.0002$, one-way ANOVA with Bonferroni tests for multiple comparisons). **D**, Mean instantaneous change in membrane potential from IC recordings (R: 6.6 ± 0.3 mV/ms vs SD: 7.9 ± 0.5 mV/ms, $p = 0.0009$, one-way ANOVA with Bonferroni tests for multiple comparisons). **E**, Raw trace of the current response to -10 mV step (R, blue; SD, red). **F**, Population data for membrane resistance in the rested (R_{m-R} , blue) and sleep-deprived (R_{m-SD} , red) cortical neurons (R: 50.6 ± 6.4 M Ω , $n = 13$ neurons from 10 mice vs SD: 54.1 ± 3.2 M Ω , $n = 6$ neurons from 6 mice, $p > 0.9999$, one-way ANOVA with Bonferroni tests for multiple comparisons). 'ns', non-significant ($p > 0.05$); *, $p < 0.05$; ***, $p < 0.001$.

5.3 The equilibrium potential of the GABA-A receptor is more depolarised in the sleep-deprived cortex

Having demonstrated that sleep deprivation does indeed alter the excitability of cortical neurons, I next tested whether there were differences in synaptic GABA_AR signalling using the same approach that I had used to compare the anaesthetised and awake states (**Section 4.3**). Presynaptic GABA release was evoked using blue light and the polarity of the response was determined by measuring the change in membrane potential (**Figure 5.2A**, top panel). In the sleep-deprived cortex, GABA responses were exclusively depolarising (**Figure 5.2A**, bottom panel). By contrast, neurons in the rested cortex exhibited a mixture of responses, with approximately half exhibiting a depolarising response and half exhibiting a hyperpolarising GABA response.

To determine whether the difference in synaptic GABA polarity was caused by underlying differences in $E_{\text{GABA}_A\text{R}}$, the same voltage-clamp ramp protocol was used as in **Sections 3.4** and **4.3**. Consistent with the depolarising shift in GABA responses observed in current-clamp recordings, neurons in the sleep-deprived cortex exhibited a more depolarised $E_{\text{GABA}_A\text{R}}$ (**Figure 5.2B** and **C**). This was associated with a non-significant trend for more depolarising RMP values (**Figure 5.2D**) such that the overall $DF_{\text{GABA}_A\text{R}}$ was depolarising in the sleep-deprived condition (by ~ 7 mV), whereas the $DF_{\text{GABA}_A\text{R}}$ did not differ from zero in the rested condition (**Figure 5.2E**).

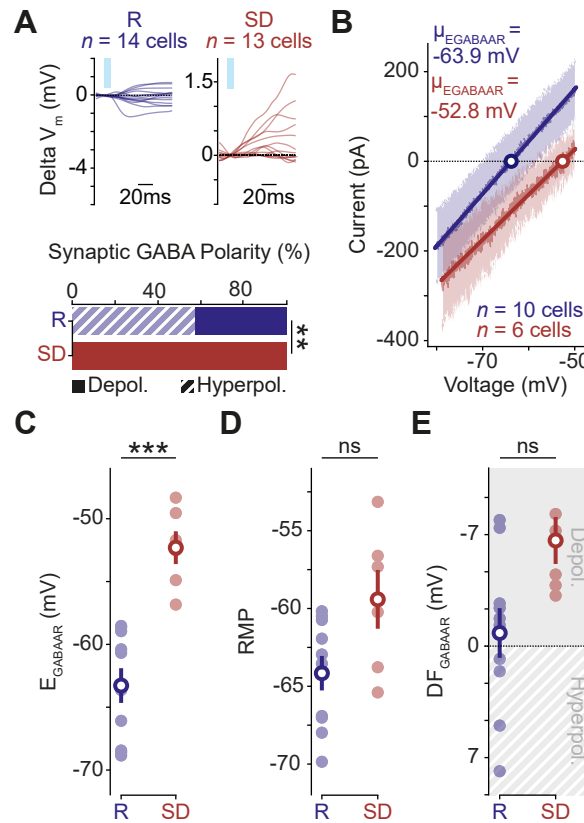


Figure 5.2: GABAergic signalling is depolarising in the sleep-deprived brain. **A**, Overlaid averaged and normalised traces of light-evoked postsynaptic GABA currents in the rested (blue, R, $n = 14$ neurons from 11 mice) and sleep-deprived cortex (red, SD, $n = 13$ neurons from 10 mice). Data for the rested group is re-used from the 'awake' group in Figure 4.2. In the rested cortex, light-evoked GABA responses can either be depolarising (Depol.) or hyperpolarising (Hyperpol.), whilst in the sleep-deprived cortex responses are exclusively depolarising (bottom panel, R: Depol. 6/14 Hyperpol. 8/14 vs SD: Depol. 13/13 Hyperpol. 0/13, $p = 0.002$, Fisher-Exact test). **B**, Averaged subtracted GABA currents recorded in VC in the rested (R, $n = 10$ neurons from 9 mice) and sleep-deprived cortex (SD, $n = 6$ neurons from 6 mice). The shaded area reflects SEM for current, solid trace reflects mean current, straight-line reflects fitted line, circle indicates the mean E_{GABAAR} . **C**, Population data of E_{GABAAR} (R: -63.3 ± 1.4 mV vs SD: -52.3 ± 1.3 mV, $p = 0.0003$, one-way ANOVA with Bonferroni tests for multiple comparisons) and RMP (**D**, R: -64.2 ± 1.1 mV vs SD: -59.4 ± 1.9 mV, $p = 0.51$, one-way ANOVA with Bonferroni tests for multiple comparisons) in the rested and sleep-deprived cortex. **E**, Population data of DF_{GABAAR} (RMP - E_{GABAAR}) for synaptic GABA_AR signalling in the rested and sleep-deprived cortex (R: -0.9 ± 1.7 mV vs SD: -7.1 ± 1.6 mV, $p = 0.32$, one-way ANOVA with Bonferroni tests for multiple comparisons). DF_{GABAAR} in the rested cortex was not significantly different to zero, consistent with an overall shunting effect ($p = 0.61$, one-sample t -test). By contrast, DF_{GABAAR} was depolarising in the sleep-deprived cortex ($p = 0.006$, one-sample t -test). 'ns', non-significant ($p > 0.05$); *, $p < 0.05$; ***, $p < 0.001$.

5.4 Blocking excitatory synaptic transmission neutralises sleep-wake differences in cortical network activity

The above findings demonstrate that acute sleep deprivation can elevate the activity profile of the cortex. To ascertain whether the greater levels of depolarising GABAergic signalling in the sleep-deprived state are a consequence of the shift in cortical network activity, I repeated the same experiment presented in **Section 4.4** and blocked excitatory synaptic transmission by antagonising glutamatergic AMPA receptors with NBQX (see detailed protocol in **Section 2.5.4**). In both the rested and sleep-deprived cortex, NBQX was highly effective in reducing spontaneous cortical activity (**Figure 5.3A**). The differences in both the resting membrane potential and membrane fluctuations were neutralised between sleep-deprived and rested cortex (**Figure 5.3B, C and D**). Across both states, there was no difference in the R_m measured in voltage-clamp (**Figure 5.3E and F**).

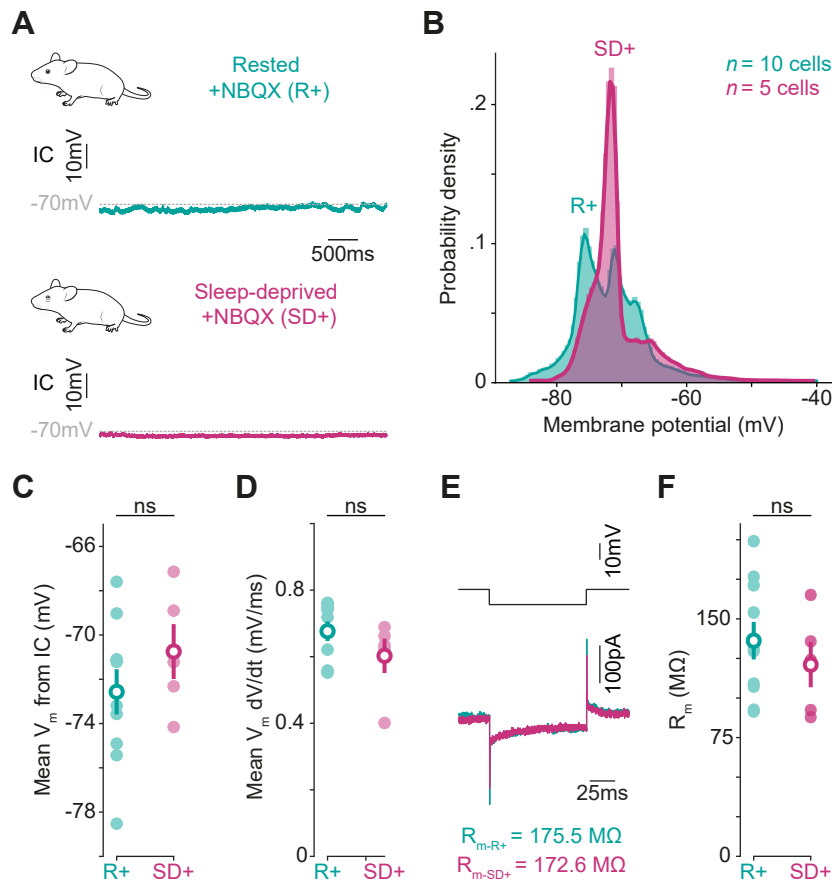


Figure 5.3: Blocking excitatory signalling neutralises sleep-wake differences in local cortical network activity. **A**, Example of perforated current-clamp (IC) recordings of spontaneous activity from a visual area 1 pyramidal neuron after a focal injection of the glutamate-receptor blocker, NBQX (100 μ M), in rested mice (R+, turquoise, top) and acutely sleep-deprived mice (SD+, magenta, bottom). **B**, Distribution of membrane potential (V_m) with probability density function in the rested ($n = 10$ neurons from 7 mice) and sleep-deprived cortex ($n = 5$ neurons from 5 mice). Data for the rested with NBQX group is re-used from 'awake +NBQX' group shown in Figure 4.3. **C**, Population data of mean V_m from IC recordings of spontaneous activity (R+: -72.6 ± 1.0 mV vs SD+: -70.8 ± 1.2 mV, $p > 0.9999$, one-way ANOVA with Bonferroni tests for multiple comparisons). **D**, Mean instantaneous change in membrane potential from IC recordings (R+: 0.68 ± 0.03 mV/ms vs SD+: 0.60 ± 0.05 mV/ms, $p > 0.9999$, one-way ANOVA with Bonferroni tests for multiple comparisons). **E**, Raw trace of the current response to -10 mV step (R+, turquoise; SD+, magenta). **F**, Population data for membrane resistance in the rested (R_{m-R+} , turquoise) and sleep-deprived (R_{m-SD+} , magenta) cortical neurons (R+: $136.2 \pm 11.9 \text{ M}\Omega$, $n = 10$ neurons from 7 mice vs SD+: $121.1 \pm 14.3 \text{ M}\Omega$, $n = 5$ neurons from 5 mice, $p > 0.9999$, one-way ANOVA with Bonferroni tests for multiple comparisons). 'ns', non-significant ($p > 0.05$).

5.5 The equilibrium potential of the GABA-A receptor remains more depolarised in the sleep-deprived cortex after blocking local network activity

By neutralising the differences in background synaptic activity with NBQX, I was then able to test the contribution of local network activity in setting the parameters for GABAergic signalling in the sleep-deprived cortex. Whereas all GABA responses were hyperpolarising in the presence of NBQX in rested cortex, GABA responses were more heterogeneous in the sleep-deprived cortex, with approximately half of the neurons still exhibiting a depolarising GABA response in the presence of NBQX (**Figure 5.4A**). Using voltage-clamp ramp protocols, I was again able to measure the parameters underlying GABA_AR function (**Figure 5.4B**). Blocking local network activity resulted in more hyperpolarising $E_{GABA_A R}$ values in both cortical states (R: -63.3 ± 1.4 mV vs R+NBQX: -78.1 ± 1.3 mV, $p < 0.0001$, *one-way ANOVA with Bonferroni tests for multiple comparisons*, as shown in **Figure 4.4**; SD: -52.3 ± 1.3 mV vs SD+NBQX: -68.7 ± 1.9 mV, $p < 0.0001$, *one-way ANOVA with Bonferroni tests for multiple comparisons*). By contrast, in the presence of NBQX, $E_{GABA_A R}$ in the sleep-deprived cortex remained significantly more depolarised than in the rested cortex (**Figure 5.4B** and **C**). Differences in RMP were neutralised by NBQX (**Figure 5.4D**), which was in line with the current-clamp recordings (**Figure 5.3**). Under conditions where the contribution of local network activity was removed, $DF_{GABA_A R}$ did not differ significantly between the sleep-deprived and rested cortex ($p = 0.18$, *one-way ANOVA with Bonferroni tests for multiple comparisons*; **Figure 5.4E**). However, the $DF_{GABA_A R}$ in the rested cortex exhibited a hyperpolarising $DF_{GABA_A R}$ ($p = 0.0014$, *one-sample t-test*), whereas $DF_{GABA_A R}$ in the sleep-deprived cortex did not differ from zero, consistent with an overall shunting effect ($p = 0.87$, *one-sample t-test*; **Figure 5.4E**). Taken together, these observations are consistent with previous *in vitro* recordings in acute brain slices, which have shown that sleep deprivation leads to depolarising shifts in $E_{GABA_A R}$ and lower $DF_{GABA_A R}$ (Alfonsa et al., 2021).

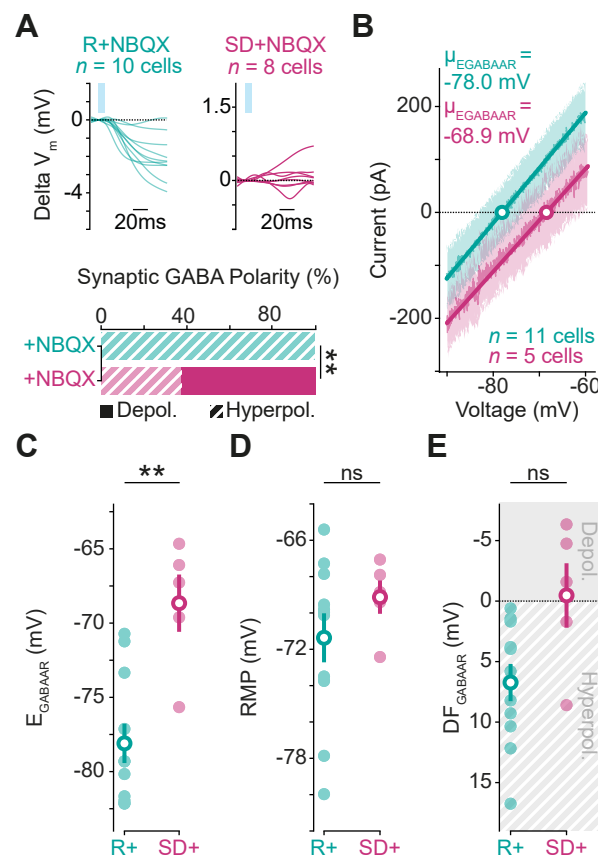


Figure 5.4: Sleep deprivation causes a depolarising shift in GABAergic signalling that is beyond the contribution of local network activity. **A**, Overlaid averaged and normalised traces of light-evoked post-synaptic GABA currents after glutamatergic signalling was antagonised with NBQX (100 μ M) in the rested (turquoise, R+, $n = 10$ neurons from 7 mice) and sleep-deprived cortex (magenta, SD+, $n = 8$ neurons from 8 mice). Data for rested with NBQX group is re-used from the 'awake +NBQX' group shown in Figure 4.4. In the rested cortex, light-evoked GABA responses are exclusively hyperpolarising (Hyperpol.) after local network activity is shut down with NBQX. By contrast, in the sleep-deprived brain, GABAergic responses remain mostly depolarising (Depol.) (bottom panel, R+: Depol. 0/10 Hyperpol. 10/10 vs SD+: Depol. 5/8 Hyperpol. 3/8, $p = 0.007$, Fisher-Exact test). **B**, Averaged subtracted GABA currents recorded in VC in the rested (R+, $n = 11$ neurons from 7 mice) and sleep-deprived cortex (SD+, $n = 5$ neurons from 5 mice). The shaded area reflects SEM for current, solid trace reflects mean current, straight-line reflects fitted line, circle indicates the mean E_{GABAAR} . **C**, Population data of E_{GABAAR} (R+: -78.1 ± 1.3 mV vs SD+: -68.7 ± 1.9 mV, $p = 0.004$, one-way ANOVA with Bonferroni tests for multiple comparisons) and RMP (**D**, R+: -71.4 ± 1.4 mV vs SD+: -69.1 ± 0.9 mV, $p > 0.54$, one-way ANOVA with Bonferroni tests for multiple comparisons) in the rested and sleep-deprived cortex. **E**, Population of DF_{GABAAR} (RMP – E_{GABAAR}) for synaptic GABA_A-mediated synaptic signalling in the rested and sleep-deprived cortex after NBQX injection (R+: 6.7 ± 1.5 mV vs SD+: -0.5 ± 2.7 mV, $p = 0.18$, one-way ANOVA with Bonferroni tests for multiple comparisons). 'ns', non-significant ($p > 0.05$); **, $p < 0.01$.

5.6 Sleep-wake history determines the set point for the equilibrium potential of the GABA-A receptor in the awake cortex

To isolate the relative contribution of network-dependent and network-independent effects, $E_{\text{GABA}_A\text{R}}$ was compared between rested and sleep-deprived cortex, before and after the addition of NBQX. $E_{\text{GABA}_A\text{R}}$ was more depolarised in the drug-free sleep-deprived cortex (**Figure 5.2C**) and this difference remained when local network activity was blocked with NBQX (**Figure 5.4C**). To calculate the NBQX-induced changes in $E_{\text{GABA}_A\text{R}}$ in the rested and sleep-deprived states, ideally I would have paired data to monitor the change in $E_{\text{GABA}_A\text{R}}$ within the same neuron. Unfortunately, it proved technically too difficult to obtain this paired data at a statistically-relevant sample size. As an alternative, I calculated the difference in the mean $E_{\text{GABA}_A\text{R}}$ with and without NBQX (i.e. mean $E_{\text{GABA}_A\text{R}}$ without NBQX minus mean $E_{\text{GABA}_A\text{R}}$ with NBQX) for each state. In the rested state, the difference in mean $E_{\text{GABA}_A\text{R}}$ was 14.8 mV, whereas in the sleep-deprived state the difference in mean $E_{\text{GABA}_A\text{R}}$ was 16.4 mV. Whilst it is not possible to determine the statistical significance of this difference, it suggests that the relative contribution of local network activity in setting $E_{\text{GABA}_A\text{R}}$ is similar between rested and sleep-deprived states. This observation would support the conclusion that the principal effect of sleep deprivation is mediated by changes in the underlying set point for $[\text{Cl}^-]_i$, consistent with previous evidence that sleep deprivation results in changes in the activity of Cl^- co-transporters that determine $E_{\text{GABA}_A\text{R}}$ (Alfonsa et al., 2021).

5.7 Discussion

In this Chapter, I explored how sleep-wake history can affect GABAergic signalling in the awake cortex. By prolonging wakefulness by three hours, I first found that background synaptic activity in the cortex is significantly elevated. In this sleep-deprived state, synaptic GABA_AR signalling becomes exclusively depolarising. Furthermore, the differences in the activity profile between rested and sleep-deprived states can be neutralised by blocking glutamatergic signalling with NBQX. In this low

activity state, GABAergic signalling in the sleep-deprived cortex remained significantly more depolarised compared to the rested cortex under the same recording conditions. Moreover, the contribution of local network activity to setting $E_{\text{GABA}_{\text{AR}}}$ appeared to be comparable, regardless of the sleep-wake history of the cortex. Taken together, these findings provide *in vivo* evidence that demonstrates that sleep history can affect the set point for GABAergic signalling in the cortex.

Synaptic signalling is known to vary across the sleep-wake cycle (Eban-Rothschild et al., 2018; Tononi and Cirelli, 2006). By using sleep deprivation, it is possible to extend the period of wakefulness and thereby exaggerate the changes in neuronal dynamics that promote a transition towards sleep. Using this approach, previous studies have shown that the increasing level of cortical excitability during wakefulness may be one mechanism that encourages the network to transition into sleep (Vyazovskiy et al., 2009). This induced elevation in local cortical activity is facilitated by factors that promote excitation including, among others, an increase in extracellular glutamate (Dash et al., 2009) and K^+ (Ding et al., 2016). The peak in this induced elevation of cortical excitability occurs after three hours of sleep deprivation, which is why this time period was chosen for my experiments (Dash et al., 2009; Vyazovskiy et al., 2009).

A recent observation made by Alfonsa and colleagues (2021) has suggested that an activity-dependent shift in GABAergic signalling represents a cellular mechanism that encodes sleep pressure in cortex. In this study, the authors show that prolonged wakefulness can induce an upregulation in NKCC1, promoting an increase in intracellular Cl^- influx and an associated depolarising shift in $E_{\text{GABA}_{\text{AR}}}$ (Alfonsa et al., 2021). This study was conducted in acute brain slices from layer 5 pyramidal neurons in somatosensory cortex, and my results from layer 2/3 pyramidal neurons confirm that $E_{\text{GABA}_{\text{AR}}}$ (-52.3 mV) is also depolarising in sleep-deprived cortex *in vivo* (**Figure 5.3**). This depolarising shift in $E_{\text{GABA}_{\text{AR}}}$ translates to an estimated shift in the intracellular Cl^- from ~ 9.7 mM to ~ 16.5 mM (calculated using the GHK equation **Section 2.6.3**). While I did not study changes in Cl^- co-transporters directly, I did observe a depolarising shift in $E_{\text{GABA}_{\text{AR}}}$ in the absence of local cortical activity (**Figure 5.4**). Specifically, in the presence of NBQX, $[\text{Cl}^-]_{\text{i}}$ in the sleep-deprived cortex was estimated to be ~ 7.2 mM ($E_{\text{GABA}_{\text{AR}}} = -68.7$ mV) compared to 3.9 mM ($E_{\text{GABA}_{\text{AR}}} = -78.1$ mV) in the rested cortex.

While an increase in NKCC1 contribution has been identified as a likely mechanism based on *in vitro* evidence (Alfonsa et al., 2021), further pharmacological and molecular studies are needed to test the contribution of this CCC to setting the depolarised $E_{\text{GABA}_{\text{A}}\text{R}}$ in the sleep-deprived cortex *in vivo*.

The effect of sleep deprivation on the cortex is not uniform and depends on the level of activity that occurs during wakefulness. This has been demonstrated by Fisher and colleagues (2016) who showed that the changes in cortical firing that occur during the wake period are related to specific behaviours. Consistent with this finding, Alfonsa and colleagues (2021) report that the depolarising shift in the $E_{\text{GABA}_{\text{A}}\text{R}}$ that occurs during prolonged wakefulness is abolished when sensory-experience is altered. In my experiments, specific behaviours during the sleep deprivation period and during the recording period were not recorded and therefore it is unknown whether these parameters differed across animals.

In addition to sleep-wake history, intracellular Cl^- homeostasis also fluctuates across the diurnal rhythm. This was originally described in neurons in the suprachiasmatic nucleus (SCN), which is considered the main regulator of circadian rhythms (Klein et al., 1991). There have been conflicting *in vitro* results as to whether the effect of $\text{GABA}_{\text{A}}\text{R}$ transmission fluctuates throughout the circadian cycle (Choi et al., 2008; De Jeu and Pennartz, 2002; Gribkoff et al., 2003, 1999; Wagner et al., 1997, 2001). However, one study has highlighted that GABA in SCN neurons may be predominantly excitatory during the dark phase, which corresponds to when mice are awake, and that this is due to a transient upregulation of NKCC1 (Choi et al., 2008). More recently, Pracucci and colleagues (2021) have shown using *in vivo* Cl^- imaging in layer 2/3 pyramidal neurons in urethane-anaesthetised mice that there is an elevation of intracellular Cl^- during the dark phase. In addition, they also show that during the dark period in which intracellular Cl^- is at its peak, cortical excitability is also elevated, as indicated by a reduced threshold to induce seizures using a chemoconvulsant. Taken together, these findings are consistent with the idea that during wakefulness, GABAergic signalling is less likely to exert hyperpolarising effects due to changes in cortical activity and co-transporter function.

In summary, the results presented in this Chapter support the conclusion that sleep-wake

history can affect the driving force for GABA_AR-mediated synaptic signalling, which reflects the levels of cortical network activity, but also network-independent effects that determine the underlying $DF_{\text{GABA}_A\text{R}}$. More broadly, these findings extend the findings of **Chapter 4** and further show how the state of local network activity can set the mode in which GABAergic transmission operates in the cortex.

Chapter 6

Optogenetic determination of resting and dynamic chloride equilibrium potentials

6.1 Introduction

The previous chapters of this thesis support the idea that a neuron's E_{Cl} can vary depending on the level of network activity, or as a result of sleep-wake-dependent changes to the Cl^- homeostatic set point. Established experimental methods for measuring E_{Cl} rely upon the activation of Cl^- -permeable receptors (e.g. $GABA_A$ Rs), either by delivering an exogenous agonist (e.g. puffing or uncaging) or by stimulating presynaptic axons to release an endogenous agonist (as explained in **Section 1.3.2**). However, these methods can be confounded by issues associated with receptor activation and the delivery of the receptor agonist. For example, it is difficult to know which populations of receptors are activated and the receptor availability can also change following activation (e.g. desensitisation) (Frosch et al., 1992; Jones and Westbrook, 1995; Karlsson et al., 2011). Moreover, the receptor's own contribution to E_{Cl} is difficult to assess (Karlsson et al., 2011; Yelhekar et al., 2016). It is also not always feasible to deliver the agonist to the target neuron, particularly in intact preparations. Furthermore, when stimulating axons to release the agonist, one must control for effects upon presynaptic mechanisms.

For these reasons, I considered alternative methods for probing E_{Cl} in an agonist-independent and neuron-specific manner. To assess how a neuron regulates its transmembrane Cl^- gradients, one ideally wants to measure E_{Cl} under a variety of conditions, both at rest and when challenged with a Cl^- load (Deisz et al., 2011; Doyon, Prescott and De Koninck, 2016; Luhmann and Prince, 1991; Misgeld et al., 1986; Thompson et al., 1988). Furthermore, sensitive measurement methods should be able to distinguish neurons that differ in their chloride-handling mechanisms. For example, several reports have suggested that parvalbumin (PV) fast-spiking interneurons exhibit different chloride homeostasis to pyramidal neurons (Banke and McBain, 2006; Martina et al., 2001; Otsu et al., 2020; Sauer and Bartos, 2010; Verheugen et al., 1999; Vida et al., 2006), but to date there has been no consensus on the contribution that cation Cl^- co-transporters (CCCs) make to these differences.

The modern optogenetic toolkit includes a range of light-activated Cl^- -selective transporters and channels (see **Section 1.3.2**). Whilst these optical constructs are primarily used to induce neuronal inhibition, it has been shown that they can also be used to manipulate intracellular Cl^- dynamics (Alfonsa et al., 2016, 2015; Lanz et al., 2020; Raimondo, Kay, Ellender and Akerman, 2012). Of particular interest here are the new generation of optically controlled Cl^- channels (Berndt et al., 2014, 2016; Govorunova et al., 2015, 2016; Wietek et al., 2015, 2016, 2014). These channels depend on the state of the transmembrane Cl^- gradient and therefore should, in principle, afford the opportunity to directly investigate E_{Cl} and the transmembrane gradient for Cl^- . Within the class of light-activated Cl^- channels, the *Guillardia theta* anion-conducting rhodopsin 2 (GtACR2) opsin is considered to have the best performance due to its high level of anion selectivity and ability to generate large photocurrents (Govorunova et al., 2015; Sineshchekov et al., 2015; Wiegert et al., 2017). Furthermore, Mahn and colleagues (2018) have modified the GtACR2, creating stGtACR2, in order to optimise its capacity to elicit neuronal inhibition. This includes a soma-targeting motif to restrict expression to the soma and prevent expression at the presynaptic terminal, which has been shown to evoke neurotransmitter release and facilitate postsynaptic excitation (Mahn et al., 2016). In addition, loxP sites have been included, allowing for expression to be controlled by Cre-lox recombination within defined cell types.

The first objective of this Chapter was to validate a novel optical method for probing E_{Cl} . Here I test whether stGtACR2, a soma-localised optically-activated Cl^- channel, can be used to perform E_{Cl} measurements in pyramidal neurons by comparing the stGtACR2 equilibrium potential to the equilibrium potential of GABA_ARs. In addition, I present how this tool can be used to quantify the contribution of KCC2 to E_{Cl} dynamics. One of the major limitations of other methods for probing E_{Cl} is that they are not easily applied in the *in vivo* brain, or are not specific to studying intracellular Cl^- . In an attempt to address this, the second objective was to determine whether this novel optical method could be used to measure E_{Cl} in the cortex *in vivo*. Having established this novel optical method in pyramidal neurons, the third objective was to assess whether the method is sufficiently sensitive to detect cell-type differences in steady-state intracellular Cl^- . To test this, I explored whether it was possible to use the stGtACR2 to confirm the previously reported differences in E_{Cl} between pyramidal neurons and PV interneurons. Finally, the fourth objective of this Chapter was to explore whether the stGtACR2 could measure differences in KCC2-mediated Cl^- extrusion between the two cell types.

6.2 A novel optogenetic method for measuring neuronal chloride dynamics

The optically-activated stGtACR2 is thought to be selective for Cl^- and therefore its equilibrium potential ($E_{stGtACR2}$) is expected to reflect the equilibrium potential for Cl^- (E_{Cl}). Experimentally, an approximation of E_{Cl} can be made by pharmacologically isolating the equilibrium potential for the GABA_AR ($E_{GABA_A R}$), which is predominately permeable to Cl^- (see **Section 1.2.1**). To test how $E_{stGtACR2}$ compares to $E_{GABA_A R}$, I selectively expressed the stGtACR2 construct in layer 2/3 pyramidal neurons in somatosensory cortex of juvenile mice (for detailed methods see **Section 2.2.3**). This was achieved by performing *in vivo* intracerebral stereotaxic injections of an adeno-associated virus (AAV) encoding a floxed stGtACR2 construct into the cortex of juvenile (four weeks postnatal, P28) CaMKII α -cre mice (**Figure 6.1A**). In this mouse line, Cre recombinase is selectively expressed in pyramidal neurons, which will restrict cleavage of the loxP sites and subsequent expression of the stGtACR2 only to this cell type (**Figure 6.1B**). After at least two weeks following viral injection, acute coronal brain

slices were prepared (see detailed procedures in **Section 2.2.5**).

As the stGtACR2 is linked to the Fusion-Red fluorescent reporter protein (Mahn et al., 2018), individual layer 2/3 pyramidal neurons expressing this Cl^- channel could be visualised under fluorescent light. I was therefore able to perform targeted whole-cell patch-clamp recordings (see detailed procedures in **Section 2.4.1**) on stGtACR2-expressing cortical neurons which, when injected with depolarising current, demonstrated a firing pattern consistent with that of pyramidal neurons (**Figure 6.1C**). To evoke stGtACR2 photocurrents, the recorded neuron was illuminated with blue light pulses (470 nm laser) delivered through the microscope's objective. E_{stGtACR2} was measured by performing voltage steps and generating a current-voltage (IV) plot of both the baseline current and the current measured during stGtACR2 activation ('stGtACR2 current'; **Figure 6.1D**, see detailed explanation of protocol in **Section 2.6.7**). In the same neurons, somatic GABA_A Rs were activated by focal application of exogenous GABA via a patch pipette connected to a picospritzer. To isolate $E_{\text{GABA}_A\text{R}}$ activation, GABA_A Rs were blocked by adding CGP55845 (10 μM) to the perfusate. A similar voltage step protocol was used to measure $E_{\text{GABA}_A\text{R}}$ (**Figure 6.1E**). To explore whether the relationship between E_{stGtACR2} and $E_{\text{GABA}_A\text{R}}$ was consistent across a range of intracellular Cl^- concentrations, whole-cell recordings were performed using one of three different pipette solutions, consisting of either 4 mM, 20 mM or 70 mM Cl^- . Across a population of neurons, E_{stGtACR2} and $E_{\text{GABA}_A\text{R}}$ were found to be significantly correlated (**Figure 6.1F**), supporting the idea that E_{stGtACR2} can be used to estimate somatic E_{Cl^-} .

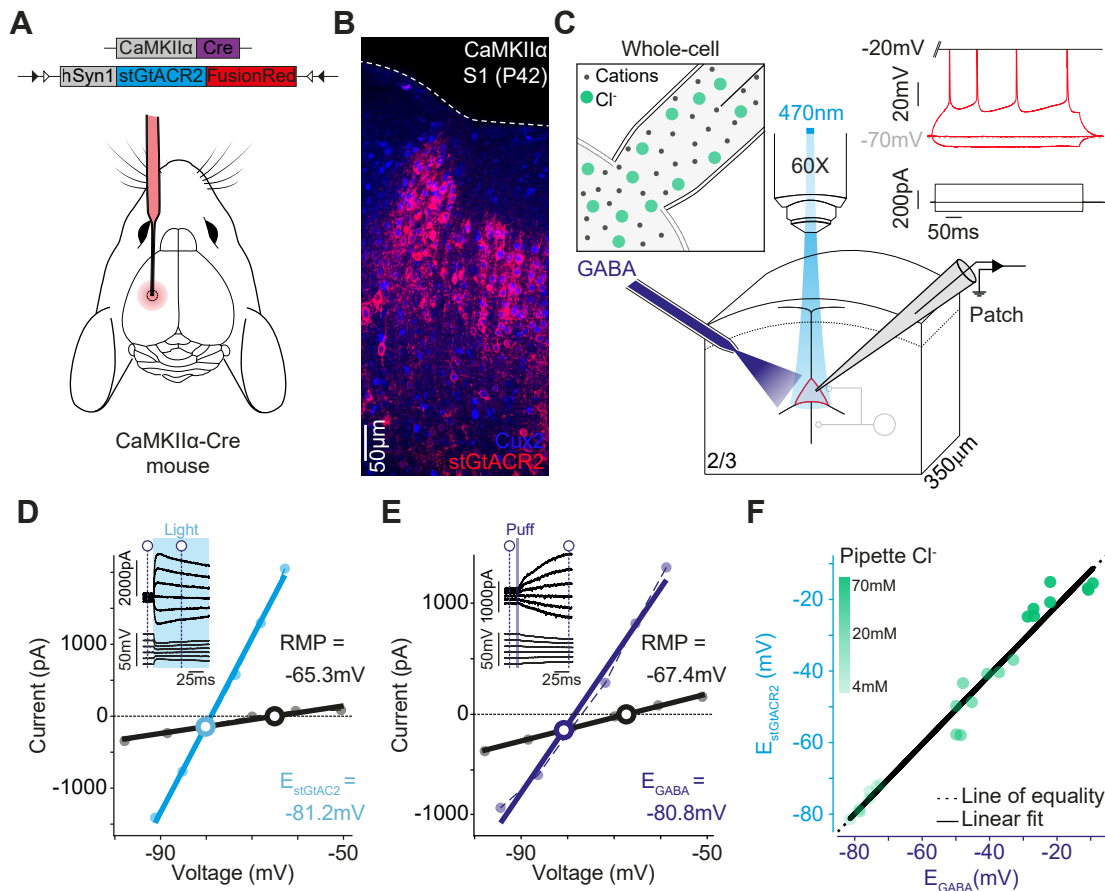


Figure 6.1: The equilibrium potential of the light-activated stGtACR2 is equivalent to the chloride-permeable GABA-A receptor. **A**, Postnatal intracerebral AAV injections were performed in the primary somatosensory cortex (S1) of homozygous juvenile (four weeks postnatal) CaMKII α -cre mice, to allow for viral transduction and expression of stGtACR2 in layer 2/3 pyramidal neurons. **B**, Confocal microscope image of a 100 μ m brain slice showing stGtACR2 (fused to FusionRed, red) and expressed in Cux2+ layer 2/3 pyramidal neurons (blue) in S1, two weeks after injection. **C**, Schematic of the experimental setup in which targeted whole-cell patch-clamp recordings were performed from stGtACR2-expressing layer 2/3 pyramidal neurons. Activation of stGtACR2 was achieved by delivering blue light (470 nm laser) via the microscope objective. To compare to $E_{GABA_{AAR}}$, focal applications of exogenous GABA were used to activate somatic GABA_AR. Inset (right corner), current-clamp recording of the firing pattern elicited from a stGtACR2-expressing pyramidal neuron following current injection. **D**, Current-voltage (IV) plot showing the relationship between the stGtACR2-induced current (cyan, 100 ms light activation) and baseline current (black) across different voltage steps. Both the resting membrane potential (RMP) and equilibrium potential for stGtACR2 ($E_{stGtACR2}$) are indicated. Inset shows electrophysiological trace with the current response and estimated voltage after series resistance correction. Points at which the baseline and stGtACR2 currents were measured are indicated (vertical blue lines). **E**, IV plot showing $E_{GABA_{AAR}}$ from focal applications of exogenous GABA puffed on the soma. **F**, $E_{stGtACR2}$ and $E_{GABA_{AAR}}$ are highly correlated (linear fit, black solid line, $r = 0.99$, $n = 24$ neurons from 7 mice, $p < 0.0001$, Pearson correlation coefficient) and equivalent (dashed black line) across a range of pipette Cl⁻ concentrations.

6.3 Quantifying chloride extrusion capacity using optically-evoked chloride fluxes

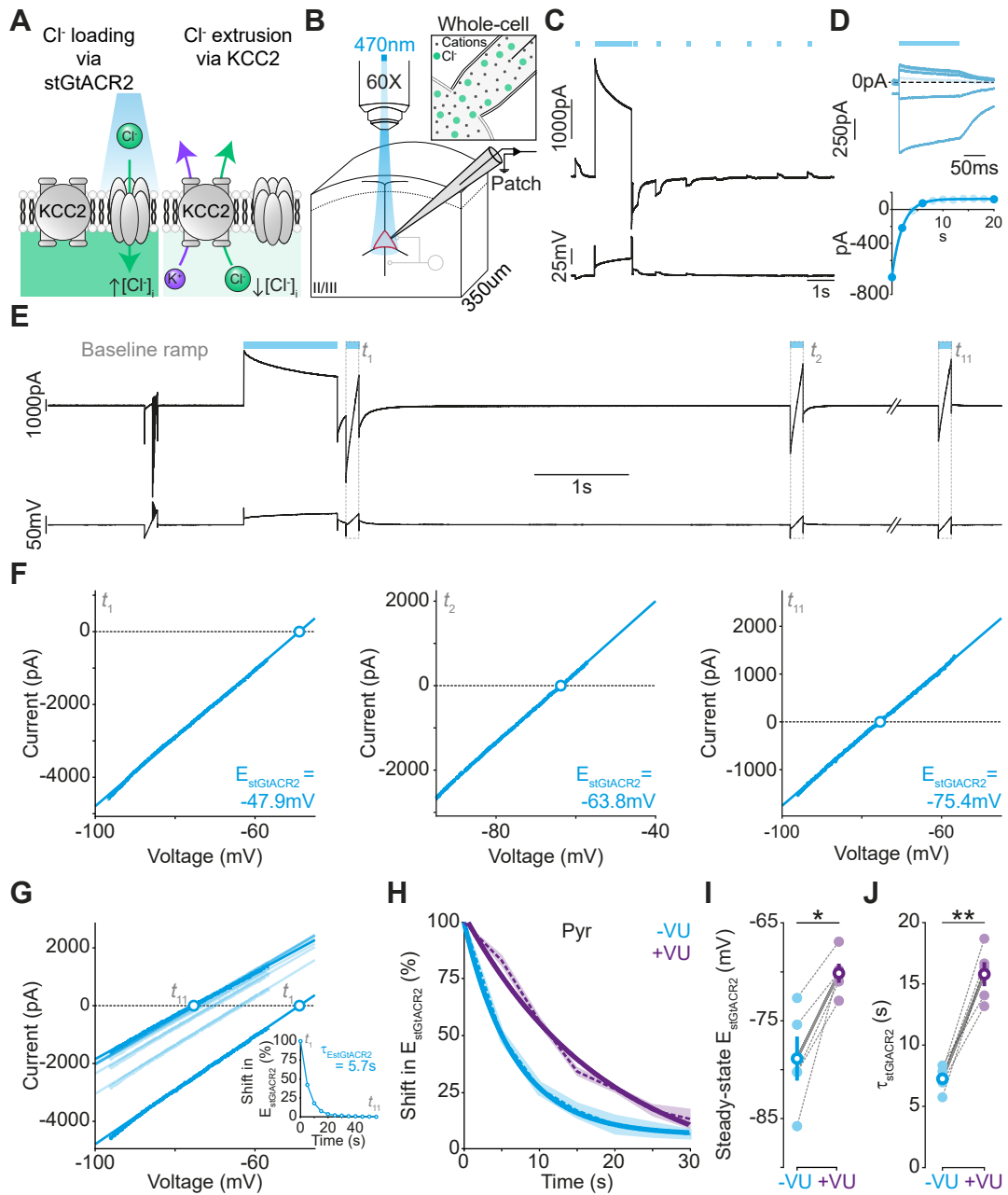
Previous work has shown that large Cl^- influxes can cause a transient increase in $[\text{Cl}^-]_i$, which leads to a transient depolarising shift in E_{Cl} , as illustrated in **Figure 6.2A** (Jin et al., 2005; Raimondo, Kay, Ellender and Akerman, 2012; Staley and Proctor, 1999). The rate at which E_{Cl} then recovers to its steady-state, can then be used as a way to quantify Cl^- extrusion mechanisms, with KCC2 being the predominant mechanism (Deisz et al., 2011; Doyon et al., 2011; Doyon, Prescott and De Koninck, 2016; Lanz et al., 2020; Raimondo, Kay, Ellender and Akerman, 2012). Previous experiments examined such E_{Cl} dynamics by activating GABA_A Rs via application of GABA or a GABA_A R-selective agonist. However, it is difficult to control the amount of agonist delivered, the pool of receptors that are activated by the agonist, or agonist-dependent changes to the receptors.

An optogenetic approach to quantifying Cl^- extrusion could avoid these agonist-related issues and allow the Cl^- fluxes to be tightly controlled using light. To investigate this possibility, I performed whole-cell patch-clamp recordings in voltage-clamp mode (pipette solution, 4 mM Cl^-) in stGtACR2-expressing pyramidal neurons (**Figure 6.2B**). I adopted a loading protocol that was similar to that used by Raimondo and colleagues (2012), and used blue light to activate stGtACR2 whilst increasing the driving force for Cl^- by depolarising the membrane potential with a voltage step (**Figure 6.2C**). The effect of this Cl^- loading protocol on subsequent stGtACR2 photocurrents could then be tracked sequentially (**Figure 6.2D**). Immediately after the loading protocol, stGtACR2 photocurrents were strongly depolarising (negative going) but then decreased in amplitude, and ultimately reversed to their original hyperpolarising state (positive going) over a period of ~50 seconds.

The depolarising shift in the stGtACR2 current indicates that the optical loading protocol is able to depolarise E_{stGtACR2} . To confirm this, I repeated the same loading protocol but used sequential voltage ramps to determine E_{stGtACR2} (**Figure 6.2E**). E_{stGtACR2} was determined by subtracting the current response to a voltage ramp before (defined as the 'Baseline ramp') and during stGtACR2 activation with blue light (**Figure**

6.2F). As expected, E_{stGtACR2} was depolarised immediately after the loading step and recovered to hyperpolarised values during the subsequent 50 seconds (**Figure 6.2G**). This approach makes it possible to define a neuron's Cl^- extrusion dynamics by calculating the rate at which E_{stGtACR2} recovers over time, as indicated by the decay constant, tau (τ_{stGtACR2} ; see detailed explanation in **Section 2.6.8**). To isolate the contribution of KCC2 to the Cl^- extrusion mechanisms, I added the KCC2-specific antagonist VU0463271 to the perfusate and repeated the same loading protocol in the same neuron. By comparing recovery curves before and after application of VU, the contribution of KCC2 could be determined optogenetically (**Figure 6.2H**). Overall, it was evident that KCC2 has a significant influence on setting both the steady-state of E_{stGtACR2} (**Figure 6.2I**), as well as on the rate, τ_{stGtACR2} , at which cortical pyramidal neurons are able to extrude Cl^- (**Figure 6.2J**).

Figure 6.2 (following page): KCC2-mediated chloride extrusion can be measured using optical activation of stGtACR. **A**, Schematic showing how $[\text{Cl}^-]_i$ can be increased (loaded) via stGtACR2 activation and decreased (unloaded) as a result of KCC2-mediated extrusion. **B**, Experimental setup of whole-cell patch-clamp recordings (pipette solution, 4mM Cl^-) of a stGtACR2-expressing layer 2/3 pyramidal neuron in an acute brain slice from somatosensory cortex. **C**, Recording trace of the voltage-clamp protocol used to test how stGtACR2 currents are affected following intense activation (top panel shows the current response, bottom panel shows the estimated voltage after R_s correction). stGtACR2 currents are evoked by 100 ms light pulses. This is then followed by a loading pulse that consisted of prolonged (1 s) stGtACR2 activation whilst concurrently depolarising the voltage to -20 mV to increase the Cl^- driving force. Following the loading step, stGtACR2 was activated (100 ms) every second. **D**, Current responses (top) to stGtACR2 activation following the loading step. The amplitude of current response at 1 s intervals (bottom). The decay constant, tau (τ), is measured from a single exponential curve fitted to the changes in current amplitude over time (cyan line). **E**, Recording trace of the voltage-clamp protocol used to measure the percentage change in E_{stGtACR2} after inducing a Cl^- load via stGtACR2 (top, current response; bottom, estimated voltage after R_s correction). To measure the baseline current (defined as 'Baseline ramp', grey), a voltage ramp (from -120 mV to -20 mV over 150 ms) was performed before the loading step (1 s activation at -20 mV). Ramps were then repeated every 5 s whilst activating stGtACR2 (blue light illumination maintained through the entire ramp, 150 ms). The first (t_1), second (t_2) and last (t_{11}) ramps following the loading step are marked (grey, dashed boxed). **F**, Current-voltage (IV) plots of the isolated stGtACR2 current (generated from the baseline ramp and ramps during stGtACR2 activation) at t_1 , t_2 and t_{11} were used to calculate the equilibrium potential for stGtACR2 (E_{stGtACR2}). **G**, Summary IV plot showing the change in isolated stGtACR2 current from t_1 to t_{11} . Inset, percentage change in E_{stGtACR2} following the loading step over time is plotted and a single exponential is fitted to calculate the decay constant (τ_{stGtACR2}), which indicates the rate of extrusion. **H**, A summary plot showing the change in E_{stGtACR2} over time under control conditions (-VU; cyan) and under conditions when KCC2 was blocked with the selective antagonist VU0463271 (+VU; purple; 10 μM). The mean (dashed line) and standard error of the mean (SEM, shaded area) are plotted. Single exponential was fitted (thick line) to population data to calculate the decay constant for both groups. Curves were cropped to show data up to and including 30 s after the loading step for clarity. Population data showing the increase in steady-state E_{stGtACR2} (**I**, -VU: -78.86 ± 2.56 mV vs +VU: -70.12 ± 0.98 mV, $p = 0.01$, paired t -test, $n = 5$ neurons from 3 mice) and τ_{stGtACR2} (**J**, -VU: 7.29 ± 0.41 s vs +VU: 15.79 ± 0.98 s, $p = 0.001$, paired t -test, $n = 5$ neurons from 3 mice) after VU application. *, $p < 0.05$; **, $p < 0.001$.



6.4 *In vivo* optogenetic measurements of the equilibrium potential of chloride

The findings of this Chapter have so far demonstrated that stGtACR2 can be used to estimate resting E_{Cl} and E_{Cl} dynamics in pyramidal neurons *in vitro*. This thesis has already demonstrated how optogenetic control of presynaptic GABA release can be used to estimate $E_{GABA_{AR}}$ *in vivo* (see **Chapter 3**). While this approach proved to be useful when performing untargeted perforated patch-clamp recordings, I was keen to investigate whether stGtACR2 could be used as part of a targeted optical method for measuring E_{Cl} in a genetically-defined neuronal population *in vivo*. The first step in this experiment was to transduce cortical pyramidal neurons with the stGtACR2 construct. I initially tried to perform the same stereotaxic viral injections as for the *in vitro* studies, following work by Jouhanneau and colleagues (2015), who successfully performed two-photon guided *in vivo* patch-clamp recordings on virally transduced layer 2/3 pyramidal neurons. However, in my experience the damage caused by the viral injections produced an inflammatory process that compromised the two-photon imaging. As an alternative, I opted to use *in utero* electroporation to express stGtACR2 in cortical pyramidal neurons (**Figure 6.3A**, for detailed protocol see **Section 2.2.4**).

Using this electroporation approach, it was possible to selectively express stGtACR2 and Tdtomato in layer 2/3 pyramidal neurons of somatosensory cortex (**Figure 6.3B**). To record from these stGtACR2-expressing neurons, two-photon guided *in vivo* patch-clamp recordings were then performed in anaesthetised juvenile mice (**Figure 6.3C and D**, see detailed protocol in **Section 2.4.3**). The Tdtomato enabled the neurons to be easily identified with two-photon microscopy using 930 nm laser light. Meanwhile Alexa594 dye was added to the internal solution to visualise the pipette using 820 nm laser light. This allowed for both whole-cell (**Figure 6.3E**) and perforated (**Figure 6.3F**) patch-clamp recordings to be performed. Using the voltage step protocol, it was possible to measure $E_{stGtACR2}$ in both whole-cell (**Figure 6.3F**, pipette solution, 4 mM Cl^-) and perforated (**Figure 6.3G**) recording modes. Under these conditions, $E_{stGtACR2}$ was found to be hyperpolarising, consistent with the *in vitro* whole-cell recordings (**Figure 6.1**) and with the *in vivo* measurements of $E_{GABA_{AR}}$ in the anaesthetised brain using the light-evoked presynaptic release (**Chapters 3 and 4**).

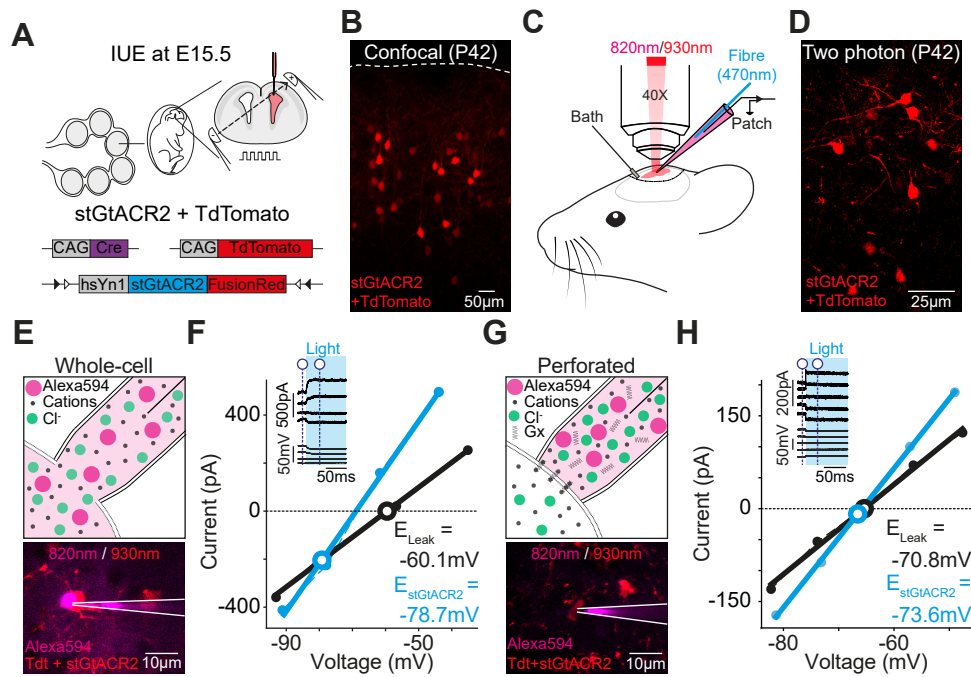


Figure 6.3: *In vivo* optogenetic measurements of equilibrium potential for chloride using stGtACR2. **A**, Schematic of *in utero* electroporation to express stGtACR2 and the fluorescent reporter, TdTomato, in mouse cortex. **B**, Confocal image of layer 2/3 pyramidal neurons (red) in primary somatosensory cortex (S1) at 6 weeks postnatal (P42). **C**, Experimental setup for two-photon targeted patch-clamp recordings. **D**, Two-photon image of TdTomato-expressing layer 2/3 pyramidal neurons. **E**, Example two-photon image during a whole-cell patch-clamp recording (pipette solution, 4 mM Cl⁻) in which the Alexa594 dye (magenta) diffuses from the pipette (white lines) into the targeted neuron. **F**, Current-voltage (IV) plot following voltage steps during which the stGtACR2 is activated with blue light (cyan, 470 nm). Inset shows electrophysiological trace with the current response and estimated voltage after series resistance correction. Measurements of the baseline and stGtACR2 currents were taken at the different voltages (dark blue lines). The intersection of the baseline current (black fitted line) with zero marks the resting membrane potential (RMP), whilst the intersection with the stGtACR2 current line (cyan fitted line) indicates the equilibrium potential for stGtACR2 ($E_{stGtACR2}$). **G**, Example two-photon image from a perforated patch-clamp recording during which the Alexa594 dye remains in the pipette. **H**, IV plot generated from voltage steps during perforated recording showing RMP and $E_{stGtACR2}$.

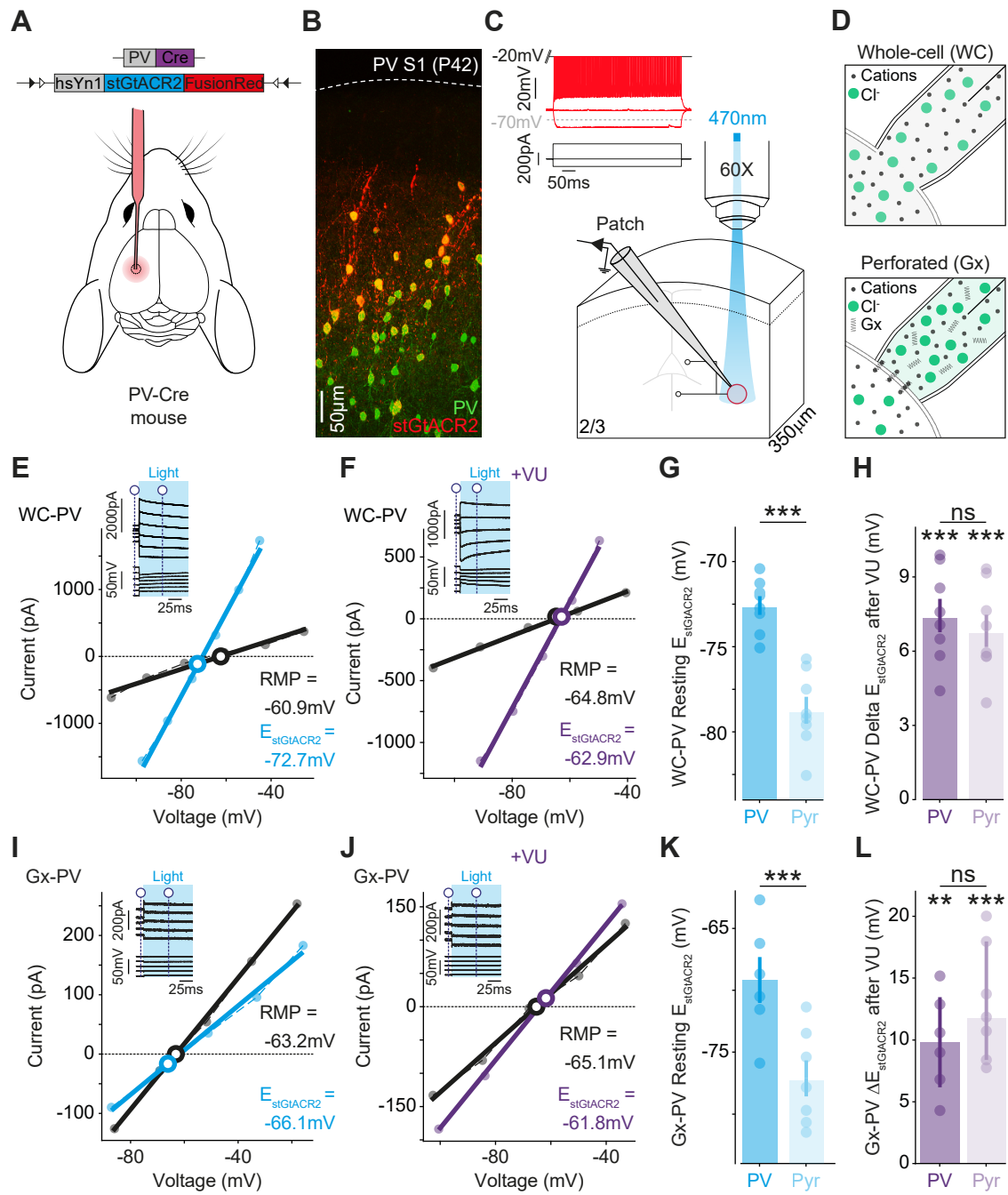
6.5 Optogenetic measurements of intracellular chloride dynamics distinguish excitatory and inhibitory neuron types

Sensitive methods for determining E_{Cl} should be able to distinguish between neurons that differ in their Cl^- handling mechanisms. For example, several reports have suggested that PV fast-spiking interneurons exhibit different chloride homeostasis to pyramidal neurons (Banke and McBain, 2006; Martina et al., 2001; Otsu et al., 2020; Sauer and Bartos, 2010; Verheugen et al., 1999; Vida et al., 2006), although there has been no consensus on the relative contribution that cation Cl^- co-transporters (CCCs) make in these different neuron types. I took advantage of the Cre-lox recombination system to express stGtACR2 in genetically-defined neuron types and investigate potential differences in resting and dynamic E_{Cl} between pyramidal neurons and PV interneurons (as discussed in **Section 6.1**). I performed stereotaxic viral injections of floxed stGtACR2 in transgenic mouse in which Cre-recombinase is only expressed in PV interneurons (**Figure 6.4A**, see detailed protocol in **Sections 2.1** and **2.2.3**). Using this method, I was able to restrict stGtACR2 expression to cortical PV interneurons (**Figure 6.4B**) and prepared acute coronal brain slices of somatosensory cortex so that I could perform patch-clamp recordings in stGtACR2-expressing PV interneurons in layer 2/3 (**Figure 6.4C**), similar to those in **Sections 6.2** and **6.3**). In these experiments I performed both whole-cell (pipette solution, 4 mM Cl^-) and gramicidin perforated patch-clamp recordings (**Figure 6.4D**). Measurements of $E_{stGtACR2}$ were made using the voltage step protocol and compared to equivalent measurements in stGtACR2-expressing pyramidal neurons. To assess the contribution of KCC2 in setting $E_{stGtACR2}$ in each neuron type, measurements were made before and after adding VU to the perfusate.

In both whole-cell and perforated patch-clamp recordings, $E_{stGtACR2}$ in PV interneurons was found to be more depolarised compared to that measured in pyramidal neurons (**Figure 6.4G** and **K**). Interestingly, under these conditions, antagonising KCC2 with VU caused a significant depolarising shift in resting $E_{stGtACR2}$ in both neuron types (**Figure 6.4F, H, J** and **L**) and there was no detectable difference in the contribution of KCC2 (**Figure 6.4H** and **L**). Together, these findings indicate that the resting E_{Cl} is different

between cortical pyramidal neurons and PV interneurons, but that the contribution of KCC2 to resting E_{Cl} is comparable between the two neuron types.

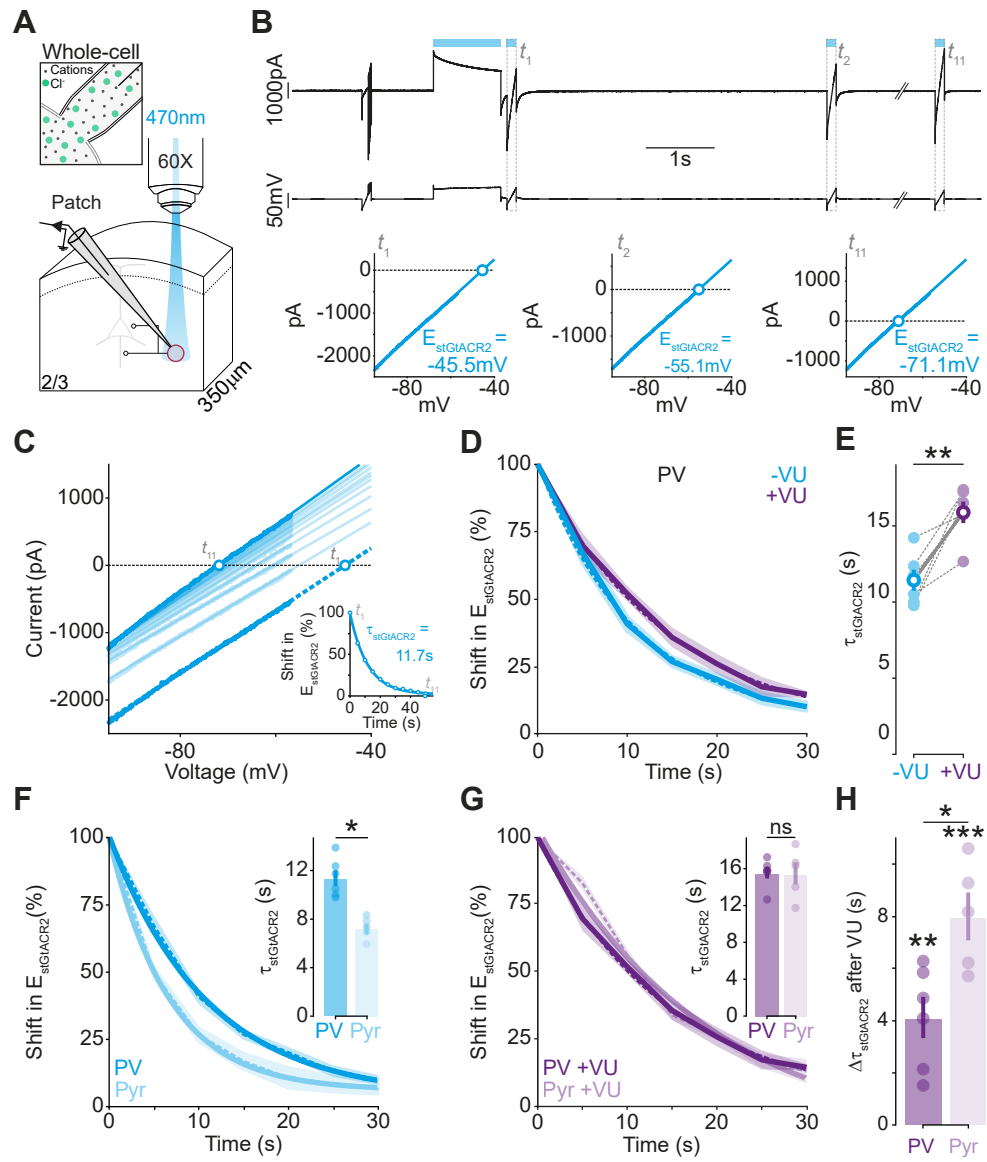
Figure 6.4 (following page): Parvalbumin interneurons exhibit depolarised optogenetic resting equilibrium potential for chloride. **A**, Homozygous mice expressing Cre recombinase in parvalbumin (PV) interneurons underwent postnatal intracerebral injections of virally packaged stGtACR2 in primary somatosensory cortex (S1). **B**, Confocal image showing selective expression of stGtACR2 (red) in layer 2/3 PV interneurons (green). **C**, *In vitro* setup in which patch-clamp recordings were performed on stGtACR2-expressing PV neurons. Inset (left corner), current-clamp recording demonstrating the action potential firing pattern expected for a fast-spiking PV interneuron after current injection. **D**, Both whole-cell (top; pipette solution, 4 mM Cl^-) and gramicidin perforated (bottom) patch-clamp recordings were performed. **E**, Current-voltage (I-V) plot generated from a whole-cell recording in which the change in stGtACR2 current (cyan) was measured across different stepped voltages to calculate the resting membrane potential (RMP) and the equilibrium potential of stGtACR2 ($E_{stGtACR2}$). **F**, IV plot from the same whole-cell recording following the application of VU (100 μ M, purple). **G**, Population data of whole-cell recordings showing more depolarised $E_{stGtACR2}$ in PV interneurons compared to pyramidal neurons (PV: -72.58 ± 1.54 mV, $n = 8$ neurons from 3 mice vs Pyr: -78.73 ± 2.24 mV, $n = 8$ neurons from 4 mice, $p < 0.0001$, *two-way ANOVA with Tukey's tests for multiple comparisons*). **H**, Population data of the change in $E_{stGtACR2}$ after KCC2 was antagonised with VU (PV: 7.44 ± 1.9 mV, $n = 8$ neurons from 3 mice vs Pyr: 6.8 ± 1.85 mV, $n = 8$ neurons from 4 mice, $p = 0.52$, *unpaired t-test*). In both PV interneurons and pyramidal neurons, there was a depolarised shift in $E_{stGtACR2}$ after VU application (PV: $p < 0.0001$, *one-sample t-test*; Pyr: $p < 0.0001$, *one-sample t-test*). The same experiment was repeated using gramicidin perforated patch-clamp recordings before and after the application of VU (**I** and **J** show example IV plots from a single PV interneuron before and after VU). **K**, Population data showing depolarised resting $E_{stGtACR2}$ in PV interneurons (PV: -69.16 ± 4.54 mV, $n = 6$ neurons from 3 mice vs Pyr: -77.12 ± 3.83 mV, $n = 7$ neurons from 5 mice, $p < 0.0001$, *two-way ANOVA with Tukey's tests for multiple comparisons*). **L**, Both neuron types showed a depolarising shift in $E_{stGtACR2}$ following VU application (PV: $p = 0.002$, *one-sample t-test*; Pyr: $p = 0.0003$, *one-sample t-test*), the difference was not significant ($p = 0.23$, *unpaired t-test*). 'ns', non-significant ($p > 0.05$); **, $p < 0.01$; ***, $p < 0.001$.



6.6 Excitatory and inhibitory cortical neurons differ in their KCC2-mediated chloride extrusion capacity

Previous work has suggested that more subtle differences in KCC2 function can be revealed when a neuron is subjected to a Cl^- load, as this reveals more of the neuron's capacity to extrude Cl^- (Doyon, Prescott and De Koninck, 2016). To test if stGtACR2 can be used to resolve more subtle cell-type differences in KCC2 activity, I compared optogenetic E_{Cl} dynamics between cortical pyramidal neurons and PV interneurons. Using the same protocol as in **Section 6.3**, I performed whole-cell patch-clamp recordings in stGtACR2-expressing PV interneurons (**Figure 6.5A**). Immediately following the loading step, E_{stGtACR2} exhibited depolarised values and gradually shifted back to hyperpolarised steady-state values over the subsequent 50 seconds (**Figure 6.5B and C**). The rate of E_{Cl} recovery following the Cl^- load was assessed by determining τ_{stGtACR2} , and compared between cell-types before (**Figure 6.5D**) and after VU (**Figure 6.5E**). This revealed that τ_{stGtACR2} was faster in pyramidal neurons than in PV interneurons (**Figure 6.5F**). Furthermore, in the presence of VU, τ_{stGtACR2} became equivalent between the two cell types (**Figure 6.5G**), with VU shifting τ_{stGtACR2} to a greater degree in the pyramidal neurons (**Figure 6.5H**).

Figure 6.5 (following page): Optogenetic equilibrium potential for chloride dynamics reveal cell-type differences in KCC2 mediated extrusion. **A**, Experimental setup in which whole-cell patch-clamp recordings (pipette solution, 4 mM Cl^-) were performed on stGtACR2-expressing parvalbumin (PV) interneurons in layer 2/3 of primary somatosensory cortex. **B**, Top panel, example trace of voltage-clamp recording (top, current recording; bottom, estimated voltage after R_s correction) measuring the changes in the equilibrium potential for stGtACR2 (E_{stGtACR2}) using voltage ramps (as described in **Figure 6.2**). Bottom panel, Current-voltage (IV) plots of the isolated stGtACR2 current (i.e. total current during stGtACR2 activation – baseline current) immediately (time point 1, t_1), at 5 s (time point 2, t_2) and at 55 s (time point 11, t_{11}) after the loading step. **C**, Summary IV plot showing the change in isolated stGtACR2 current from t_1 to t_{11} . Inset, shift in E_{stGtACR2} caused by loading step (%) over time with single exponential fitted to measure the extrusion time constant (τ_{stGtACR2}). **D**, Percentage shift in τ_{stGtACR2} in PV interneurons before (cyan) and after (purple) KCC2 was blocked with VU. **E**, Population data showing an increase in τ_{stGtACR2} after VU application in PV interneurons (-VU: 11.38 ± 0.65 s vs +VU: 15.57 ± 0.63 s, $p = 0.006$, paired t -test, $n = 6$ neurons from 3 mice). **F**, Recovery curve for PV interneurons vs pyramidal neurons (data from **Figure 6.2**) with inset showing population data (PV: 11.38 ± 0.65 s vs Pyr: 7.29 ± 0.41 s, $p = 0.01$, two-way ANOVA with Tukey's tests for multiple comparisons). **G**, Recovery curve and population data for τ_{stGtACR2} after VU application (PV: 15.57 ± 0.63 s vs Pyr: 15.79 ± 0.98 s, $p = 0.99$, two-way ANOVA with Tukey's tests for multiple comparisons). **H**, While VU caused an increase in τ_{stGtACR2} in both PV and pyramidal neurons (PV: $p = 0.004$, one-sample t -test; Pyr: $p = 0.001$, one-sample t -test), the VU-induced change in E_{stGtACR2} was greater in pyramidal neurons than PV interneurons (PV: 4.13 ± 0.79 , $n = 6$ neurons from 3 mice vs Pyr: 8.01 ± 0.92 , $n = 5$ neurons from 3 mice, $p = 0.01$, unpaired t -test). 'ns', non-significant ($p > 0.05$); *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.



6.7 Discussion

In this Chapter, I present a novel optogenetic method for studying resting and dynamic E_{Cl} using the light-activated Cl^- channel, $E_{stGtACR2}$. This new agonist-independent approach allows for Cl^- fluxes to be evoked directly in neurons of interest, thus eliminating the need to activate ligand-gated receptors within the tissue. This offers a series of advantages, including avoiding potential complications associated with activating endogenous receptors, the ability to target E_{Cl} measurements from genetically-defined neuronal cell types, and a compatibility with intact tissue preparations. To demonstrate the utility of this optogenetic approach, I show that $stGtACR2$ has an equilibrium potential ($E_{stGtACR2}$) equivalent to a known endogenous E_{Cl} channel, the $GABA_{AR}$. A key feature of this optically-controlled channel is that it can be used to actively probe $KCC2$ -mediated Cl^- extrusion by first eliciting a transient Cl^- load, and then affording sequential measurements of E_{Cl} to track recovery back to the steady-state. Whilst technically challenging, this approach can also be adapted to perform *in vivo* measurements of E_{Cl} dynamics. By harnessing the Cre-lox recombination that restricts $stGtACR2$ expression to a defined cell type, I was further able to target this approach in cortical PV interneurons and confirm reported differences in resting E_{Cl} compared to pyramidal neurons. Furthermore, I was able to show that whilst the contribution of $KCC2$ to resting E_{Cl} is comparable, measurements of dynamic E_{Cl} revealed more subtle differences in $KCC2$ -mediated E_{Cl} extrusion efficacy between the two genetically-defined cell types.

To validate the use of $E_{stGtACR2}$ as a measure of intracellular Cl^- dynamics, it was compared to $E_{GABA_{AR}}$ across a range of different set $[Cl^-]_i$ (**Figure 6.1**). The strong correlation between the $E_{stGtACR2}$ and $E_{GABA_{AR}}$ provides evidence that the $E_{stGtACR2}$ is indeed reflective of the Cl^- state of a neuron, and can therefore be used to make inferences about neuronal Cl^- dynamics. However, an important feature of $E_{GABA_{AR}}$ is that it reflects the combined equilibrium potential of both E_{Cl} , ~ -80 mV in *in vitro* slices (Ellender et al., 2014; Raimondo et al., 2013), and $E_{HCO_3^-}$, ~ -13 mV (Raimondo, Irkle, Wefelmeyer, Newey and Akerman, 2012), as shown in the GHK Equation (see **Section 1.2.1**). This is because the $E_{GABA_{AR}}$ is permeable to both Cl^- and HCO_3^- (Bormann et al., 1987; Kaila, 1994; Kaila et al., 1989; Kaila and Voipio, 1987; Kaila et al., 1993). As

stGtACR2 is thought to be a Cl^- selective channel (Govorunova et al., 2015), it was therefore surprising that E_{stGtACR2} was so similar to $E_{\text{GABA}_{\text{A}}\text{R}}$. One explanation for this finding is that stGtACR2 may also be permeable to HCO_3^- . Alternatively, another explanation could be that the contribution of HCO_3^- to the measured $E_{\text{GABA}_{\text{A}}\text{R}}$ is minimal. To test these hypotheses, E_{stGtACR2} and $E_{\text{GABA}_{\text{A}}\text{R}}$ should be compared using patch-clamp recordings in an adapted ACSF solution in which sodium bicarbonate is replaced with HEPES.

The precise temporal control of stGtACR2 activation makes it well-suited for performing sequential measurements, such that the state of E_{Cl} can be tracked following a transient Cl^- load. In these experiments, the τ_{stGtACR2} in layer 2/3 pyramidal neurons was measured to be 7.3 s, which increased to 13.8 s when KCC2 was blocked (**Figure 6.2J**). Previous *in vitro* studies across different brain preparations have made similar measurements of Cl^- recovery using different methods of inducing a Cl^- load. Staley and colleagues (1999) used applications of GABA targeted at the soma of CA1 pyramidal neurons and estimated the time constant of recovery to be 3.5 s. By contrast, Raimondo and colleagues (2012) used the light-activated Cl^- pump, halorhodopsin, to induce a load in hippocampal pyramidal neurons, and GABA applications to measure a decay constant of 14.7 s. Furthermore, Diesz and colleagues (2011) combined sharp microelectrode intracellular recordings with iontophoretic injections of Cl^- and electrically evoked inhibitory postsynaptic potentials (IPSPs) to study Cl^- extrusion kinetics. In these experiments, the authors measured a decay constant of 7.1 s, which increased to 16.7 s when furosemide, a non-selective KCC2 antagonist, was added. Finally, Jin and colleagues (2005) used voltage ramp protocols and application of muscimol, the $\text{GABA}_{\text{A}}\text{R}$ -selective agonist, to estimate a time constant of 6.7 s in layer 5 pyramidal neurons. Taken together, the average time constant measured across these four studies is 8 s, which is similar to the 7.3 s reported in this Chapter. Moreover, the closeness in the time constant estimates across preparations suggest that the effect of differences in the degree of Cl^- loading may be negligible. In support of this, Diesz and colleagues (2011) found no relationship between the magnitude of induced Cl^- load and the measured time constant.

By using the ability of stGtACR2 to measure Cl^- extrusion kinetics, I was able to identify

a difference in KCC2 function between cortical PV interneurons and pyramidal neurons. This finding extends previous work that has described a difference in the steady-state intracellular Cl^- between these cell types, with PV interneurons tending to exhibit a more depolarised $E_{\text{GABA}_A\text{R}}$ (as explained in **Section 6.1**). Importantly, the data presented in this Chapter argues that the extrusion capacity of KCC2 may indeed be different between these two cell types, which could be explained by subtle differences in transporter expression or function. While the work of Otsu and colleagues (2020) found no difference in the contribution of KCC2 to setting the steady-state intracellular Cl^- in these two cell types, they did not provide a Cl^- challenge to test co-transporter function. As argued by Doyon and colleagues (2016), more subtle differences in KCC2 function can be revealed when neurons are under a Cl^- load. In addition to identifying differences in KCC2 function between cell types, this approach has also been used to identify differences in KCC2 extrusion capacity induced by stress (Lanz et al., 2020) and epileptogenesis (Jin et al., 2005).

In interpreting these differences in Cl^- extrusion dynamics, there are other channels and transporters that need to be considered. These include the sodium-chloride-bicarbonate exchanger (NCBE), which extrudes Cl^- in exchange for moving HCO_3^- and Na^+ into the intracellular space (Grichtchenko et al., 2001; Kaila, 1994; Romero et al., 2000; Schwiening and Boron, 1994). In addition, the inward-rectifying CIC-2 channel may provide passive Cl^- extrusion during states of Cl^- load (Staley, 1994). Interestingly, Smith and colleagues (Smith et al., 1995) have shown using *in situ* hybridisation that the expression of CIC-2 mRNA was highly variable across brain regions and appeared markedly reduced in hippocampal interneurons. Whilst the expression and function of NDAE and CIC-2 in PV interneurons needs to be confirmed with modern transcriptomic techniques and functional studies, it is possible that they may contribute to the differences in Cl^- extrusion between cortical PV interneurons and pyramidal neurons.

A potential limitation of the experiments measuring Cl^- extrusion kinetics in this Chapter is that they were performed using whole-cell patch-clamp recordings. As explained in **Chapter 3**, whole-cell recordings are known to compromise the state of the transmembrane Cl^- , as the intracellular contents dialyse with the pipette solution. However, in these experiments it proved technically difficult to induce a transient Cl^-

load using perforated patch-clamp recordings. This was possibly due to R_s effects, which can limit the ability to sustain a depolarised membrane potential and increase the Cl^- driving force during the loading protocol. Furthermore, in attempting to hold the neuron at a depolarised voltage, the perforation often became compromised and broke through into the whole-cell configuration. This is consistent with what has previously been reported (Staley and Proctor, 1999). However, Raimondo and colleagues (2012) have demonstrated, in both organotypic and acute brain slices, that gramicidin perforated patch-clamp recordings can be used to induce transient Cl^- loads, which provides evidence for the technical feasibility of this approach. Future experiments may attempt to reconcile these differences in observations by attempting to replicate the findings using perforated patch-clamp recordings.

In summary, this Chapter characterises a novel optogenetic method to measure resting and dynamic E_{Cl} . By using the light-activated stGtACR2 Cl^- channel, I was able to measure E_{Cl} directly. As this approach does not depend on the use of an agonist, it avoids issues associated with receptor activation to estimate E_{Cl} . I demonstrate the versatility of this optical method by using it to measure neuronal E_{Cl} *in vivo*. Furthermore, I show that this optogenetic approach can distinguish cell type differences in E_{Cl} and has the sensitivity to reveal subtle differences in Cl^- extrusion. Taken together, these findings establish that stGtACR2 can be used effectively as a tool to accurately probe neuronal Cl^- dynamics.

Chapter 7

General Discussion

The overarching aim of this thesis was to explore how Cl^- -dependent GABA_AR synaptic signalling operates in the mammalian cortex. In this final Chapter, I will recapitulate my main experimental discoveries that have been gathered in pursuit of this aim. I will also discuss factors that need to be considered when interpreting my findings. Finally, I will extrapolate the results of my thesis into a broader context and highlight how they expand current knowledge. This will include suggestions on how the work I present in this thesis could be extended in the future.

7.1 Experimental findings

In **Chapter 3**, I presented the first *in vivo* measurements of $E_{\text{GABA}_A\text{R}}$ in cortical pyramidal neurons. To do this, I combined an established optogenetic method for evoking synaptic GABA_A ergic signalling *in vivo*, with an adapted protocol for performing gramicidin perforated patch-clamp recordings in the anaesthetised rodent cortex. Using a voltage ramp protocol, I was able to make simultaneous measurements of $E_{\text{GABA}_A\text{R}}$ and E_{Leak} , which allowed me to determine the $DF_{\text{GABA}_A\text{R}}$ and its fluctuations in real-time. By using this *in vivo* perforated patch-clamp method, I was able to confirm that KCC2 -mediated Cl^- extrusion sets $DF_{\text{GABA}_A\text{R}}$ in the cortex, whilst I found modulating extracellular impermeant anions to have a negligible effect upon the $DF_{\text{GABA}_A\text{R}}$.

In **Chapter 4**, I used this novel *in vivo* recording method to explore how GABA_AR -mediated synaptic signalling is affected by different states of cortical network

activity. Here I first characterised neuronal activity in the anaesthetised and awake cortex, which confirmed that the awake state is more synaptically active. This difference in cortical activity correlated with more depolarising $E_{\text{GABA}_{\text{A}}\text{R}}$ values and revealed that, in the awake cortex, there is a preference for shunting inhibition. By contrast, in the anaesthetised state, where cortical networks showed a lower activity profile, $\text{GABA}_{\text{A}}\text{R}$ -mediated signalling was exclusively hyperpolarising. This relationship between local network activity in the cortex and the function of $\text{GABA}_{\text{A}}\text{R}$ signalling was then confirmed by reducing local network activity by blocking glutamatergic synaptic transmission in the awake cortex and observing a hyperpolarising shift in the $\text{DF}_{\text{GABA}_{\text{A}}\text{R}}$.

In **Chapter 5**, I further explored the relationship between local cortical activity and $\text{GABA}_{\text{A}}\text{R}$ function in the awake brain. Here I manipulated the sleep-wake history of the animal in order to modify cortical activity. In mice that experienced acute sleep deprivation, I found cortical neurons to be more synaptically active, which was associated with $\text{GABA}_{\text{A}}\text{R}$ -mediated signalling becoming exclusively depolarising. To delineate the contribution of local network activity towards setting the $\text{DF}_{\text{GABA}_{\text{A}}\text{R}}$ in the sleep-deprived cortex, I reduced local network activity by blocking glutamatergic synaptic transmission. This experiment revealed that acute sleep deprivation induces a change in the set point for $\text{GABA}_{\text{A}}\text{R}$ signalling, whereas the relative contribution of local network activity to set the $\text{DF}_{\text{GABA}_{\text{A}}\text{R}}$ appears to remain relatively constant across the rested and sleep-deprived cortical states.

In **Chapter 6**, I introduced a new optical and agonist-independent method for measuring the E_{Cl} . By selectively expressing the light-activated stGtACR2 Cl^- channel in cortical neurons, I could demonstrate direct optogenetic measurements of E_{Cl} both *in vitro* and *in vivo*. The high temporal precision of this optical approach allows one to make real-time measurements of KCC2 -mediated Cl^- extrusion in genetically defined cell types. This feature allowed me to confirm that cortical PV interneurons have a higher resting E_{Cl} compared to pyramidal neurons. I then went on to show that KCC2 -mediated Cl^- extrusion is less efficient in PV interneurons, which suggests that this cell type exhibits less capacity to handle Cl^- loads.

7.2 Methodological considerations

In order to fully contextualise the results of my thesis, I first need to discuss the limitations of my methodologies. In this section, I will highlight what I believe to be the most important methodological considerations and address how they could influence the interpretation of my experimental findings.

7.2.1 Accurate control of membrane voltage

One of the unique features of patch-clamp recordings is the ability to control the membrane potential of neurons via the voltage-clamp method (Sakmann and Neher, 1984). Throughout my thesis, I use voltage-clamp recordings to allow me to estimate $E_{\text{GABA}_{\text{AR}}}$ and E_{Leak} . However, the accuracy of my measurements of these biophysical parameters relies on the ability of my perforated patch-clamp recording method to control a neuron's membrane potential, which can be impeded in various ways. First, there are errors introduced at the contact point between the recording electrode and the neuronal membrane. Any resistance at this junction will affect the ability of the patch-clamp electrode to deliver current to the neuron and therefore change the membrane potential. This resistance is referred to as access resistance or series resistance (R_s), which is how I have referred to this phenomenon throughout my thesis. In whole-cell recordings, the effect of R_s errors on voltage-clamp recordings is considered modest due to there being a large opening between the electrode and intraneuronal space, which permits the easy flow of electrical charge. However, in perforated recordings the electrical access is determined by the gramicidin cation-selective pores, which are associated with higher R_s values. For this reason, in perforated recordings, R_s errors are larger. These errors can be corrected by performing offline R_s correction (as described in **Section 2.6.1**). This includes calculating the difference between the command voltage (i.e. the voltage at which the experimenter tries to hold or 'clamp' the neuron) and the voltage loss caused by the R_s , also known as the 'voltage drop' (Traynelis, 1998). This provides an estimation of the actual voltage at which the neuron is clamped (see the example presented in **Figure 3.5**). In my recordings, I corrected for 90% of the R_s error. As the mean R_s across all of my recordings was $\sim 60 \text{ M}\Omega$, this means that only $\sim 6 \text{ M}\Omega$ of the total R_s was not corrected. Furthermore, as $E_{\text{GABA}_{\text{AR}}}$ tends to be

close to E_{Leak} , the patch-clamp amplifier would be injecting small currents at the critical point for estimating $E_{\text{GABA}_{\text{AR}}}$, and therefore the effect of R_s would be expected to be small. Consistent with these observations, I found no relationship between the estimates of $E_{\text{GABA}_{\text{AR}}}$ and R_s , which suggests that after correction, the effect of R_s is negligible.

A related source of error in voltage-clamp recordings is associated with changes in R_m , which can affect the control of the neuron's membrane potential. As stated by Ohm's law (see **Equation 1.2** in **Section 1.1.2**), when R_m is reduced, a neuron requires a greater amount of current (either injected from the patch-clamp amplifier or via synaptic inputs) to alter the neuron's membrane potential. My recordings measured significant differences in R_m , which is the reciprocal of total membrane conductance g_m , across cortical states. For example, pyramidal neurons in the awake rested cortex exhibited a mean R_m of $\sim 50 \text{ M}\Omega$, whereas in the anaesthetised state, these neurons exhibited a mean R_m of $\sim 87 \text{ M}\Omega$ (**Figure 4.1**). When local network activity was reduced in the awake cortex with NBQX, the R_m increased to $\sim 136 \text{ M}\Omega$, confirming that a lower R_m is associated with higher levels synaptic activity (**Figure 4.3**). An obvious question that arises from these observations is: how do the differences in R_m impact the estimates of $E_{\text{GABA}_{\text{AR}}}$ across the different cortical states? The effect of low R_m could affect the ability of voltage-clamp recordings to control a neuron's membrane potential (Ma et al., 2014; Zhou et al., 2009). This is because with a lower R_m , the patch-clamp amplifier would need to inject a larger amount of current to change the membrane potential. This effect of a low R_m is likely to be exaggerated when the command voltage is moved further from E_{Leak} , and therefore the current injection required increases. Whilst I did not control for this error directly, I was able to support the findings from my voltage-clamp recordings by also performing current-clamp recordings, which are not affected by the effects of command voltage. In all cases, the current-clamp recordings supported the $DF_{\text{GABA}_{\text{AR}}}$ values that were observed in the voltage-clamp recordings. For example, in neurons of the awake rested cortex where R_m is low, current-clamp recordings confirmed that evoked GABA responses are relatively depolarising (**Figure 4.2**), consistent with the voltage-clamp recordings that showed $E_{\text{GABA}_{\text{AR}}}$ tended to be above E_{Leak} . By contrast, after R_m increased following the reduction in local network activity with NBQX, the GABA responses recorded in current-clamp were exclusively hyperpolarising, consistent with the voltage-clamp measurements that showed $E_{\text{GABA}_{\text{AR}}}$

was more hyperpolarised than E_{Leak} .

Another way in which R_m may affect voltage-clamp measurements is more relevant to the ramp protocols that I used. In the ramp protocols, the command voltage was changed rapidly over time (~ 0.7 mV/ms). As R_m is directly proportional to the membrane time constant, differences in R_m would equate to differences in the time taken for the membrane to respond to current (assuming the membrane capacitance remains the same). This is less likely to occur in the voltage step protocol, as there is sufficient time (500 ms) for the membrane potential to reach a steady-state. To gauge the scale of this potential source of error, I compared $E_{\text{GABA}_{\text{AR}}}$ measurements made from the same cells using the voltage ramp and step protocols, and observed that the two correlated with one another (**Figure 3.5**). Whilst this observation supports the fact that the voltage ramp protocols provide estimates of $E_{\text{GABA}_{\text{AR}}}$, future studies could systematically quantify how R_m affects measurements made using voltage ramp protocols. One way to do this would be to compare $E_{\text{GABA}_{\text{AR}}}$ measurements made from different variations of voltage ramps, which include changing the direction (i.e. from depolarised to hyperpolarised) and voltage range of the ramp (i.e. using a narrower range that is closer to E_{Leak}). This could be complimented with computational modelling in which the relationship between the R_m , $E_{\text{GABA}_{\text{AR}}}$ and other biophysical parameters could be systematically examined.

During voltage-clamp recordings in cortical neurons, there is the additional challenge of controlling the voltage of smaller cellular compartments, such as the dendrites. This is commonly referred to as ‘space-clamp errors’ (Spruston et al., 1993; To et al., 2022; Williams and Mitchell, 2008). Neurons receive the majority of their synaptic inputs at their dendrites, with each presynaptic neuron capable of forming multiple postsynaptic contacts that can be distributed across the somatodendritic morphology (Buhl et al., 1994; Häusser et al., 2000; Redman and Walmsley, 1983; Somogyi et al., 1998). Due to the cable properties of neurons, voltage-clamp at the soma becomes less effective as one moves out into the dendritic tree (Armstrong and Gilly, 1992; Poleg-Polsky and Diamond, 2011; Rall, 2011; To et al., 2022; Velte and Miller, 1996; Williams and Mitchell, 2008). Using *in vitro* experiments, Williams and Mitchell (2008) estimate that more than 50 % of the somatic voltage-clamp is lost at the point one reaches dendrites 60 μm from the soma,

such that most measurements of synaptic currents are biased towards those received at the somatic and perisomatic compartments (Williams and Mitchell, 2008). In line with this reasoning, I used an ‘Optopatcher’ pipette holder (Katz et al., 2013), which allowed me to deliver light close to the pipette tip and soma of the recorded neuron. For these reasons, I expect that the GABA_AR-mediated conductances that I sampled are those that tend to target the soma, where voltage-clamp performance is best. In addition, there is a concordance between my measurements of membrane responses in current-clamp and the estimates of $E_{\text{GABA}_A\text{R}}$ in voltage-clamp (see above). Therefore, whilst I cannot fully exclude their effects, space-clamp errors do not represent a major concern and it is reasonable to assume that the relative effects I observe are unlikely to change.

7.2.2 Selection bias in recorded neurons

A major limitation of single-cell electrophysiological recordings is that the throughput is typically low. This is exaggerated further with *in vivo* patch-clamp recordings in awake animals, where the success rate for whole-cell recordings (i.e. the chance of a successful recording after inserting a micropipette into the cortex) is typically between 30-50 % (Jordan, 2021). For gramicidin perforated patch-clamp recordings in the awake cortex, which relies upon greater stability and good perforation, my experience is that the success rate is 5-10 %.

This low throughput means that only a select number of cortical neurons are sampled and will be used to make inferences about GABA_AR-mediated signalling within the cortical network. This is potentially problematic in the sense that there may be a selection bias towards a small number of cells in which all of the technical factors align (e.g. successful gigaseal, adequate perforation, stable recording, etc), whilst cells that do not satisfy these criteria are excluded (Rahmati et al., 2021). Cl⁻ imaging approaches are better suited to sample large numbers of cells and can therefore provide a broader overview of the resting state of intracellular Cl⁻ in the cortex (Boffi et al., 2018; Pracucci et al., 2021; Rahmati et al., 2021; Sulis Sato et al., 2017). However, for the reasons discussed in **Section 1.3.1**, Cl⁻ imaging methods are limited in their temporal resolution, they lack the sensitivity to detect GABA signalling at synapses, and they provide no information on $DF_{\text{GABA}_A\text{R}}$. Therefore, whilst I cannot exclude a potential contribution of selection bias in my electrophysiological recordings, it remains the case that the effects

that I observe across different conditions are true for the neurons that I studied.

One way to potentially improve the throughput of gramicidin perforated patch-clamp recordings is to utilise an automated approach (Allen et al., 2018; Anecchino et al., 2017; Li et al., 2017; Stoy et al., 2017). These robotic technologies have been touted to improve the yield of manual awake *in vivo* patch-clamp recordings by ~5 % (Kodandaramaiah et al., 2012) and can even be used to perform two-photon targeted recordings (Anecchino et al., 2017). To date, this technology has only been used to perform whole-cell recordings, but it is conceivable that it could be modified to perform gramicidin perforated patch-clamp recordings to probe GABAergic signalling.

7.2.3 Precision of the optogenetic approach

Throughout my thesis, I use optogenetic approaches to either evoke presynaptic GABA release (**Chapters 3-5**) or to activate Cl⁻ fluxes directly via stGtACR2 (**Chapter 6**). Using these optogenetic methods affords cell selectivity and temporal precision. However, such approaches can generate unwanted network effects that should be considered here (Lee et al., 2020; Wiegert et al., 2017), particularly in the context of activating interneurons, which can affect cortical network activity. For example, Babl and colleagues (2019) have previously demonstrated that in transgenic mice that express ChR2 in cortical interneurons, even relatively low light stimulation (< 1 mW) can elicit inhibition approximately 1 mm from an optical fibre implanted in cortex. This could be explained by lateral connectivity between adjacent cortical areas (Andrei et al., 2021; Li et al., 2019), or technical artefacts associated with the scattering of light (Yona et al., 2016). In my *in vivo* experiments, I tried to mitigate such effects by delivering light via the specialised 'Optopatcher' pipette holder, which allows for the optic fibre to be as close to the pipette tip as possible (Katz et al., 2013). Using this device, there are two main places where the light beam can scatter. The first is as the light exits the glass micropipette, and the second is as the light passes through the cortex. In the development of the Optopatcher, Katz and colleagues (2013) attempted to quantify how much scatter is caused by the micropipette and the cortex. They found that when tested in saline, the light beam emitted from the Optopatcher can extend 1 mm from pipette tip. By contrast, when the Optopatcher was placed in a brain slice, the extent of the beam is reduced to ~250 μ m (Katz et al., 2013). Whilst it is difficult to predict how the light

would scatter in a pulsating and haemodynamically active intact brain, this indicates that the Optopatcher would limit optical scatter by only delivering light at the pipette tip.

The non-specific activation of cortical interneurons within the cortex may also affect the postsynaptic response that is measured. Evoking GABA release from interneurons is expected to trigger postsynaptic GABA-mediated synaptic inhibition. However, it could also generate a polysynaptic response within cortical circuits, which might result in surrounding pyramidal neurons becoming disinhibited and, in that way, also contributing to an increase in postsynaptic excitation (Mahrach et al., 2020; Murphy and Miller, 2009; Ozeki et al., 2009; Tsodyks et al., 1997). In other words, the response measured after optogenetic stimulation could conceivably be a mixture of inhibitory and excitatory synaptic conductances.

In my experiments, I am able to confirm that the light-evoked response has a strong GABA_AR-mediated component and is not compromised by glutamatergic signalling. First, I show that by antagonising KCC2, which causes an increase in intracellular Cl⁻, I am able to modulate the driving force of the light-evoked response (**Figure 3.6**). Second, I show that even in the presence of NBQX that shuts down local excitation, I am able to reliably evoke a postsynaptic response through activating GABA-release interneurons (**Figure 4.4**). Furthermore, I observed no change in the amplitude of the light-evoked postsynaptic conductance following the addition of NBQX (**Section 4.5**), which confirms that the contribution of glutamatergic signalling to this postsynaptic response is negligible. Together, these observations establish that the responses I measured are primarily reflective of synaptic inhibition mediated through postsynaptic GABA_ARs on the recorded neuron.

In this section I have attempted to address what I believe to be the most important limitations of my experimental design. In the next section I present my interpretation of the findings and highlight how they make a contribution to current knowledge.

7.3 Implications of findings

7.3.1 Gramicidin perforated patch-clamp recordings can measure GABA-A receptor function *in vivo*

The first contribution of my thesis was to establish gramicidin perforated patch-clamp recordings *in vivo*, which allowed me to measure biophysical parameters related to GABA_AR function *in vivo*. I first demonstrate this new type of *in vivo* recording in the anaesthetised cortex (**Chapter 3**) and then go on to show its utility in studying GABA_AR function in different cortical states (**Chapters 3 and 5**). Finally, I show that this approach can be combined with multiphoton imaging to record from specific neurons in cortex (**Chapter 6**). These perforated recordings represent a new variation upon existing patch-clamp methods for single-cell recordings *in vivo* (Kitamura et al., 2008; Margrie et al., 2002, 2003; Wang et al., 2016), with the added benefit of being able to study the parameters that depend upon the neuron's native transmembrane Cl⁻ gradient.

In developing this perforated recording technique, I have been able to make the first *in vivo* measurements of DF_{GABA_AR} which, until now, have only ever been performed in *in vitro* preparations. Whilst previous imaging studies have acquired *in vivo* measurements of intracellular Cl⁻ in both the anaesthetised (Pracucci et al., 2021; Sulis Sato et al., 2017) and awake cortex (Boffi et al., 2018), the DF_{GABA_AR} *in vivo* has never been reported. This highlights an important advantage of the gramicidin perforated recordings, as they allow for simultaneous measurements of E_{GABA_AR} and E_{Leak}, which makes estimates of the DF_{GABA_AR} possible. Another advantage of the gramicidin recordings, especially when combined with the optogenetic approach to activate GABAergic signalling, is that the measurements of E_{GABA_AR} are at the activated synapse. This is not the case with Cl⁻ imaging approaches, which estimate Cl⁻ as an average over an entire compartment, such as the soma or dendrite (Rahmati et al., 2021; Sulis Sato et al., 2017).

One could argue that with the advances in imaging tools for studying the brain, which offer far superior spatial resolution and improving temporal resolution, the use of such electrophysiological methods may become obsolete. The development of genetically-encoded voltage indicators are allowing for measurements of supra- and sub-threshold neuronal activity *in vivo* with a much higher throughput than can be

achieved than with patch-clamp recordings (Abdelfattah et al., 2019; Liu et al., 2022; Piatkevich et al., 2019; Villette et al., 2019). However, the development of fluorescence resonance energy transfer (FRET)-based optical tools for measuring intracellular Cl^- *in vivo* are not yet as advanced as the voltage-indicators and suffer from additional technical challenges, such as requiring multiple lasers at different wavelengths for activation and controlling for the effects of pH (Arosio and Ratto, 2014; Bregestovski et al., 2009; Lodovichi et al., 2022). Moreover, in order to be able to study the driving force for Cl^- , there would need to be a simultaneous measurement of the membrane potential. To achieve this, one could consider an ‘all-optical’ approach similar to those that have been developed to study and probe neuronal activity *in vivo* (Packer et al., 2013, 2015; Russell et al., 2022; Zhang et al., 2018). A potential all-optical approach to measure the Cl^- driving force could include combining a voltage indicator to measure neuronal activity with an optical approach to activate Cl^- flux, like I show with stGtACR2 (Chapter 6).

7.3.2 State-dependent regulation of chloride homeostasis sets the GABA-A receptor driving force

Through establishing the ability to measure $\text{DF}_{\text{GABA}_A\text{R}}$ *in vivo*, I was led to my second major contribution, which was to demonstrate that the state of cortical network activity can directly impact GABA_AR -mediated synaptic signalling. As explained in **Section 1.2.5**, a relationship between GABA_AR conductance and neuronal Cl^- homeostasis had been predicted from modelling studies (Doyon et al., 2011) and supported by *in vitro* experiments (Yelhekar et al., 2017). Moreover, Doyon and colleagues (2016) elaborated on this prediction by suggesting that neurons under high conductance conditions *in vivo* are likely to have a higher resting Cl^- level compared to neurons in *in vitro* preparations (as illustrated in **Figure 1.9**).

The results I present in **Chapters 4 and 5** confirm this prediction, and show that, across different *in vivo* cortical states characterised by different levels of synaptic activity, the function of GABA_AR -mediated signalling can vary significantly. In the anaesthetised cortex, GABA_AR function is predominately hyperpolarising (**Figure 4.2**), whereas in the awake rested cortex, GABA_AR function is predominately shunting (**Figures 4.2 and 5.2**). Furthermore, reducing the level of local network activity by blocking excitatory

glutamatergic signalling with NBQX, I was able to delineate how much cortical activity contributes towards the set point for GABA_AR function (**Figures 4.4** and **5.4**). This was particularly useful when exploring how acute sleep deprivation affects GABAergic signalling in the awake cortex, as it allowed me to separate the contribution of activity from other sleep deprivation-induced changes in Cl⁻ homeostatic mechanisms, such as an increase in NKCC1 function (Alfonsa et al., 2021).

Due to this intrinsic link between cortical state and GABA_AR function, one can view GABAergic signalling as operating within a ‘dynamic landscape’ in which the receptor’s effects upon the neuronal membrane reflects the level of activity within the local network (illustrated in **Figure 7.1**). Furthermore, my observations may prove important for future modelling studies, which would now be able to adjust the mode of inhibition according to the cortical state that is being simulated. This would help to address the bias in the literature, which assumes that synaptic inhibition is hyperpolarising in cortex (as shown in **Figure 1.8** and **Table 1.1**).

In **Section 1.2.4**, I introduced current ideas about how different modes of GABA_AR signalling can affect neuronal computation. The results of my experimental work in this thesis further show that in the awake rested cortex, the state during which the brain is most likely to be actively engaged with the surrounding environment, there is a clear preference for shunting inhibition. This raises the question: how does shunting inhibition contribute to cortical processing in the awake brain? Whilst this question has not been answered directly, especially in relation to cortical networks, there is evidence to suggest that shunting inhibition offers key computational advantages. First, shunting inhibition has faster temporal kinetics compared to hyperpolarising inhibition (as illustrated in **Figure 1.7**), which would promote faster integration of incoming synaptic inputs (Gulledge and Stuart, 2003; Staley and Mody, 1992). Second, computational studies have shown that shunting inhibition can modulate a neuron’s sensitivity to incoming excitatory input by affecting neuronal gain modulation, especially in the presence of high levels of background synaptic activity (Ayaz and Chance, 2009; Holt and Koch, 1997; Murphy and Miller, 2003; Prescott and De Koninck, 2003; Volman et al., 2010).

Future studies may look to directly explore how shunting inhibition affects cortical

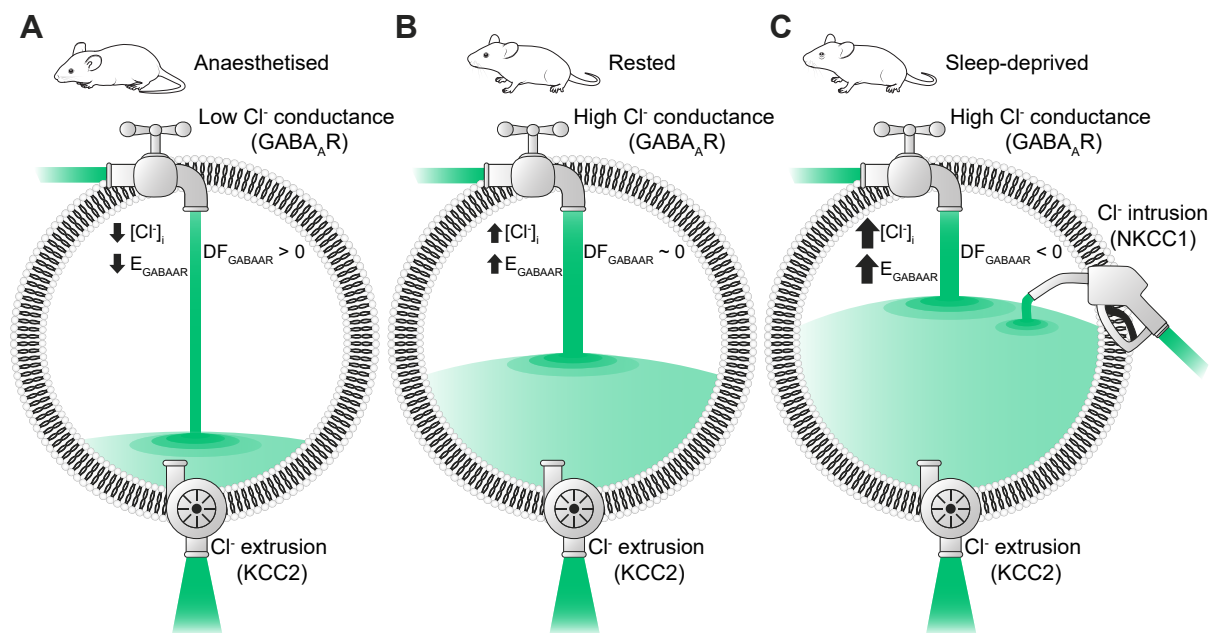


Figure 7.1: State-dependent regulation of chloride homeostasis sets the GABA driving force. **A**, In the anaesthetised cortex, low levels of synaptic activity lead to low Cl^- conductance through GABA_A receptors (GABA_ARs). In this state, intracellular concentration of chloride ($[\text{Cl}^-]_i$) is kept low by KCC2-mediated Cl^- extrusion. As the equilibrium potential for GABA ($E_{\text{GABA}_A\text{R}}$) is hyperpolarised, the driving force for GABA_AR ($\text{DF}_{\text{GABA}_A\text{R}}$) is kept above zero (see **Equation 1.6**) and, therefore, activation of the GABA_AR has a hyperpolarising effect on the neuronal membrane potential. **B**, In the rested awake cortex, high levels of synaptic inhibitory activity elevate $[\text{Cl}^-]_i$ causing a depolarising shift in $E_{\text{GABA}_A\text{R}}$. In this state, as the $\text{DF}_{\text{GABA}_A\text{R}}$ is close to zero, activation of GABA_ARs will predominately have a shunting effect on the neuronal membrane. **C**, In the sleep-deprived cortex, the upregulation of NKCC1 expression has been shown to further increase $[\text{Cl}^-]_i$ so that $E_{\text{GABA}_A\text{R}}$ is significantly more depolarised (Alfonso et al., 2021). In this state, $\text{DF}_{\text{GABA}_A\text{R}}$ is below zero and activation of GABA_AR is depolarising.

networks. This could include assessing cortical function during a specific behavioural task, whilst attempting to actively manipulate E_{Cl} within the neuronal networks that are engaged. Specifically, it would be interesting to test the effect on cortical processing if the intracellular Cl^- was lowered in cortical neurons and the mode of inhibition shifted towards being predominantly hyperpolarising in the awake cortex. Currently, there are molecular and optogenetic tools available that have been shown to reduce intracellular Cl^- . These include overexpressing KCC2 (Cheung et al., 2022; Magloire et al., 2018) or by using 'Cl-OUT', which can optically reverse the driving force by removing Cl^- from the intracellular space (Alfonsa et al., 2016). However, these experiments would be technically challenging in different ways. If one were to use a KCC2 overexpression approach, it would not be possible to show how modulating GABA_AR function affects task performance within the same animal as the KCC2 would be continuously active. It may, therefore, be difficult to determine subtle differences in cortical processing caused

by changing the mode of inhibition, versus the inherent variability in task performance between mice. By contrast, whilst the 'Cl-OUT' optogenetic approach (Alfonsa et al., 2016) would allow for greater temporal control of the manipulation and allow for within animal comparisons, it would rely on adequate expression of two opsins within a specific population of neurons. Furthermore, both of these Cl⁻ manipulations are also likely to change other intrinsic properties of the cortical neurons. For example, in order to reverse the Cl⁻ driving force, 'Cl-OUT' hyperpolarises the neuronal membrane potential. It would therefore be difficult to isolate the contribution of changing Cl⁻ to cortical processing from other parameters affecting neuronal function that are shifting at the same time. An alternative approach would be to use computational modelling in which one can simulate different cortical networks with different $E_{\text{GABA}_{\text{A}}\text{R}}$ values, whilst maintaining other parameters constant. For example, one could test how varying $E_{\text{GABA}_{\text{A}}\text{R}}$ affects the ability of a network to process or discriminate different types of stimuli.

Each of the cortical states that I have studied in this thesis has distinct activity patterns, which are reflected in single cortical neurons (**Figures 4.1, 4.3, 5.1 and 5.3**). However, what I did not explore was the fluctuations in activity that occur within each of these cortical states, which include oscillations between periods of higher activity and lower activity (as described in **Section 1.1.1**). All of my measures of synaptic activity and $E_{\text{GABA}_{\text{A}}\text{R}}$ function were made by taking an average across the period of time within each state. This approach is limited as it does not account for fluctuations in cortical activity within the cortical state. This is most obvious in the urethane-anaesthetised cortex, which oscillates between up and down states (**Figures 4.1A**). This represents an important omission, as previous *in vivo* patch-clamp studies have revealed that synaptic activity and intrinsic properties of cortical neurons vary significantly across these transient cortical up and down states (Anderson, Lampl, Reichova, Carandini and Ferster, 2000; González-Rueda et al., 2018; Haider et al., 2006; Shu et al., 2003; Waters and Helmchen, 2006). For example, Haider and colleagues (2006) have previously measured changes in total synaptic conductance during anaesthesia-induced cortical up and down states and report a ~94 % increase during up states (down state: 10.90 nS vs. up state: 21.14 nS). Excitatory synaptic conductance increased by ~66 % (down state: 5.55 nS vs. up state: 9.24 nS), whereas inhibitory synaptic conductance increased by 168

% (down state: 4.25 nS vs. up state: 11.41 nS). The significant increase in inhibitory synaptic conductances during the up states would be predicted to affect $E_{\text{GABA}_A\text{R}}$ and it is therefore conceivable that this aspect of GABA_AR -mediated signalling varies between up and down states.

The relative time spent in an up and down state is related to the depth of anaesthesia, with the duration of the down states being significantly longer in deeper anaesthetised states (Torao-Angosto et al., 2021). As I did not monitor the depth of urethane-induced anaesthesia between recording sessions, my measurements in the anaesthetised cortex likely represent an average across a range of different anaesthetic depths that consist of different proportions of up and down states. In addition to the up and down states generated by urethane, which are thought to be similar to slow-wave sleep activity seen in NREM, this anaesthetic agent has also been shown to induce REM-like activity in the cortex (Clement et al., 2008; González-Rueda et al., 2018; Mondino et al., 2022). Future experiments could look to extend my observations by using simultaneous EEG recordings to standardise the depth of urethane anaesthesia, as well as tracking how GABA_AR -function varies as the anaesthetised cortex transitions between induced REM and NREM-like activity. It would also be interesting to see whether the proportion of up and down states, reflective of different anaesthetic depths, affects GABA_AR function. My hypothesis would be that a higher proportion of up states would generate a sustained increase in inhibitory synaptic conductances, coupled with a depolarised membrane potential, which would favour depolarising $E_{\text{GABA}_A\text{R}}$.

Similarly, one could consider different arousal states as equivalent to up and down states in the awake brain (Constantinople and Bruno, 2011; Harris and Thiele, 2011; Poulet and Crochet, 2019). As the cortex becomes aroused, which can be monitored by tracking pupil diameter (McGinley et al., 2015; Nestvogel and McCormick, 2022), locomotion (McGinley et al., 2015; Nestvogel and McCormick, 2022) and whisking (Poulet and Petersen, 2008; Yamashita et al., 2013), the membrane potential depolarises, reflective of an increase in synaptic activity in the cortex. A key difference to cortical up states is that these states of increased arousal are ~ 10 fold longer in duration and can last seconds at a time (McGinley et al., 2015). *In vivo* whole-cell patch-clamp studies have also shown an increase in synaptic conductances during responses to sensory

stimuli, which are expected to occur whilst the animal is aroused, with an increase in inhibitory synaptic conductances predominating (Adesnik, 2017; Haider et al., 2013). Together, these changes during the aroused state would also be conducive towards a more depolarised $E_{\text{GABA}_A\text{R}}$.

7.3.3 Optogenetic methods offer key advantages in studying neuronal chloride dynamics

The third contribution of my thesis is to introduce a novel optogenetic approach for probing resting and dynamic chloride equilibrium potentials. By using the light-activated Cl^- channel stGtACR2, I demonstrate how this optical method meets important prerequisites for studying neuronal Cl^- , whilst offering advantages over traditional approaches. The most important of these advantages is that this optical method is agonist-independent and thereby avoids the limitations associated with relying upon GABA_AR activation (as discussed in **Section 6.1**). Using stGtACR2 also increases the specificity of E_{Cl} measurements, as it can be targeted to the cell type of interest (Mahn et al., 2018; Wiegert et al., 2017).

Another important advantage of this optogenetic method is that it offers greater temporal control of Cl^- fluxes. I demonstrate the utility of this feature by showing that stGtACR2 can measure Cl^- extrusion capacity in genetically-defined neuronal cell-types (**Figures 6.2 and 6.5**). Previous approaches to measure Cl^- extrusion have required the use of high Cl^- pipette solutions (Deisz et al., 2011; Luhmann and Prince, 1991; Misgeld et al., 1986; Otsu et al., 2020). These high Cl^- pipette solutions may further distort Cl^- sensitive second-messenger pathways, such as the With-No-Lysine kinase (WNK) signalling, which are directly involved in regulating neuronal Cl^- homeostasis (Bazúa-Valenti et al., 2015; Lüscher et al., 2020; Murillo-de Ozares et al., 2020; Valdivieso and Santa-Coloma, 2019). By contrast, stGtACR2 offers an ability to evoke transient Cl^- loads, which are less likely to affect Cl^- sensitive intracellular pathways.

Another important feature of this method is its potential utility for use in intact tissues. I show that stGtACR2 can be used to determine E_{Cl} in defined neurons of the intact brain. Whilst this was merely a demonstration, future studies could look to extend the capabilities of this technique. This could include using stGtACR2 to measure

KCC2-mediated Cl^- extrusion *in vivo*. Whilst this has been attempted using Cl^- imaging (Boffi et al., 2018), this examined steady-state differences in intracellular Cl^- following genetic knockout of KCC2. Using stGtACR2 would offer the ability to study native KCC2-mediated Cl^- extrusion in real-time with greater temporal resolution. In addition, stGtACR2 could be used to assess whether the relationship between intracellular Cl^- and local cortical network activity also exists in other cell types (as discussed in **Section 7.3.2**). In this light, a recent *in vivo* Cl^- -imaging study has suggested that Cl^- dynamics in astrocytes varies across cortical states and may directly contribute towards neuronal GABA_AR-mediated inhibition in the cortex (Untiet et al., 2022). The finding of this study further shows that Cl^- dynamics are regulated by diverse and interconnected cellular systems within the cortex. The optical method I present provides a tool to be able to further explore these cell-type specific differences in Cl^- dynamics and their contribution to cortical function.

7.3.4 Neuronal cell-types vary in their chloride set point and handling capacity

The fourth and final major contribution of my thesis highlights that intracellular Cl^- dynamics are cell-type specific. By utilising my novel optogenetic method to probe E_{Cl} with stGtACR2, I observed that between PV interneurons and pyramidal neurons there are significant differences in the E_{Cl} set point (**Figure 6.4**) and more subtle differences in the KCC2-mediated extrusion dynamics (**Figure 6.5**). The question this raises is: what is the function of these cell-specific differences? Vida and colleagues (2006) have attempted to address this question with computational modelling, where they demonstrate that more depolarised $E_{\text{GABA}_A\text{R}}$ and a bias towards shunting inhibition in hippocampal PV interneurons makes this neuronal population well-suited to generate gamma oscillations associated with memory consolidation processes. In addition, Sauer and Bartos (2010) argue that the more depolarised $E_{\text{GABA}_A\text{R}}$ in PV interneurons has a developmental role. Specifically, depolarising GABAergic signalling in PV interneurons could facilitate the recruitment of perisomatic-targeting interneurons during early development, which may aid in enhancing connectivity by depolarising pyramidal neurons, whom also have high intracellular Cl^- at this stage. As the brain matures and $E_{\text{GABA}_A\text{R}}$ becomes hyperpolarised in pyramidal neurons, PV interneurons maintain a

higher intracellular Cl^- in order to refine spike timing through shunting inhibition (Ben-Ari, 2002; Rivera et al., 1999; Sauer and Bartos, 2010). Whilst these speculations made from *in silico* and *in vitro* studies are appealing, there remains a need to test how cell-specific differences in E_{Cl} dynamics affect cortical function. The ideal experiment would be similar to the one proposed in **Section 7.3.2**, but would require the selective manipulation of intracellular Cl^- in PV interneurons, to assess what effects this has on a cortical-dependent task. The major challenge here, as mentioned above, is finding tools that are able to selectively manipulate Cl^- in specified cell types, without also compromising other parameters that underlie network function.

In addition to the steady-state difference in E_{Cl} between PV interneurons and pyramidal neurons, I discovered that KCC2-mediated Cl^- extrusion is less efficient in PV interneurons. What are the possible implications of this observation for cortical network function? By having less efficient Cl^- extrusion, PV interneurons are conceivably more vulnerable to Cl^- loads and short-term ionic plasticity in which GABAergic signalling could become excitatory (as explained in **Section 1.2.6**). This is particularly relevant in the context of seizures where GABA_AR-mediated synaptic inhibition is increased in order to ‘restrain’ the propagation of increased levels of activity (Trevelyan and Schevon, 2013; Trevelyan et al., 2006, 2007). In this context, PV interneurons may be the first to load with Cl^- , thereby causing a depolarising shift in the effect of GABAergic inputs onto these cells and increasing their excitatory drive. When this occurs, it would push the PV interneurons closer to a saturation point, known as ‘depolarisation block’, at which point their activity stops and seizure activity is allowed to propagate (Ahmed et al., 2014; Cammarota et al., 2013; Kim and Nykamp, 2017; Toyoda et al., 2015; Ziburkus et al., 2006). A recent *in vitro* study by Călin and colleagues (2021) has shown that by preventing this depolarising block in PV interneurons, it is possible to reduce the extent of seizure activity. Therefore, it is interesting to consider how boosting KCC2 extrusion in PV interneurons may prevent the accelerated activity-dependent excitatory shift in GABAergic signalling in this cell type during seizure propagation. One experiment to test this would again require the selective overexpression of KCC2 in PV interneurons and the use of an acute *in vivo* seizure model, to test whether this manipulation has any measurable impact on the propagation of seizure activity in cortex.

7.4 Concluding remarks

Through this thesis, I hope to have convinced the reader that the 'landscape' in which the GABA_AR can function in the mammalian cortex is highly dynamic. This is evident in how GABAergic signalling appears to be strongly influenced by the cortical state and cell type in which it operates. I believe these findings will have important implications for how GABAergic signalling is conceptualised and will hopefully serve as inspiration for future research into how synaptic inhibition contributes towards cortical processing.

Bibliography

- Abdelfattah, A. S., Kawashima, T., Singh, A., Novak, O., Liu, H., Shuai, Y., Huang, Y.-C., Campagnola, L., Seeman, S. C., Yu, J., Zheng, J., Grimm, J. B., Patel, R., Friedrich, J., Mensh, B. D., Paninski, L., Macklin, J. J., Murphy, G. J., Podgorski, K., Lin, B.-J., Chen, T.-W., Turner, G. C., Liu, Z., Koyama, M., Svoboda, K., Ahrens, M. B., Lavis, L. D. and Schreiter, E. R. (2019), 'Bright and photostable chemigenetic indicators for extended in vivo voltage imaging', *Science* **365**(6454), 699–704.
- Abe, Y., Furukawa, K., Itoyama, Y. and Akaike, N. (1994), 'Glycine response in acutely dissociated ventromedial hypothalamic neuron of the rat: new approach with gramicidin perforated patch-clamp technique', *Journal of Neurophysiology* **72**(4), 1530–1537.
- Adesnik, H. (2017), 'Synaptic Mechanisms of Feature Coding in the Visual Cortex of Awake Mice', *Neuron* **95**(5), 1147–1159.
- Aeschbach, D., Postolache, T. T., Sher, L., Matthews, J. R., Jackson, M. A. and Wehr, T. A. (2001), 'Evidence from the waking electroencephalogram that short sleepers live under higher homeostatic sleep pressure than long sleepers', *Neuroscience* **102**(3), 493–502.
- Agnes, E. J., Luppi, A. I. and Vogels, T. P. (2020), 'Complementary Inhibitory Weight Profiles Emerge from Plasticity and Allow Flexible Switching of Receptive Fields', *Journal of Neuroscience* **40**(50), 9634–9649.
- Ahmed, O. J., Kramer, M. A., Truccolo, W., Naftulin, J. S., Potter, N. S., Eskandar, E. N., Cosgrove, G. R., Blum, A. S., Hochberg, L. R. and Cash, S. S. (2014), 'Inhibitory single neuron control of seizures and epileptic traveling waves in humans', *BMC Neuroscience* **15**(1), 1.
- Akam, T. and Kullmann, D. M. (2010), 'Oscillations and Filtering Networks Support Flexible Routing of Information', *Neuron* **67**(2), 308–320.
- Akerman, C. J. and Cline, H. T. (2006), 'Depolarizing GABAergic Conductances Regulate the Balance of Excitation to Inhibition in the Developing Retinotectal Circuit In Vivo', *Journal of Neuroscience* **26**(19), 5117–5130.
- Albrecht, U. (2002), 'Invited Review: Regulation of mammalian circadian clock genes', *Journal of Applied Physiology* **92**(3), 1348–1355.
- Alfonsa, H., Brodersen, P., Newey, S. E., Yamagata, T., Panayi, M. C., Bannerman, D. M., Vyazovskiy, V. V. and Akerman, C. J. (2021), 'Intraneuronal chloride levels encode tiredness in cortex', *bioRxiv* pp. 1–40.
- Alfonsa, H., Lakey, J. H., Lightowlers, R. N. and Trevelyan, A. J. (2016), 'Cl-out is a novel cooperative optogenetic tool for extruding chloride from neurons', *Nature Communications* **7**(1), 1–9.
- Alfonsa, H., Merricks, E. M., Codadu, N. K., Cunningham, M. O., Deisseroth, K., Racca, C. and Trevelyan, A. J. (2015), 'The Contribution of Raised Intraneuronal Chloride to Epileptic Network Activity', *Journal of Neuroscience* **35**(20), 7715–7726.
- Alger, B. E. and Nicoll, R. A. (1979), 'GABA-mediated biphasic inhibitory responses in hippocampus', *Nature* **281**(5729), 315–317.
- Allen, B. D., Moore-Kochlacs, C., Bernstein, J. G., Kinney, J. P., Scholvin, J., Seoane, L. F., Chronopoulos, C., Lamantia, C., Kodandaramaiah, S. B., Tegmark, M. and Boyden,

- E. S. (2018), 'Automated in vivo patch-clamp evaluation of extracellular multielectrode array spike recording capability', *Journal of Neurophysiology* **120**(5), 2182–2200.
- Alvarez-Leefmans, F. J. and Delpire, E. (2009), Thermodynamics and Kinetics of Chloride Transport in Neurons, in 'Physiology and Pathology of Chloride Transporters and Channels in the Nervous System: From Molecules to Diseases', Academic Press, Cambridge, Massachusetts, pp. 81–110.
- Andersen, P., Dingledine, R., Gjerstad, L., Langmoen, I. A. and Laursen, A. M. (1980), 'Two different responses of hippocampal pyramidal cells to application of gamma-amino butyric acid.', *The Journal of Physiology* **305**(1), 279–296.
- Anderson, J., Lampl, I., Reichova, I., Carandini, M. and Ferster, D. (2000), 'Stimulus dependence of two-state fluctuations of membrane potential in cat visual cortex', *Nature Neuroscience* **3**(6), 617–621.
- Anderson, J. S., Carandini, M. and Ferster, D. (2000), 'Orientation Tuning of Input Conductance, Excitation, and Inhibition in Cat Primary Visual Cortex', *Journal of Neurophysiology* **84**(2), 909–926.
- Andrei, A. R., Debes, S., Chelaru, M., Liu, X., Rodarte, E., Spudich, J. L., Janz, R. and Dragoi, V. (2021), 'Heterogeneous side effects of cortical inactivation in behaving animals', *eLife* **10**, e66400.
- Annechino, L. A., Morris, A. R., Copeland, C. S., Agabi, O. E., Chadderton, P. and Schultz, S. R. (2017), 'Robotic Automation of In Vivo Two-Photon Targeted Whole-Cell Patch-Clamp Electrophysiology', *Neuron* **95**(5), 1048–1055.
- Armstrong, C. M. and Gilly, W. F. (1992), Access resistance and space clamp problems associated with whole-cell patch clamping, in 'Methods in Enzymology', Vol. 207 of *Ion Channels*, Academic Press, pp. 100–122.
- Arosio, D. and Ratto, G. M. (2014), 'Twenty years of fluorescence imaging of intracellular chloride', *Frontiers in Cellular Neuroscience* **8**(258), 1–12.
- Arosio, D., Ricci, F., Marchetti, L., Gualdani, R., Albertazzi, L. and Beltram, F. (2010), 'Simultaneous intracellular chloride and pH measurements using a GFP-based sensor', *Nature Methods* **7**(7), 516–518.
- Aserinsky, E. and Kleitman, N. (1953), 'Regularly Occurring Periods of Eye Motility, and Concomitant Phenomena, During Sleep', *Science* **118**(3062), 273–274.
- Aston-Jones, G. and Bloom, F. E. (1981), 'Activity of norepinephrine-containing locus coeruleus neurons in behaving rats anticipates fluctuations in the sleep-waking cycle', *Journal of Neuroscience* **1**(8), 876–886.
- Atallah, B. V., Bruns, W., Carandini, M. and Scanziani, M. (2012), 'Parvalbumin-Expressing Interneurons Linearly Transform Cortical Responses to Visual Stimuli', *Neuron* **73**(1), 159–170.
- Ayaz, A. and Chance, F. S. (2009), 'Gain Modulation of Neuronal Responses by Subtractive and Divisive Mechanisms of Inhibition', *Journal of Neurophysiology* **101**(2), 958–968.
- Ayaz, A., Stäuble, A., Hamada, M., Wulf, M.-A., Saleem, A. B. and Helmchen, F. (2019), 'Layer-specific integration of locomotion and sensory information in mouse barrel cortex', *Nature Communications* **10**(1), 2585.

- Babl, S. S., Rummell, B. P. and Sigurdsson, T. (2019), 'The Spatial Extent of Optogenetic Silencing in Transgenic Mice Expressing Channelrhodopsin in Inhibitory Interneurons', *Cell Reports* **29**(5), 1381–1395.e4.
- Bandtlow, C. E. and Zimmermann, D. R. (2000), 'Proteoglycans in the Developing Brain: New Conceptual Insights for Old Proteins', *Physiological Reviews* **80**(4), 1267–1290.
- Banke, T. G. and McBain, C. J. (2006), 'GABAergic Input onto CA3 Hippocampal Interneurons Remains Shunting throughout Development', *Journal of Neuroscience* **26**(45), 11720–11725.
- Baranyi, A., Szente, M. B. and Woody, C. D. (1993), 'Electrophysiological characterization of different types of neurons recorded in vivo in the motor cortex of the cat. II. Membrane parameters, action potentials, current-induced voltage responses and electrotonic structures', *Journal of Neurophysiology* **69**(6), 1865–1879.
- Barmashenko, G., Hefft, S., Aertsen, A., Kirschstein, T. and Köhling, R. (2011), 'Positive shifts of the GABAA receptor reversal potential due to altered chloride homeostasis is widespread after status epilepticus', *Epilepsia* **52**(9), 1570–1578.
- Barry, P. H. and Lynch, J. W. (1991), 'Liquid junction potentials and small cell effects in patch-clamp analysis', *The Journal of Membrane Biology* **121**(2), 101–117.
- Bazúa-Valenti, S., Chávez-Canales, M., Rojas-Vega, L., González-Rodríguez, X., Vázquez, N., Rodríguez-Gama, A., Argáiz, E. R., Melo, Z., Plata, C., Ellison, D. H., García-Valdés, J., Hadchouel, J. and Gamba, G. (2015), 'The Effect of WNK4 on the Sodium Chloride Cotransporter Is Modulated by Intracellular Chloride', *Journal of the American Society of Nephrology* **26**(8), 1781–1786.
- Ben-Ari, Y. (2002), 'Excitatory actions of gaba during development: the nature of the nurture', *Nature Reviews Neuroscience* **3**(9), 728–739.
- Ben-Ari, Y., Cherubini, E., Corradetti, R. and Gaiarsa, J. L. (1989), 'Giant synaptic potentials in immature rat CA3 hippocampal neurones.', *The Journal of Physiology* **416**(1), 303–325.
- Berglund, K., Schleich, W., Wang, H., Feng, G., Hall, W. C., Kuner, T. and Augustine, G. J. (2008), 'Imaging synaptic inhibition throughout the brain via genetically targeted Clomeleon', *Brain Cell Biology* **36**(1), 101–118.
- Bernander, O., Douglas, R. J., Martin, K. A. and Koch, C. (1991), 'Synaptic background activity influences spatiotemporal integration in single pyramidal cells.', *Proceedings of the National Academy of Sciences* **88**(24), 11569–11573.
- Berndt, A., Lee, S. Y., Ramakrishnan, C. and Deisseroth, K. (2014), 'Structure-Guided Transformation of Channelrhodopsin into a Light-Activated Chloride Channel', *Science* **344**(6182), 420–424.
- Berndt, A., Lee, S. Y., Wietek, J., Ramakrishnan, C., Steinberg, E. E., Rashid, A. J., Kim, H., Park, S., Santoro, A., Frankland, P. W., Iyer, S. M., Pak, S., Åhrlund Richter, S., Delp, S. L., Malenka, R. C., Josselyn, S. A., Carlén, M., Hegemann, P. and Deisseroth, K. (2016), 'Structural foundations of optogenetics: Determinants of channelrhodopsin ion selectivity', *Proceedings of the National Academy of Sciences* **113**(4), 822–829.
- Bettler, B., Kaupmann, K., Mosbacher, J. and Gassmann, M. (2004), 'Molecular Structure and Physiological Functions of GABAB Receptors', *Physiological Reviews* **84**(3), 835–867.

- Bharioke, A., Munz, M., Brignall, A., Kosche, G., Eizinger, M. F., Ledergerber, N., Hillier, D., Gross-Scherf, B., Conzelmann, K.-K., Macé, E. and Roska, B. (2022), 'General anesthesia globally synchronizes activity selectively in layer 5 cortical pyramidal neurons', *Neuron* **110**(12), 2024–2040.
- Bindman, L. J., Meyer, T. and Prince, C. A. (1988), 'Comparison of the electrical properties of neocortical neurones in slices in vitro and in the anaesthetized rat', *Experimental Brain Research* **69**(3), 489–496.
- Bitzenhofer, S. H., Ahlbeck, J. and Hanganu-Opatz, I. L. (2017), 'Methodological Approach for Optogenetic Manipulation of Neonatal Neuronal Networks', *Frontiers in Cellular Neuroscience* **11**, 1–13.
- Blaesse, P., Airaksinen, M. S., Rivera, C. and Kaila, K. (2009), 'Cation-Chloride Cotransporters and Neuronal Function', *Neuron* **61**(6), 820–838.
- Boffi, J. C., Knabbe, J., Kaiser, M. and Kuner, T. (2018), 'KCC2-dependent Steady-state Intracellular Chloride Concentration and pH in Cortical Layer 2/3 Neurons of Anesthetized and Awake Mice', *Frontiers in Cellular Neuroscience* **12**, 1–14.
- Borbély, A. A. (1982), 'A two process model of sleep regulation', *Human Neurobiology* **1**(3), 195–204.
- Borbély, A. A., Daan, S., Wirz-Justice, A. and Deboer, T. (2016), 'The two-process model of sleep regulation: a reappraisal', *Journal of Sleep Research* **25**(2), 131–143.
- Borg-Graham, L. J., Monier, C. and Frégnac, Y. (1998), 'Visual input evokes transient and strong shunting inhibition in visual cortical neurons', *Nature* **393**(6683), 369–373.
- Bormann, J. (2000), 'The 'ABC' of GABA receptors', *Trends in Pharmacological Sciences* **21**(1), 16–19.
- Bormann, J., Hamill, O. P. and Sakmann, B. (1987), 'Mechanism of anion permeation through channels gated by glycine and gamma-aminobutyric acid in mouse cultured spinal neurones.', *The Journal of Physiology* **385**(1), 243–286.
- Bos, R., Sadlaoud, K., Boulenguez, P., Buttigieg, D., Liabeuf, S., Brocard, C., Haase, G., Bras, H. and Vinay, L. (2013), 'Activation of 5-HT_{2A} receptors upregulates the function of the neuronal K-Cl cotransporter KCC2', *Proceedings of the National Academy of Sciences* **110**(1), 348–353.
- Boyden, E. S., Zhang, F., Bamberg, E., Nagel, G. and Deisseroth, K. (2005), 'Millisecond-timescale, genetically targeted optical control of neural activity', *Nature Neuroscience* **8**(9), 1263–1268.
- Bregestovski, P., Waseem, T. and Mukhtarov, M. (2009), 'Genetically encoded optical sensors for monitoring of intracellular chloride and chloride-selective channel activity', *Frontiers in Molecular Neuroscience* **2**, 1–13.
- Brickley, S. G., Cull-Candy, S. G. and Farrant, M. (1999), 'Single-channel properties of synaptic and extrasynaptic GABA_A receptors suggest differential targeting of receptor subtypes', *The Journal of Neuroscience* **19**(8), 2960–2973.
- Briz, C. G., Navarrete, M., Esteban, J. A. and Nieto, M. (2017), 'In Utero Electroporation Approaches to Study the Excitability of Neuronal Subpopulations and Single-cell Connectivity', *Journal of Visualized Experiments* **120**, e55139.
- Brown, E. N., Lydic, R. and Schiff, N. D. (2010), 'General Anesthesia, Sleep, and Coma', *New England Journal of Medicine* **363**(27), 2638–2650.

- Brown, R. E., Basheer, R., McKenna, J. T., Strecker, R. E. and McCarley, R. W. (2012), 'Control of Sleep and Wakefulness', *Physiological Reviews* **92**(3), 1087–1187.
- Buhl, E. H., Halasy, K. and Somogyi, P. (1994), 'Diverse sources of hippocampal unitary inhibitory postsynaptic potentials and the number of synaptic release sites', *Nature* **368**(6474), 823–828.
- Bukoski, A., Steyn-Ross, D. A., Pickett, A. F. and Steyn-Ross, M. L. (2018), 'Anesthesia modifies subthreshold critical slowing down in a stochastic Hodgkin-Huxley-like model with inhibitory synaptic input', *Physical Review E* **97**(6), 062403.
- Burman, R. J., Raimondo, J. V., Jefferys, J. G. R., Sen, A. and Akerman, C. J. (2020), 'The transition to status epilepticus: how the brain meets the demands of perpetual seizure activity', *Seizure* **75**(1), 137–144.
- Burman, R. J., Rosch, R. E., Wilmshurst, J. M., Sen, A., Ramantani, G., Akerman, C. J. and Raimondo, J. V. (2022), 'Why won't it stop? The dynamics of benzodiazepine resistance in status epilepticus', *Nature Reviews Neurology* **18**(7), 1–14.
- Burman, R. J., Selfe, J. S., Lee, J. H., van den Berg, M., Calin, A., Codadu, N. K., Wright, R., Newey, S. E., Parrish, R. R., Katz, A. A., Wilmshurst, J. M., Akerman, C. J., Trevelyan, A. J. and Raimondo, J. V. (2019), 'Excitatory GABAergic signalling is associated with benzodiazepine resistance in status epilepticus', *Brain* **142**(11), 3482–3501.
- Cajal, S. R. y. (1911), *Histologie du système nerveux de l'homme et des vertébrés*, Vol. 2, A. Maloine (Wellcome Collection), Paris.
- Callaway, E. M. (1998), 'Local circuits in primary visual cortex of the macaque monkey', *Annual Review of Neuroscience* **21**, 47–74.
- Callaway, E. M. (2004), 'Feedforward, feedback and inhibitory connections in primate visual cortex', *Neural Networks* **17**(5), 625–632.
- Cammarota, M., Losi, G., Chiavegato, A., Zonta, M. and Carmignoto, G. (2013), 'Fast spiking interneuron control of seizure propagation in a cortical slice model of focal epilepsy', *The Journal of Physiology* **591**(4), 807–822.
- Cardin, J. A. (2018), 'Inhibitory Interneurons Regulate Temporal Precision and Correlations in Cortical Circuits', *Trends in Neurosciences* **41**(10), 689–700.
- Cardin, J. A., Carlén, M., Meletis, K., Knoblich, U., Zhang, F., Deisseroth, K., Tsai, L.-H. and Moore, C. I. (2009), 'Driving fast-spiking cells induces gamma rhythm and controls sensory responses', *Nature* **459**(7247), 663–667.
- Caspers, H. and Speckmann, E.-J. (1972), 'Cerebral pO₂, pCO₂ and pH: Changes During Convulsive Activity and their Significance for Spontaneous Arrest of Seizures', *Epilepsia* **13**(5), 699–725.
- Chance, F. S., Abbott, L. F. and Reyes, A. D. (2002), 'Gain Modulation from Background Synaptic Input', *Neuron* **35**(4), 773–782.
- Cheung, D. L., Cooke, M. J., Goulton, C. S., Chaichim, C., Cheung, L. F., Khoshaba, A., Nabekura, J. and Moorhouse, A. J. (2022), 'Global transgenic upregulation of KCC2 confers enhanced diazepam efficacy in treating sustained seizures', *Epilepsia* **63**(1), e15–e22.
- Choi, H. J., Lee, C. J., Schroeder, A., Kim, Y. S., Jung, S. H., Kim, J. S., Kim, D. Y., Son, E. J., Han, H. C., Hong, S. K., Colwell, C. S. and Kim, Y. I. (2008), 'Excitatory Actions of GABA in the Suprachiasmatic Nucleus', *Journal of Neuroscience* **28**(21), 5450–5459.

- Clement, E. A., Richard, A., Thwaites, M., Ailon, J., Peters, S. and Dickson, C. T. (2008), 'Cyclic and Sleep-Like Spontaneous Alternations of Brain State Under Urethane Anaesthesia', *PLOS ONE* **3**(4), e2004.
- Constanti, A. and Nistri, A. (1976), 'A comparative study of the action of gamma-aminobutyric acid and piperazine on the lobster muscle fibre and the frog spinal cord', *British Journal of Pharmacology* **57**(3), 347–358.
- Constantinople, C. M. and Bruno, R. M. (2011), 'Effects and Mechanisms of Wakefulness on Local Cortical Networks', *Neuron* **69**(6), 1061–1068.
- Coull, J. A. M., Beggs, S., Boudreau, D., Boivin, D., Tsuda, M., Inoue, K., Gravel, C., Salter, M. W. and De Koninck, Y. (2005), 'BDNF from microglia causes the shift in neuronal anion gradient underlying neuropathic pain', *Nature* **438**(7070), 1017–1021.
- Crochet, S. and Petersen, C. C. H. (2006), 'Correlating whisker behavior with membrane potential in barrel cortex of awake mice', *Nature Neuroscience* **9**(5), 608–610.
- Crunelli, V. and Hughes, S. W. (2010), 'The slow (1 Hz) rhythm of non-REM sleep: a dialogue between three cardinal oscillators', *Nature Neuroscience* **13**(1), 9–17.
- Cunningham, M. D. and Enna, S. J. (1996), 'Evidence for pharmacologically distinct GABAB receptors associated with cAMP production in rat brain', *Brain Research* **720**(1), 220–224.
- Currin, C. B. and Raimondo, J. V. (2021), 'Computational models reveal how chloride dynamics determine the optimal distribution of inhibitory synapses to minimise dendritic excitability', *PLOS Computational Biology* **18**(9), 1–32.
- Currin, C. B., Trevelyan, A. J., Akerman, C. J. and Raimondo, J. V. (2020), 'Chloride dynamics alter the input-output properties of neurons', *PLOS Computational Biology* **16**(5), e1007932.
- Călin, A., Ilie, A. S. and Akerman, C. J. (2021), 'Disrupting Epileptiform Activity by Preventing Parvalbumin Interneuron Depolarization Block', *Journal of Neuroscience* **41**(45), 9452–9465.
- Dash, M. B., Douglas, C. L., Vyazovskiy, V. V., Cirelli, C. and Tononi, G. (2009), 'Long-Term Homeostasis of Extracellular Glutamate in the Rat Cerebral Cortex across Sleep and Waking States', *Journal of Neuroscience* **29**(3), 620–629.
- Davies, C. H., Davies, S. N. and Collingridge, G. L. (1990), 'Paired-pulse depression of monosynaptic GABA-mediated inhibitory postsynaptic responses in rat hippocampus', *The Journal of Physiology* **424**(1), 513–531.
- De Jeu, M. and Pennartz, C. (2002), 'Circadian Modulation of GABA Function in the Rat Suprachiasmatic Nucleus: Excitatory Effects During the Night Phase', *Journal of Neurophysiology* **87**(2), 834–844.
- Deisz, R. A., Lehmann, T.-N., Horn, P., Dehnicke, C. and Nitsch, R. (2011), 'Components of neuronal chloride transport in rat and human neocortex: Neuronal chloride transport in rat and human cortex', *The Journal of Physiology* **589**(6), 1317–1347.
- Delpire, E., Baranczak, A., Waterson, A. G., Kim, K., Kett, N., Morrison, R. D., Scott Daniels, J., David Weaver, C. and Lindsley, C. W. (2012), 'Further optimization of the K-Cl cotransporter KCC2 antagonist ML077: Development of a highly selective and more potent in vitro probe', *Bioorganic & Medicinal Chemistry Letters* **22**(14), 4532–4535.

- Delpire, E. and Staley, K. J. (2014), 'Novel determinants of the neuronal chloride concentration', *The Journal of Physiology* **592**(19), 4099–4114.
- Destexhe, A. (1997), 'Conductance-Based Integrate-and-Fire Models', *Neural Computation* **9**(3), 503–514.
- Destexhe, A., Contreras, D. and Steriade, M. (1999), 'Spatiotemporal Analysis of Local Field Potentials and Unit Discharges in Cat Cerebral Cortex during Natural Wake and Sleep States', *Journal of Neuroscience* **19**(11), 4595–4608.
- Destexhe, A. and Paré, D. (1999), 'Impact of Network Activity on the Integrative Properties of Neocortical Pyramidal Neurons In Vivo', *Journal of Neurophysiology* **81**(4), 1531–1547.
- Destexhe, A., Rudolph, M. and Paré, D. (2003), 'The high-conductance state of neocortical neurons in vivo', *Nature Reviews Neuroscience* **4**(9), 739–751.
- Dijk, D. J., Beersma, D. G. M. and Daan, S. (1987), 'EEG Power Density during Nap Sleep: Reflection of an Hourglass Measuring the Duration of Prior Wakefulness', *Journal of Biological Rhythms* **2**(3), 207–219.
- Ding, F., O'Donnell, J., Xu, Q., Kang, N., Goldman, N. and Nedergaard, M. (2016), 'Changes in the composition of brain interstitial ions control the sleep-wake cycle', *Science* **352**(6285), 550–555.
- Donnan, F. G. (1995), 'Theory of membrane equilibria and membrane potentials in the presence of non-dialysing electrolytes. A contribution to physical-chemical physiology', *Journal of Membrane Science* **100**(1), 45–55.
- Douglas, R. J. and Martin, K. A. C. (2004), 'Neuronal circuits of the neocortex', *Annual Review of Neuroscience* **27**, 419–451.
- Douglas, R. J. and Martin, K. A. C. (2007), 'Mapping the Matrix: The Ways of Neocortex', *Neuron* **56**(2), 226–238.
- Doyon, N., Prescott, S. A., Castonguay, A., Godin, A. G., Kröger, H. and Koninck, Y. D. (2011), 'Efficacy of Synaptic Inhibition Depends on Multiple, Dynamically Interacting Mechanisms Implicated in Chloride Homeostasis', *PLOS Computational Biology* **7**(9), e1002149.
- Doyon, N., Prescott, S. A. and De Koninck, Y. (2016), 'Mild KCC2 Hypofunction Causes Inconspicuous Chloride Dysregulation that Degrades Neural Coding', *Frontiers in Cellular Neuroscience* **9**, 1–16.
- Doyon, N., Vinay, L., Prescott, S. A. and De Koninck, Y. (2016), 'Chloride Regulation: A Dynamic Equilibrium Crucial for Synaptic Inhibition', *Neuron* **89**(6), 1157–1172.
- Dubanet, O., Ferreira Gomes Da Silva, A., Frick, A., Hirase, H., Beyeler, A. and Leinekugel, X. (2021), 'Probing the polarity of spontaneous perisomatic GABAergic synaptic transmission in the mouse CA3 circuit in vivo', *Cell Reports* **36**(2), 109381.
- Dzhala, V. I., Kuchibhotla, K. V., Glykys, J. C., Kahle, K. T., Swiercz, W. B., Feng, G., Kuner, T., Augustine, G. J., Bacskai, B. J. and Staley, K. J. (2010), 'Progressive NKCC1-Dependent Neuronal Chloride Accumulation during Neonatal Seizures', *Journal of Neuroscience* **30**(35), 11745–11761.
- Dzhala, V., Valeeva, G., Glykys, J., Khazipov, R. and Staley, K. (2012), 'Traumatic Alterations in GABA Signaling Disrupt Hippocampal Network Activity in the Developing Brain', *Journal of Neuroscience* **32**(12), 4017–4031.

- Düsterwald, K. M., Currin, C. B., Burman, R. J., Akerman, C. J., Kay, A. R. and Raimondo, J. V. (2018), 'Biophysical models reveal the relative importance of transporter proteins and impermeant anions in chloride homeostasis', *eLife* **7**, e39575.
- Eban-Rothschild, A., Appelbaum, L. and de Lecea, L. (2018), 'Neuronal Mechanisms for Sleep/Wake Regulation and Modulatory Drive', *Neuropsychopharmacology* **43**(5), 937–952.
- Ebihara, S., Shirato, K., Harata, N. and Akaike, N. (1995), 'Gramicidin-perforated patch recording: GABA response in mammalian neurones with intact intracellular chloride.', *The Journal of Physiology* **484**(1), 77–86.
- Eccles, J. C. (1961), 'The Ferrier Lecture: The nature of central inhibition', *Proceedings of the Royal Society of London. Series B. Biological Sciences* **153**(953), 445–476.
- Ellender, T. J., Raimondo, J. V., Irkle, A., Lamsa, K. P. and Akerman, C. J. (2014), 'Excitatory Effects of Parvalbumin-Expressing Interneurons Maintain Hippocampal Epileptiform Activity via Synchronous Afterdischarges', *Journal of Neuroscience* **34**(46), 15208–15222.
- Engel, A. K. and Singer, W. (2001), 'Temporal binding and the neural correlates of sensory awareness', *Trends in Cognitive Sciences* **5**(1), 16–25.
- Farrant, M. and Kaila, K. (2007), The cellular, molecular and ionic basis of GABAA receptor signalling, in J. M. Tepper, E. D. Abercrombie and J. P. Bolam, eds, 'Progress in Brain Research', Vol. 160 of *Gaba and the Basal Ganglia*, Elsevier, pp. 59–87.
- Farrant, M. and Nusser, Z. (2005), 'Variations on an inhibitory theme: phasic and tonic activation of GABAA receptors', *Nature Reviews Neuroscience* **6**(3), 215–229.
- Felleman, D. J. and Van Essen, D. C. (1991), 'Distributed Hierarchical Processing in the Primate Cerebral Cortex', *Cerebral Cortex* **1**(1), 1–47.
- Feng, H.-J., Mathews, G. C., Kao, C. and Macdonald, R. L. (2008), 'Alterations of GABAA-Receptor Function and Allosteric Modulation During Development of Status Epilepticus', *Journal of Neurophysiology* **99**(3), 1285–1293.
- Ferguson, K. A. and Cardin, J. A. (2020), 'Mechanisms underlying gain modulation in the cortex', *Nature Reviews Neuroscience* **21**(2), 80–92.
- Fernandez, F. R., Rahsepar, B. and White, J. A. (2018), 'Differences in the Electrophysiological Properties of Mouse Somatosensory Layer 2/3 Neurons *In Vivo* and Slice Stem from Intrinsic Sources Rather than a Network-Generated High Conductance State', *eneuro* **5**(2), 1–18.
- Fernandez, L. M. J., Comte, J.-C., Le Merre, P., Lin, J.-S., Salin, P.-A. and Crochet, S. (2017), 'Highly Dynamic Spatiotemporal Organization of Low-Frequency Activities During Behavioral States in the Mouse Cerebral Cortex', *Cerebral Cortex (New York, N.Y.: 1991)* **27**(12), 5444–5462.
- Fisher, S. P., Cui, N., McKillop, L. E., Gemignani, J., Bannerman, D. M., Oliver, P. L., Peirson, S. N. and Vyazovskiy, V. V. (2016), 'Stereotypic wheel running decreases cortical activity in mice', *Nature Communications* **7**(1), 13138.
- Fiumelli, H. and Woodin, M. A. (2007), 'Role of activity-dependent regulation of neuronal chloride homeostasis in development', *Current Opinion in Neurobiology* **17**(1), 81–86.
- Ford, A., Castonguay, A., Cottet, M., Little, J., Chen, Z., Symons-Liguori, A., Doyle, T., Egan, T., Vanderah, T., De Koninck, Y., Tosh, D., Jacobson, K. and Salvemini, D. (2015),

- 'Engagement of the GABA to KCC2 signaling pathway contributes to the analgesic effects of A3AR agonists in neuropathic pain', *Journal of Neuroscience* **35**(15), 6057–6067.
- Franks, N. P. (2008), 'General anaesthesia: from molecular targets to neuronal pathways of sleep and arousal', *Nature Reviews Neuroscience* **9**(5), 370–386.
- Frosch, M. P., Lipton, S. A. and Dichter, M. A. (1992), 'Desensitization of GABA-activated currents and channels in cultured cortical neurons', *Journal of Neuroscience* **12**(8), 3042–3053.
- Fujiwara-Tsukamoto, Y., Isomura, Y., Imanishi, M., Ninomiya, T., Tsukada, M., Yanagawa, Y., Fukai, T. and Takada, M. (2010), 'Prototypic Seizure Activity Driven by Mature Hippocampal Fast-Spiking Interneurons', *Journal of Neuroscience* **30**(41), 13679–13689.
- Gabbiani, F., Midtgaard, J. and Knopfel, T. (1994), 'Synaptic integration in a model of cerebellar granule cells', *Journal of Neurophysiology* **72**(2), 999–1009.
- Galarreta, M. and Hestrin, S. (1998), 'Frequency-dependent synaptic depression and the balance of excitation and inhibition in the neocortex', *Nature Neuroscience* **1**(7), 587–594.
- Garduno, J., Elenes, S., Cebada, J., Becerra, E. and Garcia, U. (2002), 'Expression and Functional Characterization of GABA Transporters in Crayfish Neurosecretory Cells', *Journal of Neuroscience* **22**(21), 9176–9184.
- Glykys, J., Dzhalal, V., Egawa, K., Balena, T., Saponjian, Y., Kuchibhotla, K. V., Bacskai, B. J., Kahle, K. T., Zeuthen, T. and Staley, K. J. (2014), 'Local Impermeant Anions Establish the Neuronal Chloride Concentration', *Science* **343**(6171), 670–675.
- Glykys, J., Dzhalal, V., Egawa, K., Kahle, K. T., Delpire, E. and Staley, K. (2017), 'Chloride Dysregulation, Seizures, and Cerebral Edema: A Relationship with Therapeutic Potential', *Trends in Neurosciences* **40**(5), 276–294.
- Glykys, J. and Staley, K. J. (2016), 'Developmental Decrease of Neuronal Chloride Concentration Is Independent of Trauma in Thalamocortical Brain Slices', *PLOS ONE* **11**(6), e0158012.
- Gonzalez-Burgos, G., Fish, K. N. and Lewis, D. A. (2011), 'GABA Neuron Alterations, Cortical Circuit Dysfunction and Cognitive Deficits in Schizophrenia', *Neural Plasticity* **2011**, e723184.
- González-Rueda, A., Pedrosa, V., Feord, R. C., Clopath, C. and Paulsen, O. (2018), 'Activity-Dependent Downscaling of Subthreshold Synaptic Inputs during Slow-Wave-Sleep-like Activity In Vivo', *Neuron* **97**(6), 1244–1252.e5.
- Goodkin, H. P. (2005), 'Status Epilepticus Increases the Intracellular Accumulation of GABAA Receptors', *Journal of Neuroscience* **25**(23), 5511–5520.
- Goodkin, H. P., Joshi, S., Mtchedlishvili, Z., Brar, J. and Kapur, J. (2008), 'Subunit-Specific Trafficking of GABAA Receptors during Status Epilepticus', *Journal of Neuroscience* **28**(10), 2527–2538.
- Govorunova, E. G., Sineshchekov, O. A., Janz, R., Liu, X. and Spudich, J. L. (2015), 'Natural light-gated anion channels: A family of microbial rhodopsins for advanced optogenetics', *Science* **349**(6248), 647–650.
- Govorunova, E. G., Sineshchekov, O. A. and Spudich, J. L. (2016), 'Proteomonas sulcata ACR1: A Fast Anion Channelrhodopsin', *Photochemistry and Photobiology* **92**(2), 257–263.

- Gradinaru, V., Zhang, F., Ramakrishnan, C., Mattis, J., Prakash, R., Diester, I., Goshen, I., Thompson, K. R. and Deisseroth, K. (2010), 'Molecular and Cellular Approaches for Diversifying and Extending Optogenetics', *Cell* **141**(1), 154–165.
- Gribkoff, V. K., Pieschl, R. L. and Dudek, F. E. (2003), 'GABA Receptor-Mediated Inhibition of Neuronal Activity in Rat SCN In Vitro: Pharmacology and Influence of Circadian Phase', *Journal of Neurophysiology* **90**(3), 1438–1448.
- Gribkoff, V. K., Pieschl, R. L., Wisialowski, T. A., Park, W. K., Strecker, G. J., de Jeu, M. T. G., Pennartz, C. M. A. and Dudek, F. E. (1999), 'A Reexamination of the Role of GABA in the Mammalian Suprachiasmatic Nucleus', *Journal of Biological Rhythms* **14**(2), 126–130.
- Grichtchenko, I. I., Choi, I., Zhong, X., Bray-Ward, P., Russell, J. M. and Boron, W. F. (2001), 'Cloning, Characterization, and Chromosomal Mapping of a Human Electroneutral Na⁺-driven Cl-HCO₃Exchanger*', *Journal of Biological Chemistry* **276**(11), 8358–8363.
- Grienberger, C., Milstein, A. D., Bittner, K. C., Romani, S. and Magee, J. C. (2017), 'Inhibitory suppression of heterogeneously tuned excitation enhances spatial coding in CA1 place cells', *Nature Neuroscience* **20**(3), 417–426.
- Grimley, J. S., Li, L., Wang, W., Wen, L., Beese, L. S., Hellinga, H. W. and Augustine, G. J. (2013), 'Visualization of Synaptic Inhibition with an Optogenetic Sensor Developed by Cell-Free Protein Engineering Automation', *Journal of Neuroscience* **33**(41), 16297–16309.
- Guet-McCreight, A. and Skinner, F. K. (2019), 'Using computational models to predict in vivo synaptic inputs to interneuron specific 3 (IS3) cells of CA1 hippocampus that also allow their recruitment during rhythmic states', *PLOS ONE* **14**(1), e0209429.
- Gulledge, A. T. and Stuart, G. J. (2003), 'Excitatory Actions of GABA in the Cortex', *Neuron* **37**(2), 299–309.
- Gulácsi, A., Lee, C. R., Sík, A., Viitanen, T., Kaila, K., Tepper, J. M. and Freund, T. F. (2003), 'Cell Type-Specific Differences in Chloride-Regulatory Mechanisms and GABAA Receptor-Mediated Inhibition in Rat Substantia Nigra', *Journal of Neuroscience* **23**(23), 8237–8246.
- Haider, B., Duque, A., Hasenstaub, A. R. and McCormick, D. A. (2006), 'Neocortical Network Activity In Vivo Is Generated through a Dynamic Balance of Excitation and Inhibition', *Journal of Neuroscience* **26**(17), 4535–4545.
- Haider, B., Häusser, M. and Carandini, M. (2013), 'Inhibition dominates sensory responses in the awake cortex', *Nature* **493**(7430), 97–100.
- Haider, B. and McCormick, D. A. (2009), 'Rapid Neocortical Dynamics: Cellular and Network Mechanisms', *Neuron* **62**(2), 171–189.
- Harris, J. J., Jolivet, R. and Attwell, D. (2012), 'Synaptic Energy Use and Supply', *Neuron* **75**(5), 762–777.
- Harris, K. D. and Shepherd, G. M. G. (2015), 'The neocortical circuit: themes and variations', *Nature Neuroscience* **18**(2), 170–181.
- Harris, K. D. and Thiele, A. (2011), 'Cortical state and attention', *Nature Reviews Neuroscience* **12**(9), 509–523.
- Herstel, L. J. and Wierenga, C. J. (2021), 'Network control through coordinated inhibition', *Current Opinion in Neurobiology* **67**, 34–41.

- Hewitt, S. A., Wamsteeker, J. I., Kurz, E. U. and Bains, J. S. (2009), 'Altered chloride homeostasis removes synaptic inhibitory constraint of the stress axis', *Nature Neuroscience* **12**(4), 438–443.
- Hille, B. (2001a), Classical Biophysics of the Squid Giant Axon, in 'Ion Channels of Excitable Membranes', 9 edn, Sinauer, Sunderland, Massachusetts, pp. 25–60.
- Hille, B. (2001b), Elementary Properties of Ions in Solution, in 'Ion Channel of Excitable Membranes', 3 edn, Sinauer, Sunderland, Massachusetts, pp. 340–342.
- Hille, B. (2001c), Introduction, in 'Ion Channels of Excitable Membranes', 3 edn, Sinauer, Sunderland, Massachusetts, pp. 1–22.
- Hippenmeyer, S., Vrieseling, E., Sigrist, M., Portmann, T., Laengle, C., Ladle, D. R. and Arber, S. (2005), 'A Developmental Switch in the Response of DRG Neurons to ETS Transcription Factor Signaling', *PLOS Biology* **3**(5), e159.
- Hladky, S. B. and Haydon, D. A. (1984), Ion Movements in Gramicidin Channels, in F. Bronner, ed., 'Current Topics in Membranes and Transport', Vol. 21 of *Ion Channels: Molecular and Physiological Aspects*, Academic Press, pp. 327–372.
- Hobson, J. A. and Pace-Schott, E. F. (2002), 'The cognitive neuroscience of sleep: neuronal systems, consciousness and learning', *Nature Reviews Neuroscience* **3**(9), 679–693.
- Hodge, R. D., Bakken, T. E., Miller, J. A., Smith, K. A., Barkan, E. R., Graybuck, L. T., Close, J. L., Long, B., Johansen, N., Penn, O., Yao, Z., Eggermont, J., Höllt, T., Levi, B. P., Shehata, S. I., Aevermann, B., Beller, A., Bertagnolli, D., Brouner, K., Casper, T., Cobbs, C., Dalley, R., Dee, N., Ding, S.-L., Ellenbogen, R. G., Fong, O., Garren, E., Goldy, J., Gwinn, R. P., Hirschstein, D., Keene, C. D., Keshk, M., Ko, A. L., Lathia, K., Mahfouz, A., Maltzer, Z., McGraw, M., Nguyen, T. N., Nyhus, J., Ojemann, J. G., Oldre, A., Parry, S., Reynolds, S., Rimorin, C., Shapovalova, N. V., Somasundaram, S., Szafer, A., Thomsen, E. R., Tieu, M., Quon, G., Scheuermann, R. H., Yuste, R., Sunkin, S. M., Lelieveldt, B., Feng, D., Ng, L., Bernard, A., Hawrylycz, M., Phillips, J. W., Tasic, B., Zeng, H., Jones, A. R., Koch, C. and Lein, E. S. (2019), 'Conserved cell types with divergent features in human versus mouse cortex', *Nature* **573**(7772), 61–68.
- Hodgkin, A. L. and Huxley, A. F. (1952), 'A quantitative description of membrane current and its application to conduction and excitation in nerve', *The Journal of Physiology* **117**(4), 500–544.
- Holt, G. R. and Koch, C. (1997), 'Shunting Inhibition Does Not Have a Divisive Effect on Firing Rates*', *Neural Computation* **9**(5), 1001–1013.
- Howard, M. A., Burger, R. M. and Rubel, E. W. (2007), 'A Developmental Switch to GABAergic Inhibition Dependent on Increases in Kv1-Type K⁺ Currents', *Journal of Neuroscience* **27**(8), 2112–2123.
- Huberfeld, G., Wittner, L., Clemenceau, S., Baulac, M., Kaila, K., Miles, R. and Rivera, C. (2007), 'Perturbed Chloride Homeostasis and GABAergic Signaling in Human Temporal Lobe Epilepsy', *Journal of Neuroscience* **27**(37), 9866–9873.
- Häusser, M., Spruston, N. and Stuart, G. J. (2000), 'Diversity and Dynamics of Dendritic Signaling', *Science* **290**(5492), 739–744.
- Ilie, A., Raimondo, J. V. and Akerman, C. J. (2012), 'Adenosine Release during Seizures Attenuates GABAA Receptor-Mediated Depolarization', *Journal of Neuroscience* **32**(15), 5321–5332.

- Isaacson, J. S. and Scanziani, M. (2011), 'How Inhibition Shapes Cortical Activity', *Neuron* **72**(2), 231–243.
- Isomura, Y., Sugimoto, M., Fujiwara-Tsukamoto, Y., Yamamoto-Muraki, S., Yamada, J. and Fukuda, A. (2003), 'Synaptically Activated Chloride Accumulation Responsible for Depolarizing GABAergic Responses in Mature Hippocampal Neurons', *Journal of Neurophysiology* **90**(4), 2752–2756.
- Jacobs, B. L. and Fornal, C. A. (1999), 'Activity of Serotonergic Neurons in Behaving Animals', *Neuropsychopharmacology* **21**(1), 9–15.
- Jarolimek, W., Lewen, A. and Misgeld, U. (1999), 'A Furosemide-Sensitive Potassium Chloride Cotransporter Counteracts Intracellular Chloride Accumulation and Depletion in Cultured Rat Midbrain Neurons', *Journal of Neuroscience* **19**(12), 4695–4704.
- Jean-Xavier, C., Mentis, G. Z., O'Donovan, M. J., Cattaert, D. and Vinay, L. (2007), 'Dual personality of GABA/glycine-mediated depolarizations in immature spinal cord', *Proceedings of the National Academy of Sciences* **104**(27), 11477–11482.
- Jia, D. W., Costa, R. P. and Vogels, T. P. (2021), 'Developmental depression-facilitation shift controls excitation-inhibition balance', *Communications Biology* **5**(873), 1–24.
- Jin, X., Huguenard, J. R. and Prince, D. A. (2005), 'Impaired Chloride Extrusion in Layer V Pyramidal Neurons of Chronically Injured Epileptogenic Neocortex', *Journal of Neurophysiology* **93**(4), 2117–2126.
- Johansson, S., Yelhekar, T. D. and Druzin, M. (2016), 'Commentary: Chloride Regulation: A Dynamic Equilibrium Crucial for Synaptic Inhibition', *Frontiers in Cellular Neuroscience* **10**, 1–3.
- Johnson, D. and Wu, S. M.-S. (1995a), Electrical Properties of the Excitable Membrane, in 'Foundations of Cellular Neurophysiology', 1 edn, The MIT Press, Cambridge, Massachusetts, pp. 39–53.
- Johnson, D. and Wu, S. M.-S. (1995b), Ion Movement in Excitable Cells, in 'Foundations of Cellular Neurophysiology', The MIT Press, Cambridge, Massachusetts, pp. 9–38.
- Jones, B. E. (2005), 'From waking to sleeping: neuronal and chemical substrates', *Trends in Pharmacological Sciences* **26**(11), 578–586.
- Jones, M. V. and Westbrook, G. L. (1995), 'Desensitized states prolong GABAA channel responses to brief agonist pulses', *Neuron* **15**(1), 181–191.
- Jordan, R. (2021), 'Optimized protocol for in vivo whole-cell recordings in head-fixed, awake behaving mice', *STAR Protocols* **2**(1), 100347.
- Jouhanneau, J.-S. and Poulet, J. F. A. (2019), 'Multiple Two-Photon Targeted Whole-Cell Patch-Clamp Recordings From Monosynaptically Connected Neurons in vivo', *Frontiers in Synaptic Neuroscience* **11**, 1–13.
- Jouvet, M. (1967), 'Neurophysiology of the states of sleep.', *Physiological Reviews* **47**(2), 117–177.
- Kaila, K. (1994), 'Ionic basis of GABAA receptor channel function in the nervous system', *Progress in Neurobiology* **42**(4), 489–537.
- Kaila, K., Lamsa, K., Smirnov, S., Taira, T. and Voipio, J. (1997), 'Long-Lasting GABA-Mediated Depolarization Evoked by High-Frequency Stimulation in Pyramidal

- Neurons of Rat Hippocampal Slice Is Attributable to a Network-Driven, Bicarbonate-Dependent Postassium Transient', *The Journal of Neuroscience* **17**(20), 7662–7672.
- Kaila, K., Pasternack, M., Saarikoski, J. and Voipio, J. (1989), 'Influence of GABA-gated bicarbonate conductance on potential, current and intracellular chloride in crayfish muscle fibres.', *The Journal of Physiology* **416**(1), 161–181.
- Kaila, K., Price, T. J., Payne, J. A., Puskarjov, M. and Voipio, J. (2014), 'Cation-chloride cotransporters in neuronal development, plasticity and disease', *Nature Reviews Neuroscience* **15**(10), 637–654.
- Kaila, K. and Voipio, J. (1987), 'Postsynaptic fall in intracellular p H induced by GABA-activated bicarbonate conductance', *Nature* **330**(6144), 163–165.
- Kaila, K., Voipio, J., Paalasmaa, P., Pasternack, M. and Deisz, R. A. (1993), 'The role of bicarbonate in GABAA receptor-mediated IPSPs of rat neocortical neurones', *The Journal of Physiology* **464**, 273–289.
- Kapur, J. and Coulter, D. A. (1995), 'Experimental Status Epilepticus Alters Gamma-Aminobutyric Acid Type A Receptor Function in CA1 Pyramidal Neurons', *Annals of neurology* **38**(6), 893–900.
- Karlsson, U., Druzin, M. and Johansson, S. (2011), 'Chloride concentration changes and desensitization of GABAA and glycine receptors', *Journal of General Physiology* **138**(6), 609–626.
- Katz, Y., Yizhar, O., Staiger, J. and Lampl, I. (2013), 'Optopatcher—An electrode holder for simultaneous intracellular patch-clamp recording and optical manipulation', *Journal of Neuroscience Methods* **214**(1), 113–117.
- Kelley, M. R., Deeb, T. Z., Brandon, N. J., Dunlop, J., Davies, P. A. and Moss, S. J. (2016), 'Compromising KCC2 transporter activity enhances the development of continuous seizure activity', *Neuropharmacology* **108**, 103–110.
- Khalilov, I., Minlebaev, M., Mukhtarov, M., Juzekaeva, E. and Khazipov, R. (2017), 'Postsynaptic GABA(B) Receptors Contribute to the Termination of Giant Depolarizing Potentials in CA3 Neonatal Rat Hippocampus', *Frontiers in Cellular Neuroscience* **11**, 1–11.
- Kilb, W. (2021), 'When Are Depolarizing GABAergic Responses Excitatory?', *Frontiers in Molecular Neuroscience* **14**(1), 1–12.
- Kim, C. M. and Nykamp, D. Q. (2017), 'The influence of depolarization block on seizure-like activity in networks of excitatory and inhibitory neurons', *Journal of Computational Neuroscience* **43**(1), 65–79.
- Kirmse, K., Kummer, M., Kovalchuk, Y., Witte, O. W., Garaschuk, O. and Holthoff, K. (2015), 'GABA depolarizes immature neurons and inhibits network activity in the neonatal neocortex in vivo', *Nature Communications* **6**(1), 7750.
- Kitamura, K., Judkewitz, B., Kano, M., Denk, W. and Häusser, M. (2008), 'Targeted patch-clamp recordings and single-cell electroporation of unlabeled neurons in vivo', *Nature Methods* **5**(1), 61–67.
- Klein, D., Moore, R. and Reppert, S. (1991), Introduction, in 'Suprachiasmatic Nucleus: The Mind's Clock', Oxford University Press, New York, pp. 3–12.
- Kleinberg, M. E. and Finkelstein, A. (1984), 'Single-length and double-length channels

- formed by nystatin in lipid bilayer membranes', *The Journal of Membrane Biology* **80**(3), 257–269.
- Klett, N. J. and Allen, C. N. (2017), 'Intracellular Chloride Regulation in AVP+ and VIP+ Neurons of the Suprachiasmatic Nucleus', *Scientific Reports* **7**(1), 10226.
- Klett, N. J., Cravetchi, O. and Allen, C. N. (2022), 'Long-Term Imaging Reveals a Circadian Rhythm of Intracellular Chloride in Neurons of the Suprachiasmatic Nucleus', *Journal of Biological Rhythms* **37**(1), 110–123.
- Kodandaramaiah, S. B., Franzesi, G. T., Chow, B. Y., Boyden, E. S. and Forest, C. R. (2012), 'Automated whole-cell patch-clamp electrophysiology of neurons in vivo', *Nature Methods* **9**(6), 585–587.
- Kullmann, D. M., Ruiz, A., Rusakov, D. M., Scott, R., Semyanov, A. and Walker, M. C. (2005), 'Presynaptic, extrasynaptic and axonal GABAA receptors in the CNS: where and why?', *Progress in biophysics and molecular biology* **87**(1), 33–46.
- Kuner, T. and Augustine, G. J. (2000), 'A Genetically Encoded Ratiometric Indicator for Chloride: Capturing Chloride Transients in Cultured Hippocampal Neurons', *Neuron* **27**(3), 447–459.
- Kyrozis, A. and Reichling, D. B. (1995), 'Perforated-patch recording with gramicidin avoids artifactual changes in intracellular chloride concentration', *Journal of Neuroscience Methods* **57**(1), 27–35.
- Kätzel, D., Zemelman, B. V., Buetfering, C., Wölfel, M. and Miesenböck, G. (2011), 'The columnar and laminar organization of inhibitory connections to neocortical excitatory cells', *Nature Neuroscience* **14**(1), 100–107.
- Lamsa, K., Kullmann, D. and Woodin, M. (2010), 'Spike-timing dependent plasticity in inhibitory circuits', *Frontiers in Synaptic Neuroscience* **2**, 1–13.
- Lamsa, K. and Taira, T. (2003), 'Use-Dependent Shift From Inhibitory to Excitatory GABAA Receptor Action in SP-O Interneurons in the Rat Hippocampal CA3 Area', *Journal of Neurophysiology* **90**(3), 1983–1995.
- Langevin, L. M., Mattar, P., Scardigli, R., Roussigné, M., Logan, C., Blader, P. and Schuurmans, C. (2007), 'Validating in utero electroporation for the rapid analysis of gene regulatory elements in the murine telencephalon', *Developmental Dynamics* **236**(5), 1273–1286.
- Lanz, A., Gordon, G. and Bains, J. (2020), 'Spatiotemporal chloride dynamics in hypothalamic CRHPVN neurons', *bioRxiv* pp. 1–31. Publisher: bioRxiv.
- Latimer, K. W., Rieke, F. and Pillow, J. W. (2019), 'Inferring synaptic inputs from spikes with a conductance-based neural encoding model', *eLife* **8**, e47012.
- Ledri, M., Madsen, M. G., Nikitidou, L., Kirik, D. and Kokaia, M. (2014), 'Global Optogenetic Activation of Inhibitory Interneurons during Epileptiform Activity', *Journal of Neuroscience* **34**(9), 3364–3377.
- Lee, C., Lavoie, A., Liu, J., Chen, S. X. and Liu, B.-h. (2020), 'Light Up the Brain: The Application of Optogenetics in Cell-Type Specific Dissection of Mouse Brain Circuits', *Frontiers in Neural Circuits* **14**, 1–14.
- Lee, H. H. C., Deeb, T. Z., Walker, J. A., Davies, P. A. and Moss, S. J. (2011), 'NMDA receptor activity downregulates KCC2 resulting in depolarizing GABAA receptor-mediated currents', *Nature Neuroscience* **14**(6), 736–743.

- Lee, H. H., Jurd, R. and Moss, S. J. (2010), 'Tyrosine phosphorylation regulates the membrane trafficking of the potassium chloride co-transporter KCC2', *Molecular and Cellular Neuroscience* **45**(2), 173–179.
- Leinekugel, X., Medina, I., Khalilov, I., Ben-Ari, Y. and Khazipov, R. (1997), 'Ca²⁺ Oscillations Mediated by the Synergistic Excitatory Actions of GABAA and NMDA Receptors in the Neonatal Hippocampus', *Neuron* **18**(2), 243–255.
- Li, L., Ouellette, B., Stoy, W. A., Garren, E. J., Daigle, T. L., Forest, C. R., Koch, C. and Zeng, H. (2017), 'A robot for high yield electrophysiology and morphology of single neurons in vivo', *Nature Communications* **8**(1), 15604.
- Li, N., Chen, S., Guo, Z. V., Chen, H., Huo, Y., Inagaki, H. K., Chen, G., Davis, C., Hansel, D., Guo, C. and Svoboda, K. (2019), 'Spatiotemporal constraints on optogenetic inactivation in cortical circuits', *eLife* **8**, e48622.
- Li, X., Zhou, J., Chen, Z., Chen, S., Zhu, F. and Zhou, L. (2008), 'Long-term expressional changes of Na⁺ -K⁺ -Cl⁻ co-transporter 1 (NKCC1) and K⁺ -Cl⁻ co-transporter 2 (KCC2) in CA1 region of hippocampus following lithium-pilocarpine induced status epilepticus (PISE)', *Brain Research* **1221**, 141–146.
- Lillis, K. P., Kramer, M. A., Mertz, J., Staley, K. J. and White, J. A. (2012), 'Pyramidal cells accumulate chloride at seizure onset', *Neurobiology of Disease* **47**(3), 358–366.
- Lin, J. S. (2000), 'Brain structures and mechanisms involved in the control of cortical activation and wakefulness, with emphasis on the posterior hypothalamus and histaminergic neurons', *Sleep Medicine Reviews* **4**(5), 471–503.
- Liou, J.-y., Smith, E. H., Bateman, L. M., Bruce, S. L., McKhann, G. M., Goodman, R. R., Emerson, R. G., Schevon, C. A. and Abbott, L. (2020), 'A model for focal seizure onset, propagation, evolution, and progression', *eLife* **9**, e50927.
- Liu, B.-h., Li, Y.-t., Ma, W.-p., Pan, C.-j., Zhang, L. I. and Tao, H. W. (2011), 'Broad Inhibition Sharpens Orientation Selectivity by Expanding Input Dynamic Range in Mouse Simple Cells', *Neuron* **71**(3), 542–554.
- Liu, Z., Lu, X., Villette, V., Gou, Y., Colbert, K. L., Lai, S., Guan, S., Land, M. A., Lee, J., Assefa, T., Zollinger, D. R., Korympidou, M. M., Vlasits, A. L., Pang, M. M., Su, S., Cai, C., Froudarakis, E., Zhou, N., Patel, S. S., Smith, C. L., Ayon, A., Bizouard, P., Bradley, J., Franke, K., Clandinin, T. R., Giovannucci, A., Tolias, A. S., Reimer, J., Dieudonné, S. and St-Pierre, F. (2022), 'Sustained deep-tissue voltage recording using a fast indicator evolved for two-photon microscopy', *Cell* **185**(18), 1–48.
- Lodovichi, C., Ratto, G. M., Trevelyan, A. J. and Arosio, D. (2022), 'Genetically encoded sensors for Chloride concentration', *Journal of Neuroscience Methods* **368**, 109455.
- Loomis, A. L., Harvey, E. N. and Hobart, G. (1935), 'Potential Rhythms of the Cerebral Cortex During Sleep', *Science* **81**(2111), 597–598.
- LoTurco, J. J., Owens, D. F., Heath, M. J. S., Davis, M. B. E. and Kriegstein, A. R. (1995), 'GABA and glutamate depolarize cortical progenitor cells and inhibit DNA synthesis', *Neuron* **15**(6), 1287–1298.
- Luhmann, H. J., Kirischuk, S. and Kilb, W. (2014), 'Comment on "Local impermeant anions establish the neuronal chloride concentration"', *Science* **345**(6201), 1130–1130.
- Luhmann, H. J. and Prince, D. A. (1991), 'Postnatal maturation of the GABAergic system in rat neocortex', *Journal of Neurophysiology* **65**(2), 247–263.

- Lundbæk, J. A., Maer, A. M. and Andersen, O. S. (1997), 'Lipid Bilayer Electrostatic Energy, Curvature Stress, and Assembly of Gramicidin Channels', *Biochemistry* **36**(19), 5695–5701.
- Lüscher, B. P., Vachel, L., Ohana, E. and Muallem, S. (2020), 'Chloride as a bona fide signaling ion', *American Journal of Physiology-Cell Physiology* **318**(1), C125–C136.
- Ma, B., Xu, G., Wang, W., Enyeart, J. J. and Zhou, M. (2014), 'Dual patch voltage clamp study of low membrane resistance astrocytes in situ', *Molecular Brain* **7**(1), 18.
- Magloire, V., Cornford, J., Lieb, A., Kullmann, D. M. and Pavlov, I. (2018), 'KCC2 overexpression prevents the paradoxical seizure-promoting action of somatic inhibition', *Nature Communications* **10**(1), 1225.
- Mahadevan, V. and Woodin, M. A. (2016), 'Regulation of neuronal chloride homeostasis by neuromodulators: Neuromodulation of neuronal chloride homeostasis', *The Journal of Physiology* **594**(10), 2593–2605.
- Mahn, M., Gibor, L., Patil, P., Malina, K. C.-K., Oring, S., Printz, Y., Levy, R., Lampl, I. and Yizhar, O. (2018), 'High-efficiency optogenetic silencing with soma-targeted anion-conducting channelrhodopsins', *Nature Communications* **9**(1), 1–15.
- Mahn, M., Prigge, M., Ron, S., Levy, R. and Yizhar, O. (2016), 'Biophysical constraints of optogenetic inhibition at presynaptic terminals', *Nature Neuroscience* **19**(4), 554–556.
- Mahrach, A., Chen, G., Li, N., van Vreeswijk, C. and Hansel, D. (2020), 'Mechanisms underlying the response of mouse cortical networks to optogenetic manipulation', *eLife* **9**, e49967.
- Maloney, K. J., Mainville, L. and Jones, B. E. (2002), 'c-Fos expression in dopaminergic and GABAergic neurons of the ventral mesencephalic tegmentum after paradoxical sleep deprivation and recovery', *European Journal of Neuroscience* **15**(4), 774–778.
- Margrie, T. W., Brecht, M. and Sakmann, B. (2002), 'In vivo, low-resistance, whole-cell recordings from neurons in the anaesthetized and awake mammalian brain', *Pflügers Archiv* **444**(4), 491–498.
- Margrie, T. W., Meyer, A. H., Caputi, A., Monyer, H., Hasan, M. T., Schaefer, A. T., Denk, W. and Brecht, M. (2003), 'Targeted Whole-Cell Recordings in the Mammalian Brain In Vivo', *Neuron* **39**(6), 911–918.
- Marino, M., Misuri, L. and Brogioli, D. (2014), 'A new open source software for the calculation of the liquid junction potential between two solutions according to the stationary Nernst-Planck equation', *arxiv* pp. 1–5. Publisher: arXiv.
- Martina, M., Royer, S. and Paré, D. (2001), 'Cell-Type-Specific GABA Responses and Chloride Homeostasis in the Cortex and Amygdala', *Journal of Neurophysiology* **86**(6), 2887–2895.
- Marty, A. and Neher, E. (1983), Exchange of cell content with pipette solution., in 'Single-Channel Recording', Plenum Press, New York, NY, pp. 113–114.
- Marty, A. and Neher, E. (1995), Tight-Seal Whole-Cell Recording, in B. Sakmann and E. Neher, eds, 'Single-Channel Recording', Springer US, Boston, MA, pp. 31–52.
- Matsumura, M., Cope, T. and Fetisov, E. E. (1988), 'Sustained excitatory synaptic input to motor cortex neurons in awake animals revealed by intracellular recording of membrane potentials', *Experimental Brain Research* **70**(3), 463–469.

- McCarren, M. and Alger, B. E. (1985), 'Use-dependent depression of IPSPs in rat hippocampal pyramidal cells in vitro', *Journal of Neurophysiology* **53**(2), 557–571.
- McGinley, M. J., David, S. V. and McCormick, D. A. (2015), 'Cortical Membrane Potential Signature of Optimal States for Sensory Signal Detection', *Neuron* **87**(1), 179–192.
- Misgeld, U., Deisz, R. A., Dodt, H. U. and Lux, H. D. (1986), 'The Role of Chloride Transport in Postsynaptic Inhibition of Hippocampal Neurons', *Science* **232**(4756), 1413–1415.
- Mitchell, S. J. and Silver, R. A. (2003), 'Shunting Inhibition Modulates Neuronal Gain during Synaptic Excitation', *Neuron* **38**(3), 433–445.
- Mo, C.-H., Gu, M. and Koch, C. (2004), 'A Learning Rule for Local Synaptic Interactions Between Excitation and Shunting Inhibition', *Neural Computation* **16**(12), 2507–2532.
- Mohajerani, M. H. and Cherubini, E. (2005), 'Spontaneous recurrent network activity in organotypic rat hippocampal slices', *European Journal of Neuroscience* **22**(1), 107–118.
- Mondino, A., González, J., Li, D., Mateos, D., Osorio, L., Cavelli, M., Castro-Nin, J. P., Serantes, D., Costa, A., Vanini, G., Mashour, G. A. and Torterolo, P. (2022), 'Urethane anaesthesia exhibits neurophysiological correlates of unconsciousness and is distinct from sleep', *European Journal of Neuroscience* **1**, 1–19.
- Moran, R. J., Symmonds, M., Stephan, K. E., Friston, K. J. and Dolan, R. J. (2011), 'An In Vivo Assay of Synaptic Function Mediating Human Cognition', *Current Biology* **21**(15), 1320–1325.
- Morita, K., Tsumoto, K. and Aihara, K. (2005), 'Possible effects of depolarizing GABAA conductance on the neuronal input-output relationship: a modeling study', *Journal of Neurophysiology* **93**(6), 1–20.
- Moruzzi, G. and Magoun, H. W. (1949), 'Brain stem reticular formation and activation of the EEG', *Electroencephalography and Clinical Neurophysiology* **1**(1), 455–473.
- Murillo-de Ozores, A. R., Chávez-Canales, M., de los Heros, P., Gamba, G. and Castañeda-Bueno, M. (2020), 'Physiological Processes Modulated by the Chloride-Sensitive WNK-SPAK/OSR1 Kinase Signaling Pathway and the Cation-Coupled Chloride Cotransporters', *Frontiers in Physiology* **11**, 1–28.
- Murphy, B. K. and Miller, K. D. (2003), 'Multiplicative Gain Changes Are Induced by Excitation or Inhibition Alone', *Journal of Neuroscience* **23**(31), 10040–10051.
- Murphy, B. K. and Miller, K. D. (2009), 'Balanced Amplification: A New Mechanism of Selective Amplification of Neural Activity Patterns', *Neuron* **61**(4), 635–648.
- Muthukumaraswamy, S. D., Shaw, A. D., Jackson, L. E., Hall, J., Moran, R. and Saxena, N. (2015), 'Evidence that Subanesthetic Doses of Ketamine Cause Sustained Disruptions of NMDA and AMPA-Mediated Frontoparietal Connectivity in Humans', *Journal of Neuroscience* **35**(33), 11694–11706.
- Myers, V. B. and Haydon, D. A. (1972), 'Ion transfer across lipid membranes in the presence of gramicidin A: II. The ion selectivity', *Biochimica et Biophysica Acta (BBA) - Biomembranes* **274**(2), 313–322.
- Nagel, G., Ollig, D., Fuhrmann, M., Kateriya, S., Musti, A. M., Bamberg, E. and Hegemann, P. (2002), 'Channelrhodopsin-1: A Light-Gated Proton Channel in Green Algae', *Science* **296**(5577), 2395–2398.

- Nagel, G., Szellas, T., Huhn, W., Kateriya, S., Adeishvili, N., Berthold, P., Ollig, D., Hegemann, P. and Bamberg, E. (2003), 'Channelrhodopsin-2, a directly light-gated cation-selective membrane channel', *Proceedings of the National Academy of Sciences* **100**(24), 13940–13945.
- Nestvogel, D. B. and McCormick, D. A. (2022), 'Visual thalamocortical mechanisms of waking state-dependent activity and alpha oscillations', *Neuron* **110**(1), 120–138.e4.
- Nir, Y., Staba, R. J., Andrillon, T., Vyazovskiy, V. V., Cirelli, C., Fried, I. and Tononi, G. (2011), 'Regional Slow Waves and Spindles in Human Sleep', *Neuron* **70**(1), 153–169.
- Nissbrandt, H. and Engberg, G. (1996), 'The GABAB-receptor antagonist, CGP 35348, antagonises gamma-hydroxybutyrate- and baclofen-induced alterations in locomotor activity and forebrain dopamine levels in mice', *Journal of Neural Transmission* **103**(11), 1255–1263.
- Olsen, R. W. and Sieghart, W. (2009), 'GABAA receptors: Subtypes provide diversity of function and pharmacology', *Neuropharmacology* **56**(1), 141–148.
- Ormond, J. and Woodin, M. (2011), 'Disinhibition-Mediated LTP in the Hippocampus is Synapse Specific', *Frontiers in Cellular Neuroscience* **5**, 1–11.
- Ormond, J. and Woodin, M. A. (2009), 'Disinhibition mediates a form of hippocampal long-term potentiation in area CA1', *PloS One* **4**(9), e7224.
- Otsu, Y., Donneger, F., Schwartz, E. J. and Poncer, J. C. (2020), 'Cation–chloride cotransporters and the polarity of GABA signalling in mouse hippocampal parvalbumin interneurons', *The Journal of Physiology* **598**(10), 1865–1880.
- Overstreet, L. S., Jones, M. V. and Westbrook, G. L. (2000), 'Slow Desensitization Regulates the Availability of Synaptic GABAA Receptors', *Journal of Neuroscience* **20**(21), 7914–7921.
- Owens, D. F., Boyce, L. H., Davis, M. B. E. and Kriegstein, A. R. (1996), 'Excitatory GABA Responses in Embryonic and Neonatal Cortical Slices Demonstrated by Gramicidin Perforated-Patch Recordings and Calcium Imaging', *Journal of Neuroscience* **16**(20), 6414–6423.
- Ozeki, H., Finn, I. M., Schaffer, E. S., Miller, K. D. and Ferster, D. (2009), 'Inhibitory Stabilization of the Cortical Network Underlies Visual Surround Suppression', *Neuron* **62**(4), 578–592.
- Pace-Schott, E. F. and Hobson, J. A. (2002), 'The Neurobiology of Sleep: Genetics, cellular physiology and subcortical networks', *Nature Reviews Neuroscience* **3**(8), 591–605.
- Packer, A. M., Roska, B. and Häusser, M. (2013), 'Targeting neurons and photons for optogenetics', *Nature Neuroscience* **16**(7), 805–815.
- Packer, A. M., Russell, L. E., Dagleish, H. W. P. and Häusser, M. (2015), 'Simultaneous all-optical manipulation and recording of neural circuit activity with cellular resolution in vivo', *Nature Methods* **12**(2), 140–146.
- Pan-Vazquez, A., Wefelmeyer, W., Gonzalez Sabater, V., Neves, G. and Burrone, J. (2020), 'Activity-Dependent Plasticity of Axo-axonic Synapses at the Axon Initial Segment', *Neuron* **106**(2), 265–276.e6.
- Park, S.-H. and Weber, F. (2020), 'Neural and Homeostatic Regulation of REM Sleep', *Frontiers in Psychology* **11**, 1–16.

- Pavlov, I., Scimemi, A., Savtchenko, L., Kullmann, D. M. and Walker, M. C. (2011), 'Th-mediated depolarization enhances the temporal precision of neuronal integration', *Nature Communications* **2**(1), 199.
- Pfeffer, C. K., Stein, V., Keating, D. J., Maier, H., Rinke, I., Rudhard, Y., Hentschke, M., Rune, G. M., Jentsch, T. J. and Hübner, C. A. (2009), 'NKCC1-Dependent GABAergic Excitation Drives Synaptic Network Maturation during Early Hippocampal Development', *Journal of Neuroscience* **29**(11), 3419–3430.
- Piatkevich, K. D., Bensussen, S., Tseng, H.-a., Shroff, S. N., Lopez-Huerta, V. G., Park, D., Jung, E. E., Shemesh, O. A., Straub, C., Gritton, H. J., Romano, M. F., Costa, E., Sabatini, B. L., Fu, Z., Boyden, E. S. and Han, X. (2019), 'Population imaging of neural activity in awake behaving mice', *Nature* **574**(7778), 413–417.
- Poleg-Polsky, A. and Diamond, J. S. (2011), 'Imperfect Space Clamp Permits Electrotonic Interactions between Inhibitory and Excitatory Synaptic Conductances, Distorting Voltage Clamp Recordings', *PLOS ONE* **6**(4), e19463.
- Poo, C. and Isaacson, J. S. (2009), 'Odor Representations in Olfactory Cortex: "Sparse" Coding, Global Inhibition, and Oscillations', *Neuron* **62**(6), 850–861.
- Porkka-Heiskanen, T., Strecker, R. E., Thakkar, M., Bjørkum, A. A., Greene, R. W. and McCarley, R. W. (1997), 'Adenosine: A Mediator of the Sleep-Inducing Effects of Prolonged Wakefulness', *Science* **276**(5316), 1265–1268.
- Pouille, F., Marin-Burgin, A., Adesnik, H., Atallah, B. V. and Scanziani, M. (2009), 'Input normalization by global feedforward inhibition expands cortical dynamic range', *Nature Neuroscience* **12**(12), 1577–1585.
- Pouille, F. and Scanziani, M. (2001), 'Enforcement of Temporal Fidelity in Pyramidal Cells by Somatic Feed-Forward Inhibition', *Science* **293**(5532), 1159–1163.
- Pouille, F., Watkinson, O., Scanziani, M. and Trevelyan, A. J. (2013), 'The contribution of synaptic location to inhibitory gain control in pyramidal cells', *Physiological Reports* **1**(5), 1–9.
- Poulet, J. F. A. and Crochet, S. (2019), 'The Cortical States of Wakefulness', *Frontiers in Systems Neuroscience* **12**, 1–18.
- Poulet, J. F. A., Fernandez, L. M. J., Crochet, S. and Petersen, C. C. H. (2012), 'Thalamic control of cortical states', *Nature Neuroscience* **15**(3), 370–372.
- Poulet, J. F. A. and Petersen, C. C. H. (2008), 'Internal brain state regulates membrane potential synchrony in barrel cortex of behaving mice', *Nature* **454**(7206), 881–885.
- Pracucci, E., Graham, R., Alberio, L., Nardi, G., Cozzolino, O., Pillai, V., Saieva, L., Walsh, D., Landi, S., Zhang, J., Trevelyan, A. J. and Ratto, G.-M. (2021), 'Circadian rhythm in cortical chloride homeostasis underpins variation in network excitability', *bioRxiv* pp. 1–22.
- Prescott, S. A. and De Koninck, Y. (2003), 'Gain control of firing rate by shunting inhibition: Roles of synaptic noise and dendritic saturation', *Proceedings of the National Academy of Sciences* **100**(4), 2076–2081.
- Rahmati, N., Hoebeek, F. E., Peter, S. and De Zeeuw, C. I. (2018), 'Chloride Homeostasis in Neurons With Special Emphasis on the Olivocerebellar System: Differential Roles for Transporters and Channels', *Frontiers in Cellular Neuroscience* **12**, 1–23.

- Rahmati, N., Normoyle, K. P., Glykys, J., Dzhalala, V. I., Lillis, K. P., Kahle, K. T., Raiyyani, R., Jacob, T. and Staley, K. J. (2021), 'Unique Actions of GABA Arising from Cytoplasmic Chloride Microdomains', *Journal of Neuroscience* **41**(23), 4957–4975.
- Raimondo, J. V., Burman, R. J., Katz, A. A. and Akerman, C. J. (2015), 'Ion dynamics during seizures', *Frontiers in Cellular Neuroscience* **9**, 1–14.
- Raimondo, J. V., Irkle, A., Wefelmeyer, W., Newey, S. E. and Akerman, C. J. (2012), 'Genetically encoded proton sensors reveal activity-dependent pH changes in neurons', *Frontiers in Molecular Neuroscience* **5**, 1–12.
- Raimondo, J. V., Joyce, B., Kay, L., Schlagheck, T., Newey, S. E., Srinivas, S. and Akerman, C. J. (2013), 'A genetically-encoded chloride and pH sensor for dissociating ion dynamics in the nervous system', *Frontiers in Cellular Neuroscience* **7**, 1–10.
- Raimondo, J. V., Kay, L., Ellender, T. E. and Akerman, C. J. (2012), 'Optogenetic silencing strategies differ in their effects on inhibitory synaptic transmission', *Nature Neuroscience* **15**(8), 1102–1104.
- Raimondo, J. V., Markram, H. and Akerman, C. J. (2012), 'Short-term ionic plasticity at GABAergic synapses', *Frontiers in Synaptic Neuroscience* **4**, 1–9.
- Raimondo, J. V., Richards, B. A. and Woodin, M. A. (2017), 'Neuronal chloride and excitability — the big impact of small changes', *Current Opinion in Neurobiology* **43**, 35–42.
- Rall, W. (2011), Core Conductor Theory and Cable Properties of Neurons, in 'Comprehensive Physiology', John Wiley & Sons, Ltd, pp. 39–97.
- Redman, S. and Walmsley, B. (1983), 'The time course of synaptic potentials evoked in cat spinal motoneurons at identified group Ia synapses.', *The Journal of Physiology* **343**(1), 117–133.
- Reppert, S. M. and Weaver, D. R. (2001), 'Molecular analysis of mammalian circadian rhythms', *Annual Review of Physiology* **63**, 647–676.
- Rheinberger, M. B. and Jasper, H. H. (1937), 'Electrical activity of the cerebral cortex in the unanesthetized cat', *American Journal of Physiology-Legacy Content* **119**(1), 186–196.
- Rivera, C., Voipio, J., Payne, J. A., Ruusuvuori, E., Lahtinen, H., Lamsa, K., Pirvola, U., Saarma, M. and Kaila, K. (1999), 'The potassium chloride co-transporter KCC2 renders GABA hyperpolarizing during neuronal maturation', *Nature* **397**, 5.
- Rivera, C., Voipio, J., Thomas-Crusells, J., Li, H., Emri, Z., Sipilä, S., Payne, J. A., Minichiello, L., Saarma, M. and Kaila, K. (2004), 'Mechanism of Activity-Dependent Downregulation of the Neuron-Specific K-Cl Cotransporter KCC2', *Journal of Neuroscience* **24**(19), 4683–4691.
- Rockland, K. S. and Pandya, D. N. (1979), 'Laminar origins and terminations of cortical connections of the occipital lobe in the rhesus monkey', *Brain Research* **179**(1), 3–20.
- Romero, M. F., Henry, D., Nelson, S., Harte, P. J., Dillon, A. K. and Sciortino, C. M. (2000), 'Cloning and Characterization of a Na⁺-driven Anion Exchanger (NDAE1): A NEW BICARBONATE TRANSPORTER*', *Journal of Biological Chemistry* **275**(32), 24552–24559.
- Rothman, J. S., Cathala, L., Steuber, V. and Silver, R. A. (2009), 'Synaptic depression enables neuronal gain control', *Nature* **457**(7232), 1015–1018.

- Rudolph, M., Pospischil, M., Timofeev, I. and Destexhe, A. (2007), 'Inhibition Determines Membrane Potential Dynamics and Controls Action Potential Generation in Awake and Sleeping Cat Cortex', *Journal of Neuroscience* **27**(20), 5280–5290.
- Russell, L. E., Dalglish, H. W. P., Nutbrown, R., Gauld, O. M., Herrmann, D., Fişek, M., Packer, A. M. and Häusser, M. (2022), 'All-optical interrogation of neural circuits in behaving mice', *Nature Protocols* **17**(7), 1579–1620.
- Sakmann, B. and Neher, E. (1984), 'Patch Clamp Techniques for Studying Ionic Channels in Excitable Membranes', *Annual Review of Physiology* **46**, 455–472.
- Sauer, J.-F. and Bartos, M. (2010), 'Recruitment of Early Postnatal Parvalbumin-Positive Hippocampal Interneurons by GABAergic Excitation', *Journal of Neuroscience* **30**(1), 110–115.
- Schwartz, W. J. and Klerman, E. B. (2019), 'Circadian Neurobiology and the Physiologic Regulation of Sleep and Wakefulness', *Neurologic Clinics* **37**(3), 475–486.
- Schwiening, C. J. and Boron, W. F. (1994), 'Regulation of intracellular pH in pyramidal neurones from the rat hippocampus by Na(+)-dependent Cl(-)-HCO₃⁻ exchange.', *The Journal of Physiology* **475**(1), 59–67.
- Sheardown, M. J., Nielsen, E. O., Hansen, A. J., Jacobsen, P. and Honoré, T. (1990), '2,3-Dihydroxy-6-nitro-7-sulfamoyl-benzo(F)quinoxaline: a Neuroprotectant for Cerebral Ischemia', *Science* **247**(4942), 571–574.
- Shepard, G. M. (2004), Introduction to Synaptic Circuits, in 'The Synaptic Organisation of the Brain', 5 edn, Oxford University Press, New York, NY, pp. 3–14.
- Shu, Y., Hasenstaub, A., Badoual, M., Bal, T. and McCormick, D. A. (2003), 'Barrages of Synaptic Activity Control the Gain and Sensitivity of Cortical Neurons', *Journal of Neuroscience* **23**(32), 10388–10401.
- Sidiropoulou, K. and Poirazi, P. (2012), 'Predictive Features of Persistent Activity Emergence in Regular Spiking and Intrinsic Bursting Model Neurons', *PLOS Computational Biology* **8**(4), e1002489.
- Silayeva, L., Deeb, T. Z., Hines, R. M., Kelley, M. R., Munoz, M. B., Lee, H. H. C., Brandon, N. J., Dunlop, J., Maguire, J., Davies, P. A. and Moss, S. J. (2015), 'KCC2 activity is critical in limiting the onset and severity of status epilepticus', *Proceedings of the National Academy of Sciences* **112**(11), 3523–3528.
- Sineshchekov, O. A., Govorunova, E. G., Li, H. and Spudich, J. L. (2015), 'Gating mechanisms of a natural anion channelrhodopsin', *Proceedings of the National Academy of Sciences* **112**(46), 14236–14241.
- Smith, R. L., Clayton, G. H., Wilcox, C. L., Escudero, K. W. and Staley, K. J. (1995), 'Differential expression of an inwardly rectifying chloride conductance in rat brain neurons: a potential mechanism for cell-specific modulation of postsynaptic inhibition', *Journal of Neuroscience* **15**(5), 4057–4067.
- Sohal, V. S., Zhang, F., Yizhar, O. and Deisseroth, K. (2009), 'Parvalbumin neurons and gamma rhythms enhance cortical circuit performance', *Nature* **459**(7247), 698–702.
- Somogyi, P., Tamás, G., Lujan, R. and Buhl, E. H. (1998), 'Salient features of synaptic organisation in the cerebral cortex', *Brain Research Reviews* **26**(2), 113–135. Published on the World Wide Web on 3 March 1998.1'

- Spruston, N., Jaffe, D. B., Williams, S. H. and Johnston, D. (1993), 'Voltage- and space-clamp errors associated with the measurement of electrotonically remote synaptic events', *Journal of Neurophysiology* **70**(2), 781–802.
- Staley, K. (1994), 'The role of an inwardly rectifying chloride conductance in postsynaptic inhibition', *Journal of Neurophysiology* **72**(1), 273–284.
- Staley, K. J. and Mody, I. (1992), 'Shunting of excitatory input to dentate gyrus granule cells by a depolarizing GABAA receptor-mediated postsynaptic conductance', *Journal of Neurophysiology* **68**(1), 197–212.
- Staley, K. J. and Proctor, W. R. (1999), 'Modulation of mammalian dendritic GABA-A receptor function by the kinetics of chloride and bicarbonate transport', *The Journal of Physiology* **519**(3), 693–712.
- Staley, K., Soldo, B. and Proctor, W. (1995), 'Tonic mechanisms of neuronal excitation by inhibitory GABAA receptors', *Science* **269**(5226), 977–981.
- Steriade, M. (2000), 'Corticothalamic resonance, states of vigilance and mentation', *Neuroscience* **101**(2), 243–276.
- Steriade, M. (2001), *The Intact and Sliced Brain*, 1 edn, MIT Press, Cambridge, Massachusetts.
- Steriade, M., Amzica, F. and Contreras, D. (1996), 'Synchronization of fast (30–40 Hz) spontaneous cortical rhythms during brain activation', *Journal of Neuroscience* **16**(1), 392–417.
- Steriade, M., Datta, S., Paré, D., Oakson, G. and Curró Dossi, R. C. (1990), 'Neuronal activities in brain-stem cholinergic nuclei related to tonic activation processes in thalamocortical systems', *The Journal of Neuroscience* **10**(8), 2541–2559.
- Steriade, M., McCormick, D. and Sejnowski, T. (1993), 'Thalamocortical Oscillations in the Sleeping and Aroused Brain', *Science* **262**(5134), 679–685.
- Steriade, M., Nunez, A. and Amzica, F. (1993), 'A novel slow (< 1 Hz) oscillation of neocortical neurons in vivo: depolarizing and hyperpolarizing components', *Journal of Neuroscience* **13**(8), 3252–3265.
- Steriade, M., Timofeev, I. and Grenier, F. (2001), 'Natural Waking and Sleep States: A View From Inside Neocortical Neurons', *Journal of Neurophysiology* **85**(5), 1969–1985.
- Stoy, W. A., Kolb, I., Holst, G. L., Liew, Y., Pala, A., Yang, B., Boyden, E. S., Stanley, G. B. and Forest, C. R. (2017), 'Robotic navigation to subcortical neural tissue for intracellular electrophysiology in vivo', *Journal of Neurophysiology* **118**(2), 1141–1150.
- Strecker, R. E., Morairty, S., Thakkar, M. M., Porkka-Heiskanen, T., Basheer, R., Dauphin, L. J., Rainnie, D. G., Portas, C. M., Greene, R. W. and McCarley, R. W. (2000), 'Adenosinergic modulation of basal forebrain and preoptic/anterior hypothalamic neuronal activity in the control of behavioral state', *Behavioural Brain Research* **115**(2), 183–204.
- Sulis Sato, S., Artioni, P., Landi, S., Cozzolino, O., Parra, R., Pracucci, E., Trovato, F., Szczurkowska, J., Luin, S., Arosio, D., Beltram, F., Cancedda, L., Kaila, K. and Ratto, G. M. (2017), 'Simultaneous two-photon imaging of intracellular chloride concentration and pH in mouse pyramidal neurons in vivo', *Proceedings of the National Academy of Sciences* **114**(41), E8770–E8779.

- Symmonds, M., Moran, C. H., Leite, M. I., Buckley, C., Irani, S. R., Stephan, K. E., Friston, K. J. and Moran, R. J. (2018), 'Ion channels in EEG: isolating channel dysfunction in NMDA receptor antibody encephalitis', *Brain* **141**(6), 1691–1702.
- Tabata, H. and Nakajima, K. (2001), 'Efficient in utero gene transfer system to the developing mouse brain using electroporation: visualization of neuronal migration in the developing cortex', *Neuroscience* **103**(4), 865–872.
- Takeuchi, A. and Takeuchi, N. (1967), 'Anion permeability of the inhibitory post-synaptic membrane of the crayfish neuromuscular junction', *The Journal of Physiology* **191**(3), 575–590.
- Tan, A. Y. Y., Chen, Y., Scholl, B., Seidemann, E. and Priebe, N. J. (2014), 'Sensory stimulation shifts visual cortex from synchronous to asynchronous states', *Nature* **509**(7499), 226–229.
- Tanaka, T., Saito, H. and Matsuki, N. (1997), 'Inhibition of GABAA Synaptic Responses by Brain-Derived Neurotrophic Factor (BDNF) in Rat Hippocampus', *Journal of Neuroscience* **17**(9), 2959–2966.
- Terunuma, M., Xu, J., Vithlani, M., Sieghart, W., Kittler, J., Pangalos, M., Haydon, P. G., Coulter, D. A. and Moss, S. J. (2008), 'Deficits in Phosphorylation of GABAA Receptors by Intimately Associated Protein Kinase C Activity Underlie Compromised Synaptic Inhibition during Status Epilepticus', *The Journal of Neuroscience* **28**(2), 376–384.
- Thompson, S. M., Deisz, R. A. and Prince, D. A. (1988), 'Relative contributions of passive equilibrium and active transport to the distribution of chloride in mammalian cortical neurons', *Journal of Neurophysiology* **60**(1), 105–124.
- Thompson, S. M. and Gahwiler, B. H. (1989), 'Activity-dependent disinhibition. II. Effects of extracellular potassium, furosemide, and membrane potential on Cl^- in hippocampal CA3 neurons', *Journal of Neurophysiology* **61**(3), 512–523.
- Tikidji-Hamburyan, R. A. and Canavier, C. C. (2020), 'Shunting Inhibition Improves Synchronization in Heterogeneous Inhibitory Interneuronal Networks with Type 1 Excitability Whereas Hyperpolarizing Inhibition Is Better for Type 2 Excitability', *eNeuro* **7**(3), 1–22.
- Timofeev, I., Grenier, F. and Steriade, M. (2001), 'Disfacilitation and active inhibition in the neocortex during the natural sleep-wake cycle: An intracellular study', *Proceedings of the National Academy of Sciences* **98**(4), 1924–1929.
- To, M.-S., Honnuraiah, S. and Stuart, G. J. (2022), 'Voltage Clamp Errors During Estimation of Concurrent Excitatory and Inhibitory Synaptic Input to Neurons with Dendrites', *Neuroscience* **489**(1), 98–110.
- Tononi, G. and Cirelli, C. (2006), 'Sleep function and synaptic homeostasis', *Sleep Medicine Reviews* **10**(1), 49–62.
- Torao-Angosto, M., Manasanch, A., Mattia, M. and Sanchez-Vives, M. V. (2021), 'Up and Down States During Slow Oscillations in Slow-Wave Sleep and Different Levels of Anesthesia', *Frontiers in Systems Neuroscience* **15**, 1–9.
- Toyoda, I., Fujita, S., Thamattoor, A. K. and Buckmaster, P. S. (2015), 'Unit Activity of Hippocampal Interneurons before Spontaneous Seizures in an Animal Model of Temporal Lobe Epilepsy', *Journal of Neuroscience* **35**(16), 6600–6618.

- Traynelis, S. F. (1998), 'Software-based correction of single compartment series resistance errors', *Journal of Neuroscience Methods* **86**(1), 25–34.
- Trevelyan, A. J. and Schevon, C. A. (2013), 'How inhibition influences seizure propagation', *Neuropharmacology* **69**, 45–54.
- Trevelyan, A. J., Sussillo, D., Watson, B. O. and Yuste, R. (2006), 'Modular Propagation of Epileptiform Activity: Evidence for an Inhibitory Veto in Neocortex', *Journal of Neuroscience* **26**(48), 12447–12455.
- Trevelyan, A. J., Sussillo, D. and Yuste, R. (2007), 'Feedforward Inhibition Contributes to the Control of Epileptiform Propagation Speed', *Journal of Neuroscience* **27**(13), 3383–3387.
- Tsien, J. Z., Chen, D. F., Gerber, D., Tom, C., Mercer, E. H., Anderson, D. J., Mayford, M., Kandel, E. R. and Tonegawa, S. (1996), 'Subregion- and Cell Type–Restricted Gene Knockout in Mouse Brain', *Cell* **87**(7), 1317–1326.
- Tsodyks, M., Kenet, T., Grinvald, A. and Arieli, A. (1999), 'Linking Spontaneous Activity of Single Cortical Neurons and the Underlying Functional Architecture', *Science* **286**(5446), 1943–1946.
- Tsodyks, M. V., Skaggs, W. E., Sejnowski, T. J. and McNaughton, B. L. (1997), 'Paradoxical Effects of External Modulation of Inhibitory Interneurons', *Journal of Neuroscience* **17**(11), 4382–4388.
- Tyzio, R., Cossart, R., Khalilov, I., Minlebaev, M., Hübner, C. A., Represa, A., Ben-Ari, Y. and Khazipov, R. (2006), 'Maternal Oxytocin Triggers a Transient Inhibitory Switch in GABA Signaling in the Fetal Brain During Delivery', *Science* **314**(5806), 1788–1792.
- Tyzio, R., Minlebaev, M., Rheims, S., Ivanov, A., Jorquera, I., Holmes, G. L., Zilberter, Y., Ben-Ari, Y. and Khazipov, R. (2008), 'Postnatal changes in somatic gamma-aminobutyric acid signalling in the rat hippocampus', *European Journal of Neuroscience* **27**(10), 2515–2528.
- Udakis, M., Pedrosa, V., Chamberlain, S. E. L., Clopath, C. and Mellor, J. R. (2020), 'Interneuron-specific plasticity at parvalbumin and somatostatin inhibitory synapses onto CA1 pyramidal neurons shapes hippocampal output', *Nature Communications* **11**(1), 4395.
- Untiet, V., Beinlich, F. R. M., Kusk, P., Kjaerby, C., Andersen, M., Hauglund, N., Sigurdsson, B., Hirase, H., Petersen, N., Verkhratsky, A. and Nedergaard, M. (2022), 'Astrocytic chloride is brain state dependent and regulates cortical neuronal activity', *bioRxiv* pp. 1–35.
- Valdivieso, A. G. and Santa-Coloma, T. A. (2019), 'The chloride anion as a signalling effector', *Biological Reviews* **94**(5), 1839–1856.
- Valeeva, G., Tressard, T., Mukhtarov, M., Baude, A. and Khazipov, R. (2016), 'An Optogenetic Approach for Investigation of Excitatory and Inhibitory Network GABA Actions in Mice Expressing Channelrhodopsin-2 in GABAergic Neurons', *The Journal of Neuroscience* **36**(22), 5961–5973.
- van Vreeswijk, C. and Sompolinsky, H. (1996), 'Chaos in Neuronal Networks with Balanced Excitatory and Inhibitory Activity', *Science* **274**(5293), 1724–1726.
- Vattikuti, S. and Chow, C. C. (2010), 'A Computational Model for Cerebral Cortical Dysfunction in Autism Spectrum Disorders', *Biological Psychiatry* **67**(7), 672–678.

- Velte, T. J. and Miller, R. F. (1996), 'Computer simulations of voltage clamping retinal ganglion cells through whole-cell electrodes in the soma', *Journal of Neurophysiology* **75**(5), 2129–2143.
- Verheugen, J. A., Fricker, D. and Miles, R. (1999), 'Noninvasive measurements of the membrane potential and GABAergic action in hippocampal interneurons', *The Journal of Neuroscience* **19**(7), 2546–2555.
- Vida, I., Bartos, M. and Jonas, P. (2006), 'Shunting Inhibition Improves Robustness of Gamma Oscillations in Hippocampal Interneuron Networks by Homogenizing Firing Rates', *Neuron* **49**(1), 107–117.
- Viitanen, T., Ruusuvuori, E., Kaila, K. and Voipio, J. (2010), 'The potassium chloride cotransporter KCC2 promotes GABAergic excitation in the mature rat hippocampus: GABA excitation and KCC2', *The Journal of Physiology* **588**(9), 1527–1540.
- Villette, V., Chavarha, M., Dimov, I. K., Bradley, J., Pradhan, L., Mathieu, B., Evans, S. W., Chamberland, S., Shi, D., Yang, R., Kim, B. B., Ayon, A., Jalil, A., St-Pierre, F., Schnitzer, M. J., Bi, G., Toth, K., Ding, J., Dieudonné, S. and Lin, M. Z. (2019), 'Ultrafast Two-Photon Imaging of a High-Gain Voltage Indicator in Awake Behaving Mice', *Cell* **179**(7), 1590–1608.
- Vogels, T. P. and Abbott, L. F. (2009), 'Gating multiple signals through detailed balance of excitation and inhibition in spiking networks', *Nature Neuroscience* **12**(4), 483–491.
- Vogels, T. P., Sprekeler, H., Zenke, F., Clopath, C. and Gerstner, W. (2011), 'Inhibitory Plasticity Balances Excitation and Inhibition in Sensory Pathways and Memory Networks', *Science* **334**(6062), 1569–1573.
- Voipio, J., Boron, W. F., Jones, S. W., Hopfer, U., Payne, J. A. and Kaila, K. (2014), 'Comment on "Local impermeant anions establish the neuronal chloride concentration"', *Science* **345**(6201), 1130.
- Volman, V., Levine, H. and Sejnowski, T. J. (2010), 'Shunting Inhibition Controls the Gain Modulation Mediated by Asynchronous Neurotransmitter Release in Early Development', *PLOS Computational Biology* **6**(11), e1000973.
- Vyazovskiy, V. V., Olcese, U., Hanlon, E. C., Nir, Y., Cirelli, C. and Tononi, G. (2011), 'Local sleep in awake rats', *Nature* **472**(7344), 443–447.
- Vyazovskiy, V. V., Olcese, U., Lazimy, Y. M., Faraguna, U., Esser, S. K., Williams, J. C., Cirelli, C. and Tononi, G. (2009), 'Cortical Firing and Sleep Homeostasis', *Neuron* **63**(6), 865–878.
- Wagner, S., Castel, M., Gainer, H. and Yarom, Y. (1997), 'GABA in the mammalian suprachiasmatic nucleus and its role in diurnal rhythmicity', *Nature* **387**(6633), 598–603.
- Wagner, S., Sagiv, N. and Yarom, Y. (2001), 'GABA-induced current and circadian regulation of chloride in neurones of the rat suprachiasmatic nucleus', *The Journal of Physiology* **537**(Pt 3), 853–869.
- Wang, D. D. and Kriegstein, A. R. (2011), 'Blocking Early GABA Depolarization with Bumetanide Results in Permanent Alterations in Cortical Circuits and Sensorimotor Gating Deficits', *Cerebral Cortex* **21**(3), 574–587.
- Wang, X.-J. and Buzsáki, G. (1996), 'Gamma Oscillation by Synaptic Inhibition in a Hippocampal Interneuronal Network Model', *Journal of Neuroscience* **16**(20), 6402–6413.

- Wang, Y., Liu, Y.-z., Wang, S.-y. and Wang, Z. (2016), 'In vivo whole-cell recording with high success rate in anaesthetized and awake mammalian brains', *Molecular Brain* **9**(1), 86.
- Waters, J. and Helmchen, F. (2006), 'Background Synaptic Activity Is Sparse in Neocortex', *Journal of Neuroscience* **26**(32), 8267–8277.
- Wehr, M. and Zador, A. M. (2003), 'Balanced inhibition underlies tuning and sharpens spike timing in auditory cortex', *Nature* **426**(6965), 442–446.
- Wiegert, J. S., Mahn, M., Prigge, M., Printz, Y. and Yizhar, O. (2017), 'Silencing Neurons: Tools, Applications, and Experimental Constraints', *Neuron* **95**(3), 504–529.
- Wietek, J., Beltramo, R., Scanziani, M., Hegemann, P., Oertner, T. G. and Wiegert, J. S. (2015), 'An improved chloride-conducting channelrhodopsin for light-induced inhibition of neuronal activity in vivo', *Scientific Reports* **5**(1), 14807.
- Wietek, J., Broser, M., Krause, B. S. and Hegemann, P. (2016), 'Identification of a Natural Green Light Absorbing Chloride Conducting Channelrhodopsin from *Proteomonas sulcata*', *Journal of Biological Chemistry* **291**(8), 4121–4127.
- Wietek, J., Wiegert, J. S., Adeishvili, N., Schneider, F., Watanabe, H., Tsunoda, S. P., Vogt, A., Elstner, M., Oertner, T. G. and Hegemann, P. (2014), 'Conversion of Channelrhodopsin into a Light-Gated Chloride Channel', *Science* **344**(6182), 409–412.
- Wildenberg, G. A., Rosen, M. R., Lundell, J., Paukner, D., Freedman, D. J. and Kasthuri, N. (2021), 'Primate neuronal connections are sparse in cortex as compared to mouse', *Cell Reports* **36**(11), 109709.
- Wilent, W. B. and Contreras, D. (2005), 'Dynamics of excitation and inhibition underlying stimulus selectivity in rat somatosensory cortex', *Nature Neuroscience* **8**(10), 1364–1370.
- Williams, S. R. and Mitchell, S. J. (2008), 'Direct measurement of somatic voltage clamp errors in central neurons', *Nature Neuroscience* **11**(7), 790–798.
- Wilson, H. R. (1999), 'Simplified Dynamics of Human and Mammalian Neocortical Neurons', *Journal of Theoretical Biology* **200**(4), 375–388.
- Woodin, M. A., Ganguly, K. and Poo, M.-m. (2003), 'Coincident Pre- and Postsynaptic Activity Modifies GABAergic Synapses by Postsynaptic Changes in Chloride Transporter Activity', *Neuron* **39**(5), 807–820.
- Woody, C. D. and Gruen, E. (1978), 'Characterization of electrophysiological properties of intracellularly recorded neurons in the neocortex of awake cats: A comparison of the response to injected current in spike overshoot and undershoot neurons', *Brain Research* **158**(2), 343–357.
- Wright, R., Newey, S. E., Ilie, A., Wefelmeyer, W., Raimondo, J. V., Gingham, R., McIlhinney, R. J. and Akerman, C. J. (2017), 'Neuronal Chloride Regulation via KCC2 Is Modulated through a GABA Receptor Protein Complex', *The Journal of Neuroscience* **37**(22), 5447–5462.
- Wright, R., Raimondo, J. V. and Akerman, C. J. (2011), 'Spatial and Temporal Dynamics in the Ionic Driving Force for GABA-A Receptors', *Neural Plasticity* **2011**, 1–10.
- Yamada, J., Okabe, A., Toyoda, H., Kilb, W., Luhmann, H. J. and Fukuda, A. (2004), 'Chloride uptake promoting depolarizing GABA actions in immature rat neocortical neurones is mediated by NKCC1', *The Journal of Physiology* **557**(3), 829–841.

- Yamashita, T., Pala, A., Pedrido, L., Kremer, Y., Welker, E. and Petersen, C. C. H. (2013), 'Membrane Potential Dynamics of Neocortical Projection Neurons Driving Target-Specific Signals', *Neuron* **80**(6), 1477–1490.
- Yelhekar, T. D., Druzin, M. and Johansson, S. (2017), 'Contribution of Resting Conductance, GABAA-Receptor Mediated Miniature Synaptic Currents and Neurosteroid to Chloride Homeostasis in Central Neurons', *eNeuro* **4**(2), 9–17.
- Yelhekar, T. D., Druzin, M., Karlsson, U., Blomqvist, E. and Johansson, S. (2016), 'How to Properly Measure a Current-Voltage Relation?—Interpolation vs. Ramp Methods Applied to Studies of GABAA Receptors', *Frontiers in Cellular Neuroscience* **10**, 1–10.
- Yona, G., Meitav, N., Kahn, I. and Shoham, S. (2016), 'Realistic Numerical and Analytical Modeling of Light Scattering in Brain Tissue for Optogenetic Applications', *eNeuro* **3**(1), 9–15.
- Zagha, E., Casale, A. E., Sachdev, R. N. S., McGinley, M. J. and McCormick, D. A. (2013), 'Motor Cortex Feedback Influences Sensory Processing by Modulating Network State', *Neuron* **79**(3), 567–578.
- Zajac, M., Chakraborty, K., Saha, S., Mahadevan, V., Infield, D. T., Accardi, A., Qiu, Z. and Krishnan, Y. (2020), 'What biologists want from their chloride reporters – a conversation between chemists and biologists', *Journal of Cell Science* **133**(2), jcs240390.
- Zemelman, B. V., Lee, G. A., Ng, M. and Miesenböck, G. (2002), 'Selective Photostimulation of Genetically ChARGed Neurons', *Neuron* **33**(1), 15–22.
- Zeng, H. and Sanes, J. R. (2017), 'Neuronal cell-type classification: challenges, opportunities and the path forward', *Nature Reviews Neuroscience* **18**(9), 530–546.
- Zhang, F., Wang, L.-P., Brauner, M., Liewald, J. F., Kay, K., Watzke, N., Wood, P. G., Bamberg, E., Nagel, G., Gottschalk, A. and Deisseroth, K. (2007), 'Multimodal fast optical interrogation of neural circuitry', *Nature* **446**(7136), 633–639.
- Zhang, Z., Russell, L. E., Packer, A. M., Gauld, O. M. and Häusser, M. (2018), 'Closed-loop all-optical interrogation of neural circuits in vivo', *Nature Methods* **15**(12), 1037–1040.
- Zhao, S., Ting, J. T., Atallah, H. E., Qiu, L., Tan, J., Gloss, B., Augustine, G. J., Deisseroth, K., Luo, M., Graybiel, A. M. and Feng, G. (2011), 'Cell type-specific channelrhodopsin-2 transgenic mice for optogenetic dissection of neural circuitry function', *Nature Methods* **8**(9), 745–752.
- Zhou, M., Xu, G., Xie, M., Zhang, X., Schools, G. P., Ma, L., Kimelberg, H. K. and Chen, H. (2009), 'TWIK-1 and TREK-1 Are Potassium Channels Contributing Significantly to Astrocyte Passive Conductance in Rat Hippocampal Slices', *Journal of Neuroscience* **29**(26), 8551–8564.
- Ziburkus, J., Cressman, J. R., Barreto, E. and Schiff, S. J. (2006), 'Interneuron and Pyramidal Cell Interplay During In Vitro Seizure-Like Events', *Journal of Neurophysiology* **95**(6), 3948–3954.

Seen from afterward the time appears to have been
all of a piece which of course it was but how seldom
it seemed that way when it was still happening...

Completion, W.S. Merwin (1927–2019)