# The role of the Retrosplenial Cortex in Associative Memory


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Aos meus pais e aos meus avós

por todo o amor.

To my parents and to my grandparents

for all the love.

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# Abstract

Encoding of episodic memories involves associating different features of the environment and experiences. Understanding how sensory-sensory associations are encoded would shed light on the basis of how events are stored in the brain.

The retrosplenial cortex (RSC) is known to reciprocally connect to the hippocampal formation and to sensory areas. Its connectivity suggests a role in both episodic and spatial memory processes. A role in the formation and storage of sensory associations might underlie these processes and is supported by RSC-lesion and -inactivation studies.

In this thesis, I use optogenetics to inactivate the RSC while mice perform various memory paradigms to clarify the function of this structure in encoding associations to support memory. Thus, I develop a sensory associative memory task in mice which allows me to test sensory-sensory associations. Developing, for the first time, an appetitive sensory preconditioning (SPC) paradigm in mice, failed to provide robust results. So, I chose an aversive SPC paradigm as the best way to probe neutral sensory associations in mice.

I show that while control mice successfully learned the preconditioned association in the task, precise closed-loop optogenetic disruption of the RSC when mice form associations between neutral stimuli reduces performance to chance levels. Moreover, I demonstrate that this disruption is independent of spatial confound.

Using the same technique to silence RSC activity during short-term or long-term spatial learning did not affect the formation and retrieval of memory, in contrast to previous studies.

Finally, in order to truly address the question of how sensory-sensory associations are formed, I present preliminary data of a calcium imaging study aimed at understanding the mechanisms by which these associations are formed in the RSC.

In summary, I established a new sensory preconditioning task for mice allowing us to timely probe associative memory between neutral stimuli. Optogenetic silencing of RSC demonstrate the necessity of the RSC in this type of memory and combined with future functional imaging studies will help understand how the brain forms neutral associations.

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# List of abbreviations

**aCC** anterior cingulate cortex

**AD** Alzheimer's disease

**ANOVA** analysis of variance

**AP** antero-posterior

**AS** auditory stimulus

**CaMKII** $Ca^{2+}$/calmodulin-dependent protein kinase II

**CC** corpus callosum

**CS** conditioned stimulus

**DAPI** 4',6-Diamidine-2'-phenylindole dihydrochloride

**FFW** free-feeding weight

**fMRI** functional magnetic resonance imaging

**GABA** gamma-aminobutyric acid

**HCl** hydrochloric acid

**HP** hippocampus

**IP** intraperitoneal

**ITI** inter-trial interval

**LED** light-emitting diode

**LiCl** lithium chloride

**MTL** medial temporal lobe

**NMDA** N-methyl-D-aspartate

**PBS** phosphate buffer saline

**PFA** paraformaldehyde

**PSTH** peristimulus time histogram

**Rdg** dysgranular RSC

**Rga** granular RSC a

**Rgb** granular RSC b

**ROI** region of interest

**RSC** retrosplenial cortex

**SAC** sound-attenuating chamber

**SEM** standard error of the mean

**SPC** sensory preconditioning

**US** unconditioned stimulus

**VI** variable interval

**WN** white noise

# Chapter 1

# Introduction

> "All science, even the divine science, is a sublime detective story. Only it is not set to detect why a man is dead; but the darker secret of why he is alive"
>
> G.K. Chesterton

It was getting dark, although it was not yet 5pm. I could barely enjoy the views from the window. When the spires started to show up, my stop arrived. I got off the bus and got my two massive suitcases from the driver. It was cold. Cold as I had not experienced in a long time. I had no mobile phone with me and only a paper map to find my way in that new city. The city that would be my home for the next four years.

That is how I remember my first arrival at Oxford. I can picture most of the details in my mind. That is how amazing memory is. I can relive an experience that happened a long time ago. An experience that is part of who I am today.

## 1.1 Memory: a physical process

### 1.1.1 The conception of memory

Because of this sense of how memory shapes who we are, of this power to time travel, human beings have concerned themselves with defining it and understanding it since ancient times. Among the many philosophers who have approached the subject Aristotle is considered to be the first one to study it scientifically (Burnham, 1888). Before him, Plato's definition of memory helps us understand how people would look at it:

> "The power which the soul has of recovering, when by itself, some feeling which she experienced when in company with the body" - Plato

For Plato and many others, memory was not part of our physical body and was more related to the soul. Aristotle challenged that. Although Aristotle claimed that the brain is only a cooling apparatus to counteract warmth that comes from the heart, he did link memory with a physical process that happens in our bodies. For Aristotle, memory was a movement that happens when we perceive things that continues to endure in our bodies even after the stimulus is gone.

In Aristotle's view of memory, images were its core substrate and abstract ideas would only be recollected if they involved images. He was one of the first to have presented the process of associations as important for recollection to occur. On the other hand, he stated that memory that relies on associations, was a faculty of men only, not animals.

Contrary to Aristotle, St. Augustine claimed, based on empirical evidence, that animals do have the capacity to associate and to form memories. He also argued that memory was not only formed of images, but also formed of stored impressions from the other senses and factual knowledge could also be remembered:

> "Everything there is preserved separately and in categories, and each has been placed there by means of its own proper access: for example, light and all the colors, and the shapes of bodies, go through the eyes; all kinds of sounds through the ears; all smells through the access of the nostrils; all tastes through the access of the mouth; and, from the sense of touch of the body as a whole, what is hard, and what soft, what is hot or cold, gentle or harsh, heavy or light, inside or outside the body. The vast recesses of memory, and the concealed and indescribable hiding places of one sort or another, receive all this material to be recalled as required and reconsidered: and every bit of it enters the memory by its own access route, and is replaced there. It is not the sensations themselves but the impressions of those sense experiences which are there at hand ready for the person whose thoughts bring them to remembrance". (Confessions Ch. 10 - XIII, (Augustine, 2016))

In summary, to Augustine, it was clear that memories were formed of impressions of sensations that were captured by our different sensory organs. He looked at memory with great regard and tried to convey its importance in his other book *De Trinitate* (Augustine et al., 2002) where he compared memory with God, the Father. Moreover, the idea that the brain was the organ responsible for memory and mental activity had gained strength; although, for Augustine, the ventricles were the region where memory and other neural processes would lie.

Once more, with Augustine, the importance of associations for memory was highlighted:

> "If, for instance, I wish to remember what I ate yesterday evening, I have already remembered either that I ate yesterday evening, or if I do not yet remember this, I have certainly remembered something that happened at this time; if nothing else then, at least, I remember yesterday and that

> part of it at which I generally eat supper, and what it means to eat supper. For if I remembered nothing of the kind, then I could not have the will to remember what I had eaten yesterday evening". (The Trinity, Augustine, (Augustine et al., 2002))

In this example given by Augustine, we can observe how associations between concepts, events and time are important for remembrance.

Many of the theories regarding memory that come after were based upon these three philosophers. The ideas that came did not immediately make use of scientific experimentation, other than maybe some basic anatomical descriptions of the brain. Proper scientific experimentation on memory will only be seen from the $19^{\text{th}}$ Century. Still, we can find value in some philosophical views by recognising concepts that today are obvious to us.

By the time the French philosopher Descartes started thinking about the pores of the brain as the place where memory was stored, all theories had embraced the idea that memory was dependent on a physical process and that this process happened somewhere in the brain. How people described this physical process varied. Some theorists claimed that memory is dependent on *movement* and/or *vibrations* that happen in the brain. Others theorised that sensory impressions leave a *mark* or *trace*, which explained memory storage. Another theory attested that impressions left the brain with a *disposition* that allowed that impression to be more easily remembered later on.

One certainly cannot say that these ideas were completely wrong. Even now, mostly what we know is that memories depend on neurons firing action potentials, which could be defined as a *movement* of charges across a membrane. We do know they can leave a *physical trace* such as the change in synapses. Finally, we know that encountering a stimulus for the first time will change how we possibly respond to it the next time through *plasticity mechanisms*. Thus, those ideas evolved over the next

centuries but their core is still very similar.

### 1.1.2 Remembering associations

It is interesting to note that the association of ideas was occasionally raised in relation to memory processes all throughout history. How from one idea we bring that next one. How, from observing an empty glass, one could visualise beer. These associations were the target of many questions since ancient times. Although most philosophers concerned themselves with explaining only what memory is and how it happens, some of them linked associations with its processes, as Aristotle and Augustine did. In the $18^{th}$ Century the British philosopher David Hartley, went even further and tried to show that all reasoning depends on associations. At that time, philosophers and physiologists did not know exactly what elements composed the nerve matter, still some claimed that the nerve elements form complexes through associations. It was also around that time that philosophers and experimenters started asking themselves: How would these associations happen? Some of the first hypotheses indicated that memories for concepts or sensory stimuli would lie in overlapping locations in the brain. Ideas that are similar or converge to a certain point will tend to change regions/elements in the brain which are overlapping or very close together, thus being associated (Burnham, 1888). These explanations of how associations worked were combined with the observation of how emotions can affect which ideas and sensory cues we associate. They led to the development of some concepts that were later used by researchers like Donald Hebb (Hebb, 1949) to explain plasticity in the brain. One of the most common concepts, brought by William James, was:

> "The amount of activity at any given point in the brain-cortex is the sum of the tendencies of all other points to discharge into it, such tendencies being proportionate (1) to the number of times the excitement of each other point may have co-existed with that of the point in question; (2)

> to the intensity of such excitements; and (3) to the absence of any rival locality or process functionally disconnected with the first point, into which the discharges might be diverted". - William James's Law of Association (James, 1890)

William James brought in his description of associations concepts that are still very useful to understand and to study associations. They were, nevertheless, very abstract. Further advances would only be observed when the field of physiology started to better understand how the brain and its units work. Scientific descriptions of the elements that compose the brain (Cajal, 1894; Golgi, 1873) gave rise to different theories of how the brain might work. While the Reticular theory, supported by people like Golgi, claimed that neurons form a giant network of continuous membrane (López-Muñoz et al., 2006); Cajal's observations indicated that they are actually isolated units, thus giving support to the Neuron doctrine (López-Muñoz et al., 2006). These and other advances in physiology started making the descriptions of how memory work a lot more detailed and helped explain how this physical process would work. Even before Charles Sherrington described the synapse (Sherrington, 1907), theories viewed the connections between neurons as the site of learning and memory. Cajal endorsed the idea that multiplying the connections between neurons underlie memory processes while others (Tanzi, 1893) claimed that the strengthening of existing connections is what makes a memory.

With the discovery, around that same time, that the brain is composed of functionally segregated regions (Brodmann, 1909), scientists came back to the question of where memories would seat in the brain. To scientifically identify this place, further developments in physiology and psychology had to occur. We needed to be able to test memories and associations.

### 1.1.3 Studying memories and associations

Around the time William James wrote "*The Principles of Psychology*" (James, 1890), what has been considered the first experimental work on memory was published by Dr. Hermann Ebbinghaus (Ebbinghaus, 1885). Although he only experimented on himself, the set of experiments and protocols he developed laid a groundwork for future experiments to come. Ebbinghaus spent years testing himself at the same time of day on memorising series of sequential non-sense syllables formed by vowel between two consonants. The series could have varied length. He tested for how long series of syllables would need to be repeated before being fully memorised and later on he asked how long it would take him to relearn them after variables intervals. He also tested whether how strongly the syllables were associated with one another in a same series by mixing different series while keeping together syllables from the same previously learned series.

Ebbinghaus's results were later expanded and confirmed by other physiologists. They greatly influenced Müller and Pilzecker who adapted Ebbinghaus's non-sense syllables to a series of experiments where for the first time they exposed subjects to a fixed number of training repetitions before comparing the number of correctly and incorrectly remembered associations between syllables. In their experiments, they observed that syllables had a tendency to persevere in one's mind. They also noticed that associations could be disrupted if attention was directed somewhere else in the first few minutes after the presentation of the stimuli (Müller and Pilzecker, 1900). This was the initial evidence used to propose the Consolidation Theory (Lechner et al., 1999).

Before Edward Thorndike's experiments, most of the concepts and experiments had a focus in humans. Even though philosophers and experimentalists thus far had observed animal behaviour and come to some of the conclusions mentioned above, most experiments with animals were focused on understanding their sensory organs.

In 1898, this started to change with a monograph from Thorndike where he exposed a set of experiments performed with dogs, cats and chicks (Thorndike, 1898). The focus of his experiments was understanding associations and how animals are able to form them. In his work, Thorndike described the first instrumental learning paradigm. He would put a cat inside a box and in order for the cat to escape from the box and get access to food, the animal would need to pull a loop or press a lever that then opened the door. He observed that after a few trials the animals were able to do the action required faster than at the beginning of the experiment, indicating that they had formed an association. In the end, Thorndike summarises his findings as such:

> "Our work has described a method, crude but promising, and has made the beginning of an exact estimate of just what associations, simple and compound, an animal can form, how quickly he forms them, and how long he retains them."

At about the same time Thorndike was experimenting on cats and dogs, Ivan Pavlov had started his research on the digestive glands of dogs (Pavlov and Thompson, 1910). When observing the response of glands to different stimuli, he decided to quantify this response and take proper notes on the stimuli presented. These observations led to the following experiment (Pavlov and Anrep, 1940):

Dogs went through a surgical procedure for the implantation of a salivary duct that would then allow Pavlov to quantify secretion from digestive glands. He made use of the inborn reflex, an unconditioned reflex, which causes an animal, which is hungry, to salivate when presented with food. For several times, a dog was presented with the sound of a metronome followed by the presentation of food. After several paired presentations, he observed that the metronome sound "had acquired the property of stimulating salivary secretion and of evoking the motor reactions characteristic of the alimentary reflex". He called this property a conditioned reflex. He also highlighted

the fact that a decerebrated dog could not be conditioned. This linked this type of conditioning to a dependence on central nervous system activity.

Both Thorndike's and Pavlov's experiments required associations between stimuli, the main difference being that Thorndike would make use of reward to teach an association and Pavlov used an unconditioned response to measure the association between two stimuli. Moreover, Thorndike's puzzle boxes required the animals to have an instrumental response to the initial stimulus (pressing a lever) in order to get to reinforcement, while for Pavlov's conditioning, no response to the stimulus paired with food was required. This is the difference between what we now call Classical (or Pavlovian) and Instrumental conditioning (Hilgard and Marquis, 1940). These experiments were expanded by Behaviourists, such as Watson, and later on Skinner with his description of operant conditioning versus classical conditioning (Skinner, 1935). The development of memory tests and behavioural paradigms that are able to evaluate acquisition, storage and consolidation of memories is, still today, extremely important in order to help us understand the molecular, cellular and systemic processes involved in storing and remembering information to survive in an environment.

## 1.2 A Place for Memory

### 1.2.1 Episodic Memory: The memory of experiences

So far, I have introduced here a general concept of memory. But since ancient times, people have noticed that there are various ways of remembering something. To remember the death of a relative is not the same as remembering how to ride a bicycle and not the same as remembering one's name. Naturally, even though there are ideas that are common to different types of memory, some of them discussed in the sections above, there are different elements to one or the other.

Long-term memories can be subdivided into two main categories: explicit and

implicit memories. Which means we can differentiate between memories that are consciously recollected and others that are not. Before that in 1972 Endel Tulving (Tulving, 1972) proposed in his book a categorisation of explicit long-term memory that is, till today, very much used. He suggested that consciously recalled long lasting memories could be split into two main categories: episodic and semantic memories. Semantic memory is the remembrance of facts, knowledge about words and verbal symbols. For example, to remember my name, or that the day is composed of a light and a dark period or to remember who the current Prime Minister is. Although, sometimes one cannot fully disentangle these types, the focus of this study are the memories that relate to events in our lives, to our experiences of the world: what we call episodic memories (Eichenbaum, 2000). Finally, Tulving also highlights that although it is possible to find examples of both types of memories, many times these will overlap.

### 1.2.2 How and where memories are stored?

Episodic memory is the recollection of events that happen in a specific context (Eichenbaum, 2000). It stores not only the events themselves but also the spatial-temporal relationships between them (Tulving, 1972). Events are composed of several sensory elements: e.g. scents, sounds, touch, images and spatial context (Davachi, 2006). Our ability to form these types of memories will be crucial to understanding the environment and surviving in it. At the beginning of this chapter, I described one experience that I have gone through here in Oxford. Our lives are made of a collection of those and that experience was made up of several events. The arrival was an event that I experienced. It had scents, images, feelings and sounds that I remembered. That is how a memory starts. It all starts with our bodies collecting these sensations. Our different sense receptors capture vibration, motion, molecules and transform their information into electrical currents. These currents go through

our periphery and enter our brain. Somewhere in the brain these sensations and the temporal and spatial associations between them are stored. But how? And where?

There are several disorders that serve as examples of the impact of impaired episodic memories in a person's life. This impact was best observed on studies with amnesic patients such as Scoville's patient, H.M. (Scoville and Milner, 1957), which triggered a search for the brain structures responsible for memory. H.M. was a patient who had an extensive bilateral lesion to the medial temporal lobe (MTL), affecting, in great part, the hippocampus. He displayed a partial retrograde amnesia (Scoville and Milner, 1957). He remembered past events that had happened three years before the operation but no events since then. He also could not form new episodic memories, although other cognitive abilities were intact. This immediately led to the conclusion that structures in the MTL could be the site for recent episodic memories. The medial temporal lobe is a large region. Thus our "place for memory" was still quite undefined. Using brain damage to better understand and study memory was, and still is, a common method. Studies on memory loss in patients with lesions of different structures in the brain gave support to the idea that specific structures would be the storage site of certain memory types.

Already in the $19^{\text{th}}$ Century, the idea that memories reorganise over time had been proposed based on observations of people with memory loss. H.M.'s iconic case demonstrated this idea that the MTL was important for encoding and initial storage with memories then migrating somewhere else. Later on other studies reported ungraded retrograde amnesia and full anterograde amnesia in a patient with a shrunken hippocampus but spared entorhinal cortex and other MTL structures (Cipolotti et al., 2001)creating a controversy with the proposed consolidation theory. Once human lesions were better analysed and studied, researchers realised that controversies were mainly caused by the fact that lesions were not as restricted to the MTL as initially thought (Squire et al., 2004).

Animal lesions presented an advantage with regards to human studies. Lesioning methods available were more extensive and could be better restricted to a given structure. The damage caused to an animal's brain could be more controlled and could be better and more readily evaluated. A series of studies with lesions in monkeys showed that the extent of memory impairment was dependent on the lesion size and locations in the MTL (Mishkin, 1978; Zola-Morgan et al., 1994), claiming that hippocampal lesions alone did not bring about a striking memory deficit. In the beginning most lesions relied in aspiration. Further advances in lesion studies allowed damage to be constrained to smaller regions, or restricted to cells, sparing *en passant* fibres. Electrolytic lesions in the hippocampus of rats after contextual fear conditioning led to deficits only if the lesions were made one day after learning and no deficits were observed for lesions after 7, 14 or 28 days (Kim and Fanselow, 1992). Consequently, the idea that specific damage to the hippocampus caused the partially graded amnesia outcome seen in humans gained support. Still animal lesions are not completely free from controversies. Until today, scientist still discuss the role of the hippocampus in contextual fear conditioning, now with the knowledge that the rodent hippocampus can be subdivided into a dorsal and ventral parts that might be involved in different functions (Anagnostaras et al., 2002).

The theory that the hippocampus is not the last storage site and long-term memory would not be dependent on this structure (Scoville and Milner, 1957; Squire, 1987) postulates that memories are dependent on the hippocampus for a short time and later, they would be stored in neocortical circuits (Frankland and Bontempi, 2005). Still, there was evidence that the location of memory processes was still an open question. A study by Goshen and collaborators (Goshen et al., 2011) contrasts with the previous result found by Kim and Fanselow (Kim and Fanselow, 1992) for contextual fear memories and the hippocampus. By using optogenetics to timely inhibit CA1 (a region of the hippocampus) during or after contextual fear conditioning, Goshen *et*

*al.* show that acquisition and retrieval of contextual fear conditioning is affected, including remote retrieval. Moreover, they show that when they extend the inhibition period to mimic lesions or pharmacological interventions the dependence of remote memories on the hippocampus started to fade (Goshen et al., 2011).

Following studies helped define the two main views regarding hippocampal function. The first is that hippocampus is necessary for episodic memory in order to create a relational network between the different stimuli and inputs it receives (Konkel and Cohen, 2009). On the other hand, many studies provided evidence to support a role in spatial memory. In 1976, place cell in the CA1 area of the hippocampus were first described (O'Keefe, 1976). These are cells that fire preferentially when an animal is in a specific position in space. Their discovery opened up the possibility that the hippocampus might hold a cognitive map (Tolman, 1948). A classical task to probe spatial memory is the Morris water maze task. The task involves placing an animal (rat, mouse) in a pool of water and the animal has to locate a hidden platform under the surface. The animal learns the location of the platform and with each session decreases the time taken to navigate to that point. It was by developing this task, that Richard Morris showed that hippocampal aspiration lesions caused a deficit in navigation (Morris et al., 1982). People have tried to find a common ground between its two proposed functions on spatial memory/navigation and episodic memory (Eichenbaum and Cohen, 2014; Burgess et al., 2002; Leutgeb et al., 2005; Buzsáki and Moser, 2013).

For the past years, most of the studies regarding episodic memory have focused on the hippocampus. Lesion, drug inactivation studies and, nowadays, chemo and optogenetic studies have come a long way to try and clarify the role of this structure on memory. On the other hand, it has also become clearer that studies with other structures of the MTL, other subcortical and cortical regions as targets are increasingly necessary.

### 1.2.3 The role of associations in episodic memory formation

Associations have been part of the concept of memory for a long time (see Section 1.1.2). Associations are the main components of what we call an event as an event could be described as a collection of related sensory stimuli in a certain context with its spatial-temporal relationships. Stimuli and context are somehow also associated with each other. Events organised in time, associated in time, will form an episode. Thus, formation of associations between sensory stimuli, objects and contexts are extremely important to an animal's life. These stored relationships are crucial to our decision-making processes (Bornstein et al., 2017).

A number of factors can affect how associations are stored as an episode. These factors can cause the strength of the associations to vary. Besides the factors mentioned by William James in his law presented on Section 1.1.2, some other factors could be the attentional state of the subject and the behavioural relevance of the stimuli being associated (Peters et al., 2009).

Associations are important for episodic memory on two levels. First, when encoding an event and then when retrieving that memory. Here, the main interest is in the first case when our brain combines sensory information into memory representations. The region where associative memories are formed would require a constant input of information regarding the surroundings that comes from several regions of the brain. A region such as the hippocampus, which is linked with both episodic non-spatial memory and spatial memory (Battaglia et al., 2011; Burgess et al., 2002), would seem like an appropriate place for associations to take place.

The medial temporal lobe had been implicated in associative memory in several studies (Sommer et al., 2005; Sperling et al., 2003). Imaging studies in humans also highlighted a specific role for the hippocampus in episodic memory, by showing that it was largely active when forming associations between items or stimuli of an episode (Henke et al., 1997). Another experiment, now in rats, highlighted the importance

of hippocampus in binding several multimodal information together by comparing hippocampal and amygdala contributions to contextual fear memory or cued fear conditioning (Phillips and LeDoux, 1992). They showed that hippocampus is necessary for contextual fear conditioning but not for cued fear conditioning, indicating a role in binding more multiple and complex stimuli. But these experiments also highlighted a role for other structures, such as the amygdala in forming associations. More evidence came from a study by Iordanova et al. which indicated that the hippocampus is not necessary for the association of a stimuli pair (Iordanova et al., 2011). Iordanova *et al.* demonstrated that NMDA (N-methyl-D-aspartate) receptors in the hippocampus are necessary for an animal to retrieve episodes but not to retrieve simple associations between stimuli components of that episode.

Moreover, other experiments show that the associative memory network might be far more complex. Lesions to perirhinal and entorhinal cortices abolished the representation of visual associative information in a higher visual area (Higuchi and Miyashita, 1996) and another MTL region, the parahippocampal cortex, was implicated in both spatial and nonspatial contextual associations (Aminoff et al., 2007). Different regions in the medial temporal lobe are linked with either associative recollection or familiarity (Aggleton and Brown, 2006; Mayes et al., 2007). All these studies highlighted how other structures might have a role in associative memory.

Lately, another region has arisen as a potential interface between primary sensory regions and the medial temporal lobe memory system. The retrosplenial cortex (RSC) is targeted by several cortical and thalamic regions and also strongly connects with the parahippocampal region and the hippocampal formation.

## 1.3 A hub for memory: the Retrosplenial Cortex

### 1.3.1 The Anatomy of the Retrosplenial Cortex

The Retrosplenial Cortex takes its name from its localisation in the human brain. It sits behind the splenium, the largest and most caudal part of the Corpus Callosum. It was first described by Brodmann (Brodmann, 1909) and extensive anatomical studies highlighted its transitional nature from a 3-layered structure like the hippocampus to a 6-layered cortical structure (area 23) (Vogt, 1976). Lately, it has attracted increased attention after many years of being marginally studied.

In rodents, the RSC corresponds to the entire posterior cingulate cortex in humans (Broadman areas 29 and 30) and is one of the largest cortical regions in these species (Figure 1.1A and B). It has different layers depending on its mediolateral position and can be separated onto a granular region (area 29) with 4 to 5 layers and a dysgranular (Rdg) one (area 30) (Vann et al., 2009) with 6 layers. The dysgranular region is the more dorsal part of the RSC (Figure 1.1B and C). The granular RSC is marked for a more densely packed layer 2-3 and for more granule cells between layers 3 and 5 (Figure 1.1C). It can also be subdivided further into at least two subregions (Rga and Rgb; Figure 1.1B and C). This nomenclature, used in this thesis, was suggested by Van Groen and Wyss (Van Groen and Michael Wyss, 1990) and later used in many other studies (Van Groen and Wyss, 1992; Wyss and Van Groen, 1992; Van Groen et al., 2004; Pothuizen et al., 2009).

In the rat, the RSC was shown to have strong intrinsic connections within and between its different areas (Figure 1.1C). The different areas will project to all subdivisions in the parahippocampal region and to the hippocampus (Wyss and Van Groen, 1992; Sugar et al., 2011). One of its main connection is the anterior and dorsolateral nuclei of the thalamus, which is a known relay of sensory information. This is complemented by direct connections from different primary and secondary

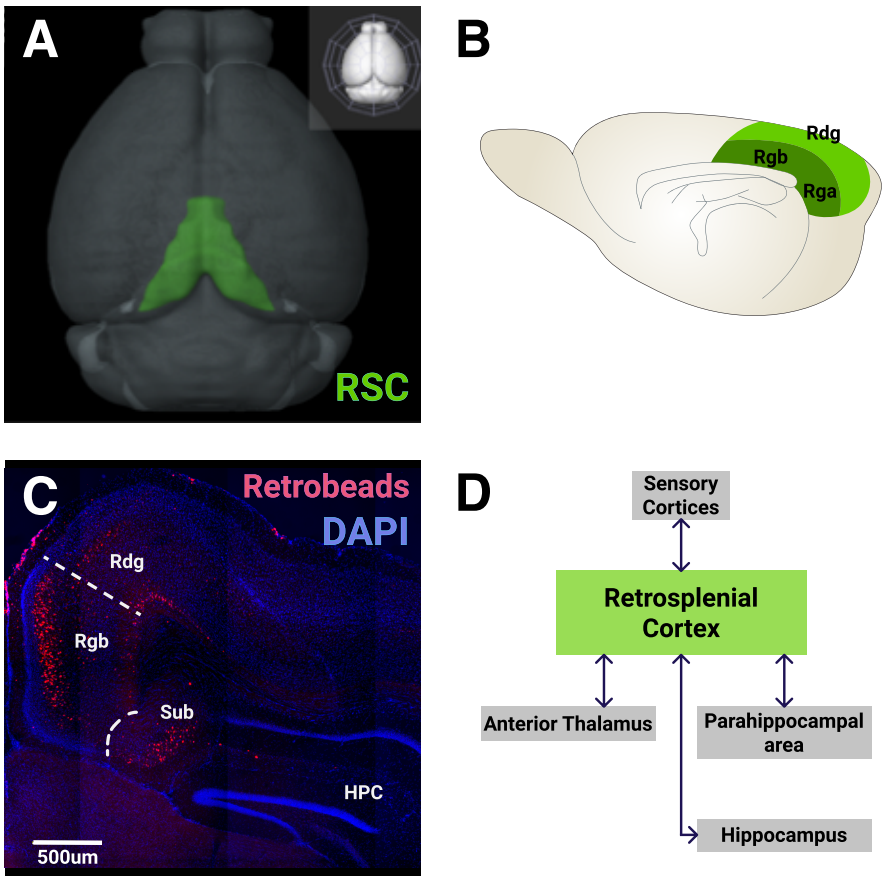



**Figure 1.1: The anatomy of the retrosplenial cortex.** (A) Sagittal view of the RSC (green) in a mouse brain. Adapted from the Allen Brain Atlas. (B) View of the RSC in the rat brain. The divisions are highlighted in shades of green. Rdg - dysgranular RSC (light green); Rga - granular RSC a and Rgb - granular RSC b (dark green). Adapted from (Vann et al., 2009). (C) Coronal slice of a mouse brain with the divisions of the RSC. Retrobeads (Red) were injected in the RSC. They mark cells that connect intrinsically and cells in the Subiculum (Sub) that connect to RSC. HPC - Hippocampus (D) Schema of connections of the RSC to the different regions.

sensory areas (Figure 1.1D). A connection with motor areas has also been described and it connects reciprocally with frontal areas (Vann et al., 2009), thus connecting the hippocampal formation with cortical regions related to sensory and executive functions.

Below it is summarised the main connections and later relate them to current known functions of the retrosplenial cortex. These connections were identified mostly in rats but also some were identified in monkeys.

#### 1.3.1.1 Intrinsic connections

Extensive intrinsic connections between all areas (Rdg, Rga and Rgb) have been described (For a review see (Sugar et al., 2011; Shibata et al., 2009)). Most connections are reciprocal but longitudinally it is observed that rostro-to-caudal connections are more common (Shibata et al., 2009). Moreover, caudal parts of the different areas are more strongly connected between each other, while rostral parts will preferentially connect with the rostral parts of the other areas.

While ipsilateral connections between the areas within the RSC mostly originate in deeper layers, the controlateral connections come from layers 2/3 and 5 of each area. Both controlateral and ipsilateral connections terminate in layers 1, 2/3 and 5 of the different RSC areas (Shibata et al., 2009).

Despite this general pattern of intrinsic connections, RSC's areas present differences in how strongly connected they are to each other (Shibata et al., 2009). Thus, indicating that these different areas might serve differently to RSC function.

#### 1.3.1.2 RSC - Hippocampal Formation

Dysgranular RSC has reciprocal connections with subiculum, postsubiculum and entorhinal cortex. It also projects to the parasubiculum and to the perirhinal cortex (Wyss and Van Groen, 1992). The projections to parasubiculum and subiculum have

a topographic organization (Sugar et al., 2011).

The granular RSC a shows reciprocal connections with postsubiculum and presubiculum. It also receives projections from a different number of hippocampal formation regions. Besides the already mentioned connection, it receives input from the CA1 and subiculum, while also projecting to parasubiculum and entorhinal cortex. The granular RSC b, on the other hand, has reciprocal connections only with the postsubiculum, where it sends some of its main projections (Van Groen and Wyss, 2003). But it does receive projections from subiculum and the CA1 area (Wyss and Van Groen, 1992). In fact, all rostro-caudal levels of the subiculum will send projections to Layer 1 of the granular area of the RSC. Subiculum connections from hippocampus to RSC will target layers I, II and III in the RSC (Sugar et al., 2011). Connections are largely excitatory but long-range GABAergic projections from CA1 to retrosplenial cortex have also been described (Jinno et al., 2007).

#### 1.3.1.3 RSC - Thalamus

One of the main inputs to the retrosplenial cortex comes from the thalamus, more specifically the anterior thalamic nuclei (Shibata, 1993, 1998). While the anterodorsal thalamic nucleus projects to the granular region of the RSC (both Rga and Rgb), only Rgb area weakly projects back to the anterodorsal nucleus (Van Groen and Michael Wyss, 1990; Van Groen and Wyss, 2003). A few projections from Rgb also reach the reticular nucleus, and the nucleus reuniens of the thalamus (Van Groen and Wyss, 2003).

The laterodorsal nucleus have lighter projections to the granular area and a stronger input to the dysgranular RSC (Van Groen and Wyss, 1992). Nevertheless it receives projections from all RSC areas that finish in different regions of the laterodorsal nucleus (Van Groen and Michael Wyss, 1990; Van Groen and Wyss, 1992). The anteromedial and lateroposterior nuclei have reciprocal connections to the dysgranular

RSC only.

These thalamic nuclei, the hippocampus and the RSC were co-activated in a study looking at c-Fos expression after spatial memory tasks (Vann et al., 2000) and anterior thalamic nuclei lesions led to deficits in the water maze reference task (Van Groen et al., 2002).

#### 1.3.1.4 RSC - Primary Sensory cortices

One of the main described reciprocal connections is with visual cortical areas (Vogt and Miller, 1983), mainly through dysgranular RSC. In the mouse, besides the RSC projection to the primary visual cortex (Makino and Komiyama, 2015), RSC also sends axons to Layer 1 and 5 of the barrel field of the somatosensory cortex (unpublished observations from our lab). Afferent connections from the auditory cortex to the granular RSC have also been described (Munoz-Lopez et al., 2010). Moreover, the RSC receives multimodal information from the primary sensory areas through area 2, a cortical region surrounded by visual, somatosensory and auditory cortices (Hishida et al., 2014). Consequently, through these anatomical connections it provides major polymodal inputs to parahippocampal areas (Burwell and Amaral, 1998).

#### 1.3.1.5 RSC - Frontal cortices

Strong reciprocal connections with anterior cingulate regions have been described with the rostral anterior cingulate cortex (aCC) connecting reciprocally with caudal RSC and rostral RSC connecting with caudal aCC (Shibata et al., 2004; Jones et al., 2005).

Very few prelimbic connections to RSC have been described (Fisk and Wyss, 1999; Shibata and Naito, 2008). However, projections from primary and secondary motor cortices (Yamawaki et al., 2016) and orbital cortex exist (Shibata and Naito, 2008).

#### 1.3.1.6 Other projections

Other efferent projections include axons sent to the rostral pontine nuclei, the caudal dorsomedial part of the striatum, raphe nuclei, the locus coeruleus, the diagonal band and the mammilary bodies (Van Groen and Wyss, 2003). Very light projections from the basal nucleus of amygdala have been described (Buckwalter et al., 2008) in a retrograde tracing study in monkeys but they were not confirmed by a different study (Aggleton et al., 2012) when using anterograde tracing.

In summary, the RSC has extensive connections with sensory, memory and executive function areas. It is possibly one of the main relays of sensory information to the hippocampus and it also projects back to primary sensory areas possibly providing contextual information or some kind of top-down modulation of sensory perception (Makino and Komiyama, 2015). Its subregions show different connectivity, indicating different roles in its functions.

For example, while granular RSC will receive major inputs from the thalamus, the dysgranular RSC will communicate with primary visual cortex (Van Groen et al., 2004; Pothuizen et al., 2009, 2010).

### 1.3.2 Retrosplenial Cortex Function

Taking into consideration the RSC connectivity, one would think that it must have a role in memory. As it is extensively connected to the medial temporal lobe structures that have so much been implicated in memory processes. Even more, it is remarkable how human lesions in the RSC showed a similar deficit as the one caused by medial temporal lobe lesions with the same graded retrograde amnesia descriptions (Valenstein et al., 1987). But that was not the first proposed function of the RSC. This region was first mentioned by Papez when he proposed the circuit, that took his name, involved in emotion, indeed, despite a few sparse efforts to understand the role of the retrosplenial cortex. It is still unclear what is its main function. Studies so far have pointed to two

possible functions that are probably related to each other. One in spatial memory and navigation and another function in episodic memory (or episodic-like memory) (For reviews see (Miller et al., 2014; Mitchell et al., 2018)). Below I describe in more detail how the RSC might take part in these processes.

#### 1.3.2.1 Spatial Memory and Navigation

Studies on human brain lesions have indicated a role of the RSC in memory but did not observe deficits in visuospatial abilities at the time (Valenstein et al., 1987). Initially, they only describe problems with graded retrograde and anterograde episodic memory. Other studies came to revealed that retrosplenial lesions caused problems with navigation (Takahashi et al., 1997; Katayama et al., 1999). This first accounts of damage to the retrosplenial cortex used the term pure topographical disorientation to describe people that were unable to find their way in the environment. But this term is quite broad and vague and there are many reasons that could lead to this same diagnosis which involve whether the sense of egocentric space has been disturbed, the allocentric spatial representations or even an inability to recognise landmarks (Aguirre and D'Esposito, 1999). Thus, it was natural to pursue the reason why RSC damage could lead to that outcome.

Evidence then showed that topographic disorientation due to damage to the RSC was more related to the inability of using allocentric cues than related to egocentric representations (Takahashi et al., 1997; Katayama et al., 1999). Lesions also led to patients failing to navigate familiar environments even when recognising individual landmarks and there are reports where they were unable to navigate new environments (Vann et al., 2009). This was corroborated by imaging studies that showed RSC activation when humans performed such tasks (For a review see (Maguire, 2001)). Moreover, RSC activity seems to be more related to the relationship between landmarks (Dilks et al., 2011) and more responsive to permanence of landmarks (Auger et al.,

2012).

Despite these studies, brain damage in humans is hard to define and specify, so observed deficits could be due to lesions spreading beyond the retrosplenial cortex. Lesion studies in rodents were necessary to confirm this RSC function. Several lesions studies in rats showed deficits in spatial navigation tasks such as the water maze (Sutherland et al., 1988; Whishaw et al., 2001; Harker and Whishaw, 2004) or T-Maze Alternation tasks (Pothuizen et al., 2010). Since, the rodent RSC is proportionally large in these species, some initial lesion studies were unable to target the whole structure or lesions hit the cingulate bundle creating confusion on the role of RSC in spatial memory. Further lesion studies using different techniques were necessary to relate the RSC with this function (Vann and Aggleton, 2002; Vann et al., 2003; Vann and Aggleton, 2004).

When excitotoxic lesions were extensive to the full RSC extent, animals displayed deficits in task that test allocentric memory (Vann and Aggleton, 2002). This was in contrast with previous studies where lesions spared most the caudal part of the RSC (Neave et al., 1994). When damage was restricted to only caudal lesions of the RSC the deficits were less pronounced (Vann et al., 2003). Electrotoxic lesions in the RSC showed its sensitivity to spatial change by removal of a landmark but not to stimulus novelty (Parron and Save, 2004). These studies (Vann and Aggleton, 2002; Parron and Save, 2004) confirmed that RSC is necessary for using landmarks to navigate. Other studies highlighted the importance of an intact RSC to solve tasks where cues are put in conflict (Pothuizen et al., 2008).

Further evidence of the role of the retrosplenial cortex in spatial memory and navigation came from studies looking at RSC activation during behaviour. c-Fos activation in the RSC was observed when rats went through a working memory version of the radial-arm maze when an animal has to visit all arms before completing a trial (Vann et al., 2000) but not when they were just running up and down one of the

arms of the maze. Interestingly, in the same study, further activation of the RSC was not detected when the maze was moved into a new room, indicating that RSC is not involved in processing novel stimuli. Also, the discovery that around 10% of cells in the retrosplenial cortex exhibit head-direction signals, highlighted the possibility of its main function being spatial related (Chen et al., 1994; Cho and Sharp, 2001). Longitudinal c-Fos experiments also showed that RSC holds a spatial memory engram that becomes stabler with learning (Milczarek et al., 2018).

A few studies led to the hypothesis that RSC role in spatial memory would involve a role for this structure in shifting between frames of reference when navigating in space. Shifting from using extramaze cues to intramaze ones. A shift from an allocentric frame of reference using landmarks to a egocentric frame. The importance of saving the relationship between landmarks and even the described head-direction signal could be used for this purpose. For example, when changing the relationship between cues (Vann and Aggleton, 2002; Pothuizen et al., 2008), or switching from performing the task in light or in the dark (Pothuizen et al., 2010).

Another way the retrosplenial cortex could be supporting spatial memory is through forming or storing contextual information. Context could be defined as a group of background cues that remain present (Miller et al., 2014). Many studies implicate the RSC in processing those background cues (Keene and Bucci, 2008a,c).

#### 1.3.2.2 Context or associations?

Valenstein's patient T.R. with lesions in the retrosplenial area leads to a possible different function for the retrosplenial cortex (Valenstein et al., 1987). T.R. presented a similar diagnosis as patient H.M. (Scoville and Milner, 1957; Milner et al., 1998) with both a retrograde and an anterograde amnesia. Despite possible issues with the actual lesion location, other studies showed that RSC lesions lead consistently to memory deficits, especially with autobiographical memory (Maguire, 2001).

In humans, the RSC is also part of what have been called the default mode network (Raichle et al., 2001). This group of structures is active during rest and most regions are then silent when performing tasks. It is thought that this network acts on internal processing of memories, where context would be an important part of it. Even though, RSC's role in this network has yet to be clarified.

The default mode network is known to be impaired in Alzheimer's disease (Buckner, 2005), which greatly affects episodic memory. Hypometabolism of the RSC has been implicated in mild cognitive impairment patients (Nestor et al., 2003) and this impairment can be noticed very early on (Minoshima et al., 1997). These observations combined with the discovery of a RSC dysfunction in an AD mouse model (Poirier et al., 2011) might indicate a role for the retrosplenial cortex in memory deficits observed in AD patients.

As mentioned in Section 1.2.2, context information is part of what forms an event. The idea that the RSC could contribute to context representation could also be related to this region's function in episodic memory. Moreover, in order to form a context representation we must connect cues from different sensory modalities and RSC's position in the brain and connectivity seems to indicate a region that could be doing exactly that.

Human and rodent studies gave great support to the idea of RSC working towards context representations. Subjects show greater RSC activation when they view objects with a strong contextual association (Bar and Aminoff, 2003). Also, reactivation of a contextual memory ensemble in the RSC was sufficient for behaviour expression (Cowansage et al., 2014). This role in context representation could be underlying both the spatial and episodic memory function of the RSC.

To form a context representation the RSC would need to combine multimodal cues (Sutherland and Rudy, 1989; Rudy and Sutherland, 1995) even when they might not be behaviourally relevant. A study showed that RSC-lesioned rats were unable to use

acquired contextual information to disambiguate between two conflicting response cues, even though, they were able to dissociate contexts and associate an outcome with a specific context and its associated cues (Nelson et al., 2014). Which indicates that the contextual representation might be stored somewhere else.

Two recent studies implicate RSC in associative learning paradigms (Robinson et al., 2014; Makino and Komiyama, 2015) and there is a growing amount of evidence that the retrosplenial cortex might have a role in forming associations between concurrent or serial stimuli (Robinson et al., 2011). There are indications that it would be more important for binding stimuli which have not yet acquired behavioural value. A feature that could underlie its function in contextual memories. The RSC privileged position combined with the evidence from these studies highlight a role for RSC in the formation of sensory associations.

## 1.4 Scope and organization of the thesis

Experimental approaches to study memory date back to 1885. Despite the development of many different ways of investigating memory, it has always been difficult to precisely target different memory processes. One of these processes is to form associations to support memory. One specific type of association that might be important for spatial, episodic and contextual memory is neutral sensory associations.

One would think that a region where multiple types of information converge to would be a good target to study the formation of associative memory which groups such types of information together. Yet, the retrosplenial cortex has been somewhat neglected as a site for the possible formation of memories other than spatially related ones. Thus, I set out to further our knowledge of how this brain region might act to aid the formation of associations.

In this thesis, I will use optogenetics, which allows me to inactivate neuronal cells

with timely precision, to understand RSC function in a combination of spatial and associative memory paradigms. Since genetic methods are most commonly used in animal research with mice and most RSC studies had been previously developed in rats, I had to first establish specific behavioural paradigms using mice as subjects.

Thus, in order to add to the discussion on whether a role of retrosplenial cortex in sensory associations would underlie the different aspects of its described functions in spatial and episodic memory and to better understand how associations might be encoded in the brain, this thesis set out to achieve the following aims:

1. Establishment of a robust sensory associative task (Chapter 3) to probe neutral associations in mice;

2. Describe the effects and confirm optogenetic silencing of retrosplenial activity with electrophysiological recordings (Chapter 4) in order to validate this technique;

3. Describe the effect of disrupting RSC activity optogenetically *in vivo* in freely moving animals while performing spatial (Chapter 5) or non-spatial behavioural paradigms (Chapter 4);

4. Probe the role of the RSC in forming associative memories when they do not involve a spatial component (Chapter 4);

5. Develop a calcium imaging study to understand how associations are formed in the brain (Chapter 4);

# Chapter 2

# General Methods

## 2.1 Experimental Procedures

### 2.1.1 Animals

All animal husbandry and experimental procedures were approved and conducted in accordance with the United Kingdom Animals (Scientific Procedures) Act 1986 under project and personal licenses from the Home Office.

Animals used in all behavioural and electrophysiological experiments were C57Bl/6 male mice (*Mus musculus*) and unless stated otherwise animals were obtained at various ages from the University of Oxford's animal facility colonies. A total of 152 male mice were used for all behavioural experiments and four for electrophysiology. Due to technical issues with LED headstage implants, 15 animals had to be excluded from the results.

For calcium imaging experiments, a total of four mice were used, all bred from a C57BL/6 background. A transgenic line constitutively expressing the fluorescent calcium indicator GCaMP6s in excitatory neurons was chosen for these experiments. These were obtained by crossing DBA-Tg(tetO-GCaMP6s)2Niell (Jackson Laboratory, Stock no. 024742) and (CaMKIIa-tTA)1Mmay mice (Jackson Laboratory, Stock no.

003010).

Whenever possible, mice were group-housed in holding cages (Mouse Cage M3, NKP cages) and were kept in a mouse housing room with a 12-hour light/dark photoperiod (light onset at 7:00 am). All procedures were conducted within the light part of the photo-period. The mouse holding room was maintained at a temperature of 23 $\pm$ 1$^o$C and humidity was set to 40 $\pm$ 10%. Mice with implants were kept in a taller and bigger cage to avoid damage to the implant.

The mice were fed low-fat mouse chow in the form of pellets and had access to food and water *ad libitum* in their home cages, unless one or the other were restricted during experimental procedures. Each mouse's free-feeding weight (FFW) was calculated from the average weight obtained from daily weighing's during three consecutive days before starting water or food regulation procedures.

### 2.1.2 Surgical procedures

Under anaesthesia, 6 to 7 weeks old mice received surgery for opsin expression through viral injection and had a light-emitting diode (LED) implanted on top of the skull.

Anaesthesia was induced via inhalation of 4% isoflurane (Zoetis) at 1 L/min. When mice were fully anaesthetized, they were placed in a stereotaxic frame (Kopf instruments). The depth of anaesthesia was monitored by checking pedal withdrawal reflex and level of isoflurane was kept at initially 0.5-1% at 0.4 L/min during surgery. Before surgery, mice received injections of meloxicam (5 mg/kg, Metacam) and vetergesic (0.1 mg/kg). A marcaine (2mg/kg) injection under the head top skin was used for local anaesthesia. The animal eyes were protected by a lubricant (Lacrilube, Allergan) to prevent them from drying during the procedure. The animal's temperature was controlled by a closed-loop temperature system (DC Temperature Controller, FHC).

#### 2.1.2.1 Injections and implant for optogenetics

*LED implant:* Custom made red (630nm wavelength) LED headstages (Newbury) were used for optogenetic illumination. Each headstage used was tested and cleaned to avoid contamination before being implanted.

*Surgical procedure:* Animals were put under anaesthesia as described above. A circular incision was made, and the skull was cleaned and scraped in preparation for the craniotomy. A 1.8 mm diameter craniotomy centred in the midline and at -2.05 mm from Bregma was made using a dental drill (Microtorque II, Circuit Medic) while keeping the *dura mater* intact. Injections of either rAAV9/CaMKIIa-Jaws-KGC-GFP-ER2 (North Carolina Vector Core) or rAAV5/CaMKII-EYFP (North Carolina Vector Core) were made using an automated injector (Nanoject II, Drummond Scientific) with micropipettes of thin-walled borosilicate glass (3.5 inches, Drummond). Mice received injections at four sites (DV: $\pm$0.3 mm; AP: $-1.8$ and $-2.3$ mm) targeting the RSC. At each site, 50 nL injections were made at different depths (from dura: $-0.8/-0.6/-0.4/-0.2$ mm) at a 50 nL/min rate. The injection needle was left in place for 4 min before being removed from each site.

Finally, red LED implants (custom-built, Figure 2.1C) were cemented (Simplex Cement, Kemdent; Superbond C&B, Sunmedical) on top of the craniotomy. After surgeries, animals were kept in a thermoregulated chamber until recovered from anaesthesia. They were then transferred to post-operative cages where chow mash and dried fruits and seeds were available. If necessary, animals were provided with jelly containing Metacam for analgesia.

#### 2.1.2.2 Chronic window implantation

*Chronic imaging windows:* Custom imaging coverslips were designed by stacking 1 $\times$ 3 mm wide and 1 $\times$ 4 mm wide round coverslips (total thickness 0.45 mm), kept together by means of an ultraviolet-cured optical glue (Norland 81).

*Surgical procedure:* For calcium imaging, instead of injections and the LED implant, mice received a cranial window over the RSC. A 3 mm craniotomy was made around the midline in the right hemisphere (position of centre relative to Bregma: 2 mm posterior and 0.5 mm lateral). An imaging window was placed, with the 3 mm wide stack fitting in the craniotomy and the 4 mm wide coverslip allowing it to be cemented to the bone. This way the coverslip replaced the cranial bone and protected the brain from potential sources of infection. Together with the cranial window, mice were implanted with custom-built titanium headplates for head-fixation. The plate allowed for head-fixation from both sides of the animals' heads and were also cemented with dental cement (C&B Super-Bond) to the clean dry skull.

### 2.1.3 Behavioural procedures

Behavioural experiments started three weeks after viral injections or when the animals were 10-weeks-old. Before any behavioural experiment started animals were handled for as long as needed for them to get used to the experimenter. Usually for at least three days. On the handling days, mice would be allowed to sit on the experimenter's hand or arm for around one minute and also be weighed so that the free-feeding weight (FFW) could be measured. This measurement was used for controlling water or food restriction according to protocols below.

#### 2.1.3.1 Food regulation procedure

On the first day of food regulation, mice were moved to a different cage where no food chow was available. Animals still had *ad libitum* access to water during all days of procedure. On the following days, mice were given around 2-4 g of crushed chow per mice depending on the weight loss. Mice were kept at 90% of free-feeding weight during the studies. Since mice were still young, they were allowed to grow 1 g/week over their free-feeding weight until they reached 14 weeks of age. After around a week

of food regulation, behavioural experiments started.

#### 2.1.3.2 Water regulation procedure

Animals had *ad libitum* access to chow in their home cages during water regulation procedures. But since, water availability is important for chow consumption, water availability was gradually decreased in order to not cause a harsh weight drop. On the first day, water bottles were removed just before the start of the dark cycle. The next morning, water was made available again throughout the light cycle and removed just before the dark cycle began. On the 3$^{rd}$ day, mice had access to water only for two hours during the light cycle (around the time when behavioural testing would be happening). On the 4$^{th}$ day, access was limited to just 1 h during the light cycle and on the 5$^{th}$ day, behavioural testing started. During testing, animals would typically receive a total amount of water of 1-1.5 mL of liquid rewards. If necessary, animals were given extra access after daily behavioural testing was completed. Mice were kept between 85 to 90% of free-feeding weight.

#### 2.1.3.3 Behavioural apparatus

*Operant boxes:* sensory preconditioning experiments were conducted on two possible setups. The first setup was composed of 8 standard operant conditioning chambers (Med Associates) enclosed in sound-attenuating chamber (SAC) outfitted with an exhaust fan (Figure 2.1A). A food well, equipped with an infrared beam to detect head entries, was mounted on one of the walls. The food well was linked to syringe pumps (Med Associates). A house light on the opposite wall served as the visual stimulus (flashed at 2 Hz) and a speaker was used to present the auditory stimuli (a 3000 Hz pure tone (85 dB) or a white noise (85 dB)). All equipment was controlled by commercially available hardware and software (MED Associates). The second setup had eight identical custom-built operant boxes (12 cm x 12 cm x 12 cm), enclosed in

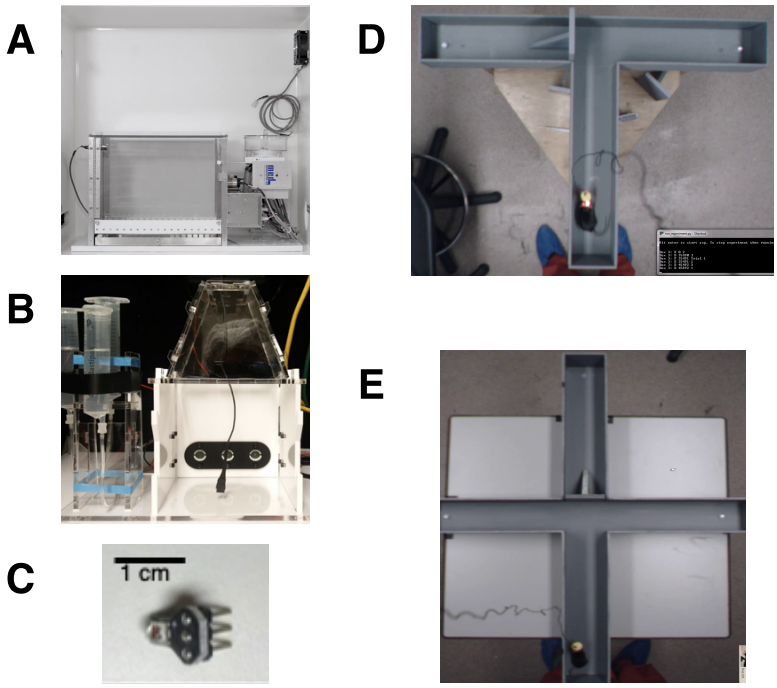



**Figure 2.1: Behavioural apparatus.** (A) Operant boxes used for sensory preconditioning experiments. Houselight was located on the left side while the magazine for reward delivery behaviours was located on the right. (B) Custom made operant box for combining optogenetics and behaviour. At the back it is possible to see three nosepokes for reward delivery. On the left, syringes filled with different solutions supply the solenoids in the back (not shown). (C) LED implant for optogenetic activation. (D) T-maze used for T-maze alternation task. It is possible to observe a mouse running down the main arm during a sample run. The implant in the mouse's head is connected and a yellow tag was placed for tracking. The left arm is blocked. (E) Cross maze used for the reference spatial memory task. A mouse is seen running down the South arm while connected for optogenetic manipulation. The opposite arm is blocked so the cross-maze turns into a T-maze. A yellow tag is present for tracking.

custom-built SAC with an exhaust fan (Figure 2.1B). They contained an extended ceiling with an aperture to allow freely moving optogenetic manipulation through an $\approx$ 15 cm long cable with a connector that would fit the implanted LEDs. The cable was connected to hardware through a 3-D printed commutator fitted with ball bearings. The back panel wall contained three nosepokes with backlight, infrared beam detection and a central hole where tubes connected to solenoid valves deliver liquid rewards. Houselights were mounted on the side walls of the SAC. All boxes were controlled through open source behavioural hardware and software (PyControl).

*T-Maze:* The T-Maze used for experiments described on Chapter 5 was a wood maze with grey non-reflective painted walls and floors (Figure 2.1D). Its main arm measured 10 x 46 cm and each side arm had 10 x 35 cm and the maze was elevated from the floor by 120 cm. Side walls were 10 cm high. Small food cups (custom made, Diameter: 1cm) could be placed at 2 cm from the end wall of each arm. Three arm blockers (10 x 15 cm) made from the same material and paint as the maze were used to block access to arms when necessary. The maze was placed in the centre of a behavioural room with standard lighting. The room had different objects positioned on each of the four walls of the room.

*Cross-Maze:* For the Cross-Maze experiments described on Chapter 5, a maze with the shape of a cross with two opposite longer arms of 10 x 45 cm and two opposite shorter ones with 10 x 35 cm was placed on top of a table in the centre of the behavioural room (Figure 2.1E). The side walls had 10 cm. The maze was painted with a non-reflective grey colour. Arm blockers (15 cm) were used to prevent access to the arm opposite to the starting arm and to keep the animal in the arm chosen once it had made a turn. Printed pattern cues were placed at 0.5 m distance from the end of the short arms.

### 2.1.4 Calcium imaging

Handling and water regulation, as described in Section 2.1.3.2, started when mice reached 13/14 weeks of age.

*Imaging setup:* A custom-built running wheel allowed head-fixed animals to run freely during the experiment. The wheel had a rotary encoder (Avago HEDM-55xx) attached to detect running speed. The encoder was connected to a PyControl breakout board through an adapter board (PyControl). A liquid delivery spout was placed in a movable spout holder for reward delivery. The spout was connected through rubber tubing to solenoid driven by a lickometer board (PyControl) which also allowed the detection of licks. All hardware was connected through a PyControl breakout board that allowed simultaneous start of behaviour and imaging software.

Calcium transients in excitatory neurons were detected using a Sutter MOM two-photon microscope controlled by ScanImage v.5.3 software. A Mai-Tai laser (SpectraPhysics; 70 fs pulsewidth, 80 MHz repetition rate) emitted a light tuned to 900 nm to excite GCaMP during imaging. A resonant scanner in bidirectional mode was used at a resolution of 512 $\times$ 512 pixels (acquisition rate at $\approx$ 30 Hz) to acquire fluorescent emission with a 16X/0.80W LWD immersion objective (Nikon) and GaAsP photomultipliers (Hamamatsu) to detect emitted photons. The field of view was 570 $\times$570 µm and was imaged up to 350 µm below the cortical surface. The microscope was fully enclosed in a custom-built dark chamber.

### 2.1.5 Histological procedures

Animals were anaesthetised with sodium pentobarbital (60 mg/kg, Pentoject) intraperitoneally and transcardially perfused initially with 0.1 M phosphate buffer saline (PBS, Sigma Life Sciences) followed by 4% paraformaldehyde (PFA, TAAB Laboratories) in 0.1 M phosphate buffer (pH 7.4). Brains were kept overnight in PFA at 4$^o$C and then changed to 0.1 M PBS and stored at 4$^o$C until histological procedures.

Perfused brains were processed in 50 $\mu$m slices using a vibratome (VT1000S, Leica) or a microtome (Leitz Wetzlar) and collected in PBS. One in every six slices was chosen for analysis and imaging. Slices were incubated in DAPI (4',6-Diamidine-2'-phenylindole dihydrochloride, Acros Organics) and mounted on microscope slides (Fisherbrand) in mounting medium (Vectashield, Vector) and covered with a coverslip (VWR) for imaging.

Images of whole brain slices for analysis of virus expression were obtained from a multichannel fluorescence microscope (EVOS FL Auto Imaging System, Life Technologies) with an automatic objective at 10X magnification. Detailed images of slices from brains used for electrophysiological recordings were obtained from a laser scanning confocal microscope (FV1000, Olympus), equipped with an air 20X/0.75 NA and an oil-immersion 40X/0.8 NA objective.

## 2.2 Data Analysis

### 2.2.1 Histological analysis

Image analysis software ImageJ (Fiji) was used to analyse and quantify viral expression area in histologically processed brain slices. Using acquired images of whole brain slices, viral expression area was manually determined and traced with ImageJ for area calculation. The RSC region and level of Bregma of a certain brain slice was determined based on a mouse brain atlas (Paxinos and Franklin, 2001). The level of bregma was attempted to match levels in the range from -3.8 to 1 mm in 0.3 mm steps for comparison of areas between. Infected areas outside of the RSC region were also traced and calculated. An effort was made to keep the observer blind to the experimental condition, but the viral constructs used lead to a different expression pattern. The opsin Jaws is expressed in the membrane due to trafficking sequences KGC and ER2 while in the controls EYFP expression is cytoplasmatic.

### 2.2.2 Behavioural analysis

Details on analysis of behavioural data are presented on its respective chapters. But in summary, custom written MATLAB and Python codes were used for analysis of operant boxes behavioural output. For spatial tasks, a chronometer was used to manually quantify latencies. All behaviour was recorded by infrared cameras to allow for monitoring even in the dark.

#### 2.2.2.1 Two-photon imaging processing and analysis

Video registration and cell detection was done using the Suite2p open-source software (Pachitariu et al., 2017). ROI selection was done initially in a semi-automatic way with Suite2p followed by manual verification. Cell detection over days was achieved with Suite2p with a manual curator. Alignment of imaging data and behaviour data was done through custom written MATLAB programs. Suite2p return raw fluorescence signal corrected for neuropil signal contamination based on the following calculation:

$$F_{\text{cell}}(t) = F(t) - F_{\text{neuropil}}(t) \times r,$$

where $F_{\text{neuropil}}(t)$ is the neuropil signal surrounding each cell, calculated by averaging the signal of all pixels with a 20 μm region from cell centre (excluding other identified cells). $r$ is the contamination ratio calculated as the ratio between the average fluorescence of blood vessels and that of neuropil.

For all subsequent analysis the change in fluorescence was used as measure of activity and was defined as:

$$\frac{\Delta f}{f}(t) = \frac{f(t) - f_0}{f_0},$$

where $f_0$ is the baseline for that acquisition, defined as all fluorescence values that fall between the $10^{\text{th}}$ the $70^{\text{th}}$ percentile of their distribution averaged across the session. $f(t)$ represents the ROI's neuropil-corrected fluorescence at time $t$.

Custom written python scripts were used for subsequent data analysis and graph plotting.

### 2.2.3 Statistical analysis

All statistical analysis was performed using SPSS Statistics software (v. 25.0.0.1, IBM Corporation). Analysis of behaviour with within subjects repeated measures factors were analyzed using GLM Repeated Measures in SPSS Statistics. Initially counterbalancing factors (stimulus identity) was added as a covariate in the model, but removed if they did not interact with Group or Experimental Condition.

# Chapter 3

# Probing associative memory

## 3.1 Introduction

The retrosplenial cortex most studied function has been with relation to spatial memory and navigation, but the fact that it is involved in non-spatial tasks (Robinson et al., 2011, 2014; Makino and Komiyama, 2015; Hattori et al., 2019) indicates that its core function might underlie both spatial and non-spatial memory. Recently, it has been proposed that the RSC is related to some kind of associative memory that supports its role in contextual and spatial memory (Miller et al., 2014; Todd and Bucci, 2015). This aligns with the response of RSC to landmarks and other spatial cues (Dilks et al., 2011; Auger et al., 2012). Also, it might explain the importance of this region to contextual memory. Hence, to be able to study retrosplenial function in forming associations, one needs to have ways of testing this type of memory. In this Chapter, I set out to establish behavioural paradigms for mice that would allow me to test associative memory while optogentically inactivating the RSC.

More than involving the RSC in associative memory in general, recently developed studies have suggested a role of this structure in sensory associative memory, specifically in binding together stimuli that have not yet acquired a behavioural significance.

The discoveries that support this idea will be examined on Chapter 4. At this point, I propose to review how the study of associations has developed, describe the establishment of sensory associative paradigms and explain the reasons for the use of the tasks presented here.

It is essential for episodic memory to store component parts of an event and to link those by spatial, temporal or other relationships (Mayes et al., 2007). Thus, it requires associations. These are formed from identifying repeating or permanent patterns and regularities from the environment. To understand how our brains form and store these relationships and the role of different structures on this process is key to explaining episodic memory.

At the Introduction of this thesis I have described experiments that were able to test and study associations. While, Ebbinghaus experiments focus on the human associative capacity (Ebbinghaus, 1885), Thorndike's and Pavlov's experiments will start to shape how we can study associations in animals (Thorndike, 1898; Pavlov and Anrep, 1940). The development of classical and operant conditioning paradigms made possible the testing of how associations between two stimuli occur. A conditioned stimulus (CS), such as a stimulus found in the environment (tone, light, etc.) could be paired with an unconditioned stimulus (US), usually associated with a positive or negative outcome and afterwards this association could be tested. These paradigms combined with lesion, and later on, inactivation studies started to expose the role of different structures on the process of associations (Keene and Bucci, 2008c; Radwanska et al., 2010; Gewirtz and Davis, 2000).

Nevertheless, there are different types of associations. Although many of the associations we make in our lives are related to a pleasurable or aversive outcome, as the ones found in the conditioning paradigms mentioned above, we also associate sensory stimuli that have low-salience in an incidental association process to in due course assign them meaning and value (Wimmer and Shohamy, 2012). For instance,

these associations might be what underlie the composition of context representation (Miller et al., 2014; Bar and Aminoff, 2003; Bar, 2007). These incidental associations can be called stimulus-stimulus associations and cannot be tested through normal conditioning only (Busquets-Garcia et al., 2018).

The development of the sensory preconditioning (SPC) paradigm by Brogden (Brogden, 1939) will allow testing of sensory-sensory associations. In his work, Brogden, first presents two sensory stimuli (S1 and S2) from different modalities (bell and light) to dogs on an initial exposure phase. Then, in the following phase, he conditions the dog's flexion reflex (CR) to one of the stimuli (S1) through an electrical shock (US). Finally, he presents the dog with the other stimulus (S2), the one that had not been paired with the shock but was paired with the conditioned stimulus (S1) in the first phase. This stimulus (S2) is able to elicit the conditioned response of the flexion reflex. He, thus, concludes that an association between light and bell (S1 and S2) must have been formed on the initial phase for this transfer to have happened. In this way, the sensory preconditioning paradigm allows us to probe the formation of an association between stimuli that are not linked with a positive or negative outcome initially. It allows us to test sensory-sensory associations (Rizley and Rescorla, 1972; Gewirtz and Davis, 2000).

One question that can be raised about the neural processes happening in SPC is whether the unpaired stimulus (S2) elicits the conditioned response because conditioning on the second phase links them to each other or if it is mediated by the association with the paired stimulus (S1). This was answered by showing that extinction of the first-order conditioning (S1-US) also extinguished the induction of conditioned response by the unpaired stimulus (S2) (Rizley and Rescorla, 1972). If SPC causes S2 to be directly linked to the CR than extinguishing the S1-CR link would not dissociate S2 and the CR.

In the originally designed SPC paradigm, Brogden used two stimuli from different

sensory modalities. Two different studies addressed the question if associations between stimuli from the same nature were possible (Lavin, 1976; Rescorla and Cunningham, 1978). They adapted taste aversion to SPC and showed that both two flavours delivered sequentially, or compounds made of two different flavours delivered as a mixture could be used in the initial phase and lead to a preconditioning effect.

This is a very well-established paradigm to study neutral sensory associations as it has been demonstrated to work on different animal models. Experiments with SPC on cats, attempted to define the time relationship between the two sensory stimuli on the preexposure phase (Hoffeld et al., 1958). They were not able to establish a function of how the time relationship would vary but later on, Rescorla experiments on rats indicated that simultaneously presented stimuli would lead to a stronger association than sequentially presented ones (Rescorla, 1980). Other experiments with rats described the neural representation of sensory-sensory associations in the orbitofrontal cortex (Sadacca et al., 2018). Moreover, recently, in humans, SPC was used to show that the hippocampus is involved in projecting value to previously acquired associations (Wimmer and Shohamy, 2012). However, to the best of my knowledge no sensory preconditioning paradigm in mice has been described.

Since the sensory-sensory association happens in a different phase from sensory-outcome association it is possible to differentiate this two types of associations. Hence, the sensory preconditioning paradigm gives the advantage of testing the retrosplenial cortex importance in forming neutral sensory associations by silencing this region specifically in the initial phase of the paradigm. In order to do that and to use the optogenetic system described in the previous chapter, it is first necessary to establish this paradigm for testing mice. Thus, here, I describe the development of two sensory preconditioning paradigms in mice: one using an appetitive conditioning and another using an aversive paradigm. I also discuss the advantages and restrictions of each of them.

## 3.2 Methods

All housing procedures for mice are described on Section 2.1.1.

### 3.2.1 Appetitive Sensory Preconditioning - Protocol I

16 C57Bl/6 male mice (15 to 16 weeks-old) underwent an appetitive sensory preconditioning paradigm. First, handling and food regulation procedures started as described on Section 2.1.3. On the 3$^{rd}$ day after food access was restricted, mice were exposed to 20% Sucrose (Sigma) for 2 days in their home cages. After that, the Pretraining phase started.

*Pretraining phase:* On the first day of pretraining, mice were put in operant boxes (MED Associates, described on Section 2.1.3.3), where the food cup was filled to the top with 20% Sucrose solution. Houselights and fan were off. Mice were left for 10 min. to explore the box and drink. If no reward consumption was observed, then mice were re-exposed after all mice had been exposed. The second day of pretraining consisted of a variable interval (VI) 60 s schedule. Mice received 14 rewarded trials with an ITI on average of 60 s (ranging from 50 to 70 s in intervals of 5 s, randomly chosen). At each trial, 40 $\mu$L of 20 % sucrose solution were delivered. Finally, the 3$^{rd}$ day consisted of a VI 120 schedule (ranging from 100 to 140 in intervals of 10s). Houselights were kept off during these VI schedule days.

*Preexposure phase:* Mice were exposed to 20 intermixed trials of sensory stimuli (Figure 3.1A). First, 10 s of auditory stimuli (3KHz tone at 85dB or white noise (WN) at 70dB) were delivered. One of the sound stimuli (the sound stimulus identity was counterbalanced) was then followed by 10 s of a visual stimulus: houselights flashing at 5 Hz. While not flashing, the houselights were kept off. ITI was on average of 120 s as used in the VI 120 s schedule. No reward was given and the access to the food cup was blocked. After six days of preexposure to stimuli, the conditioning phase started.

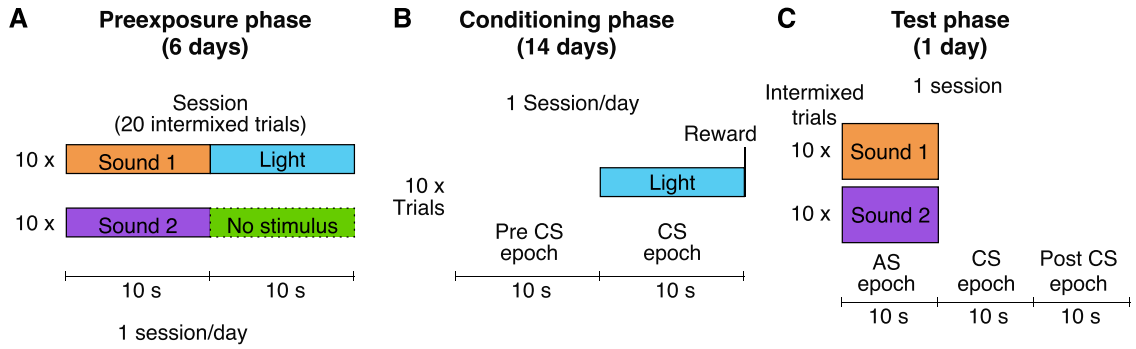



**Figure 3.1: Appetitive SPC - Protocol I.** (A) The preexposure phase consisted of presentations of sound cues with a 10 s duration. Sound 1 was followed by 10 s of a flashing light (5 Hz). Sound 2 was presented alone, and no other stimulus followed. Sound cues were counterbalanced and were either a pure tone of 3 Hz or white noise. (B) Conditioning to the flashing light (5 Hz for 10 s) with presentation of reward (40 µL sucrose solution) happening at light onset. Analysed epochs included 10 s prior to the start of the light (Pre CS epoch) and 10 s during the light presentation (CS epoch). (C) Single session test. Intermixed presentations of 10 s sound cues. Analysed epochs involved 10 s during auditory stimulus presentation (AS epoch), 10 s afterwards which corresponded to the 10 s period when light would be expected and 10 s after this period (Post CS epoch). AS - auditory stimulus; CS - conditioned stimulus.

*Conditioning phase:* Animals received 10 trials of conditioning with an ITI on average of 120 s again (Figure 3.1B). At each trial, 10 s of flashing houselights stimulus (conditioned stimulus) were followed by a 40 $\mu$L sucrose solution reward delivery at the end of the visual stimulus. When not flashing houselights were kept off again. This phase was completed once asymptotic behaviour was observed.

*Test phase:* Finally, preconditioning associations were tested under extinction by exposing the animals to 20 intermixed trials of 10 s of the sound stimuli only (ITI $\approx$ 120 s) (Figure 3.1C). 10 trials of each stimuli were delivered in total.

Beam break data from food cup approaches was collected during all three phases of the task. Conditioned responses and test responses were analysed as the average number of head entries per trial in the food magazine and the average duration of those head entries per trial during specific epochs. For conditioned responses, normalised responses were calculated as the response during the 10 s of conditioned stimulus

minus responses just before light onset.

### 3.2.2 Appetitive Sensory Preconditioning - Protocol II

16 C57Bl/6 male mice (10 to 15 weeks-old) underwent an appetitive sensory preconditioning paradigm. Same handling and food regulation procedures as on Protocol I were used. On the 3$^{rd}$ day after food access was restricted, mice were exposed to 20% Sucrose for 2 days in their home cages. After that, the Pretraining phase started.

*Pretraining phase:* On this protocol only one day of pretraining was given. In the morning, mice were put on the operant boxes (MED Associates, detailed on Section 2.1.3.3), where the food cup was filled with 20% Sucrose solution. If after the 10 min of exploration no reward consumption was observed, then mice were re-exposed at the end of the morning. Then, in the afternoon, mice received a VI 240 s schedule with 10 rewarded trials (ITI $\approx$ 240, ranging from 120 to 360 s in 24 s intervals). At each trial, 40 $\mu$L of sucrose solution were delivered. Houselights were kept off during the Pretraining phase.

*Preexposure phase:* Mice received 4 days of daily sessions of 20 intermixed trials of sensory stimuli (Figure 3.2A). The same procedure as described on Protocol I was used, except that at each trial, 30 s of auditory stimuli (3KHz at 85dB or WN at 70dB) were delivered instead of 10 s. One of the sound stimuli was then followed by 10s of a visual stimulus: houselights flashing at 5Hz. The ITI was on average of 240 s here as with the VI Schedule. Houselights were kept off when not flashing.

*Conditioning phase:* As with Protocol I, animals received 10 trials (ITI $\approx$ 240 s) of conditioning per session. On this protocol (Figure 3.2B), two daily sessions occurred over 5 days. One trial was composed of the 10 s flashing light presentation while reward (40uL of 20% sucrose solution) was delivery at 1s, 5s and 10s after light onset.

*Test phase:* Animals were tested (Figure 3.2C) by receiving 10 intermixed trials of 30 s of the sound stimuli only (ITI $\approx$ 240 s).

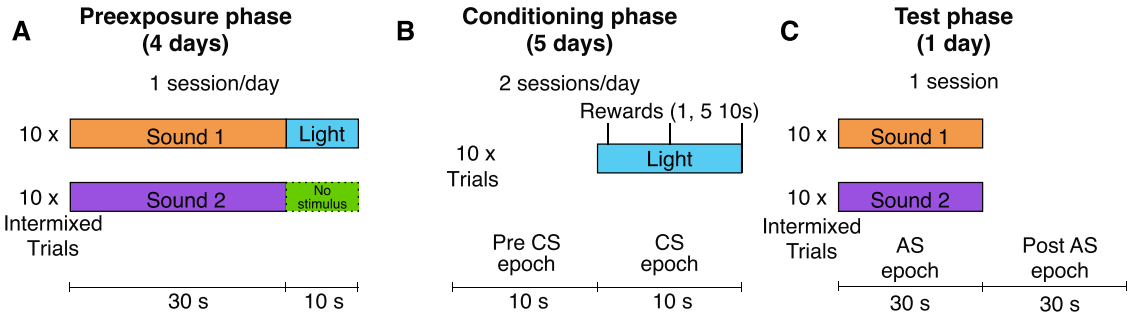



**Figure 3.2: Appetitive SPC - Changed protocol.** (A) In the preexposure phase, sound cues now had a 30 s duration. Sound 1 was followed by 10 s of a flashing light (5 Hz). No other stimulus followed Sound 2 (identity was counterbalanced between a pure tone of 3Hz and white noise, WN). (B) Changed protocol for conditioning to the flashing light (5 Hz for 10 s) where sucrose solution delivery was done 1 s, 5 s and 10 s after light onset (40 µL each). Analysed epochs included 10 s prior to the start of the light (Pre CS epoch) and 10 s during the light presentation (CS epoch). (C) Single session test. Intermixed presentations of 30 s sound cues. Analysed epochs involved 30 s during sounds (AS epoch) and 30 s afterwards (Post AS epoch), which included the 10 s period when light would be expected. AS - auditory stimulus; CS - conditioned stimulus.

*Conditioning to preconditioning cues:* For 3 days, animals received 2 daily sessions of 10 intermixed trials. At each trial, one of the sound stimuli was presented for 30 s (5 presentations of each) and a reward was delivered at the stimulus offset.

Beam break data was collected during pretraining, conditioning, test and re-conditioning phases. Responses were analysed as previously described for protocol I.

### 3.2.3 Aversive Sensory Preconditioning - Protocol I

Handling and water regulation procedures started on 8 naive male C57Bl/6 mice (13-weeks-old) as described on Section 2.1.3. All phases of the experiment took place on holding cages identical to the mice home cages but without any bedding, food or water bottle available. Compound solutions were presented at food bowls made of metal placed on either ends of the cage. Solutions were made of either single

compounds or pairs of mixed compounds. Compounds used were: 0.01 M hydrochloric acid (HCl, Fisher Scientific), 60.0 $\mu$M quinine (Aldrich Chemistry), 10% Sucrose (Sigma) and 0.15 M Saline (Sigma). When mixed, compounds were made up to retain these concentrations. Each day, mice received 30 min sessions in the morning where liquid compounds would be available and, in the afternoon, they had access to water for 30 min in their homecages.

*Preexposure phase:* For 8 days, mice received exposure to paired mixed compounds (Figure 3.3A). These were given in 2-day cycles. Each cycle was composed of day 1 when, in the morning, mice would be allowed to explore the experimental cage and were presented with 4 mL of mixed compound AX (A could be Sucrose or Saline; X could be HCl or Quinine; identities were counterbalanced) for 30 min. Then on day 2 of the cycle, mice were exposed for 30 min to 4 mL of mixed compound BY (B was the opposite of A; Y was the opposite of X; identities were counterbalanced). AX and BY were presented on opposite ends of the cage and this position was kept constant over sessions.

*Conditioning phase:* In 2-day cycles, for 6 days, mice received 30-min exposure to 4 mL of simple compounds X or Y, in the morning of alternate days (Figure 3.3B). X would be available on the same side as AX had been presented during preconditioning and Y on the side where BY had been presented. After a session where X was available, mice received an intraperitoneal (IP) injection of 0.15 M lithium chloride (LiCl, 40 mg/Kg, Sigma Aldrich). The LiCl injection caused sickness and diarrhoea. When, Y was presented during the session, mice received 0.9% Saline (Irripod, CDMedical) injection instead. Mice were monitored for 2 hours after injections. The day following the last day of conditioning, mice had a recovery day, when they were allowed 1h of access to water in the morning.

*Test:* A single choice test was given (Figure 3.3C). Animals had access to two food bowl simultaneously and could choose between drinking compound A or compound B.

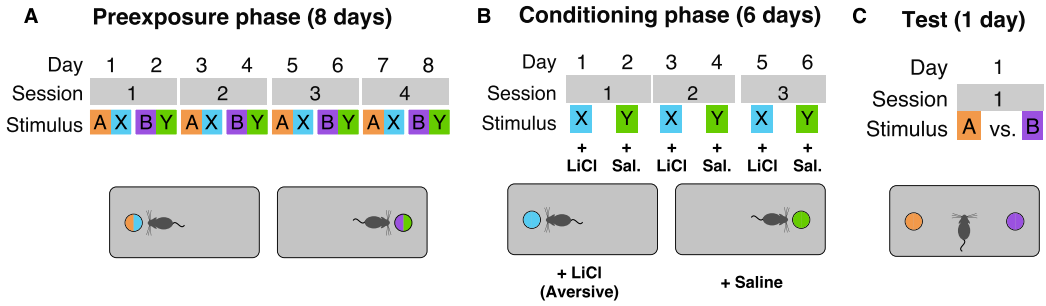



**Figure 3.3: Aversive SPC protocol.** (A) Preexposure phase protocol. Stimuli were presented on alternate days such that Session 1 corresponds to Day 1 (presentation of AX) and 2 (presentation of BY) and so on. (B) Conditioning phase protocol when mice were presented with pure solutions X and Y on alternate days. X presentations were followed by injections of lithium chloride (LiCl). Y was followed by saline (Sal.). (C) Single session choice test. Both pure solutions A and B were presented at the same time. For all phases, solutions part of the same session were presented on opposite sides of a housing cage. Stimuli used were sucrose solution, saline, quinine and hydrochloric acid and identities were counterbalanced.

Compound A was presented where AX had been previously presented and B where BY had been.

Before and after each session, food bowls where weighted and this was recorded for consumption analysis. Total consumption of each compound was estimated as weight before minus the weight after.

### 3.2.4 Aversive Sensory Preconditioning - Operant boxes

16 C57Bl/6 male mice underwent surgery for viral injection and LED implantation as described in Section 2.1.2. Eight animals had their head implants fall off and were discarded. Of the remaining, three had been injected with Jaws virus but their LED implants were not functioning, and thus, were considered as controls with the remaining four controls for the results presented here. Handling and water regulation procedures started four weeks after surgery and behaviour testing right after.

*Preexposure phase:* Each morning, mice had 30 min exposure to flavour compounds

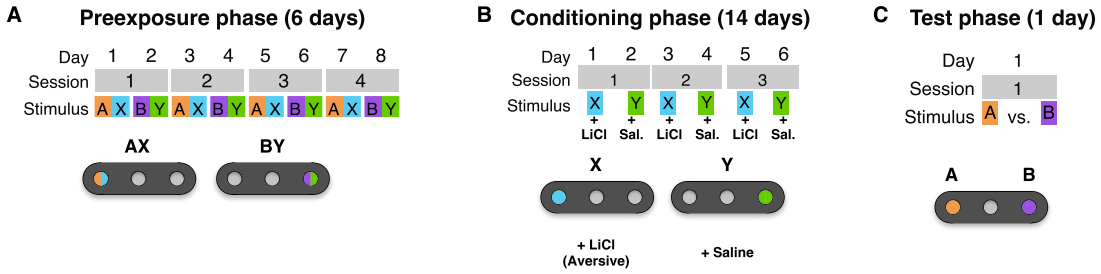



**Figure 3.4: Aversive preconditioning in operant boxes.** Aversive sensory preconditioning protocol adapted to operant boxes. The boxes had three nosepokes. Solutions were delivered from the side pokes when animals poked with ITI of 1 s. For all phases, solutions part of the same session were presented on opposite sides and identities were counterbalanced. (A) Preexposure phase with AX and BY being delivered on alternate days. (B) Conditioning phase protocol. LiCl injection were applied after sessions where X was presented, and saline injections were used after Y sessions. (C) Single session choice test between A and B.

AX or BY on alternate days for 8 consecutive days (Figure 3.4A). AX would be delivered through one of the side pokes and BY, the next day, on the opposite side poke. Pokes were lit up to indicate that reward was available. Subjects had to poke to get a 15 $\mu$L delivery of the mixed compounds. Poking triggered the rewards and they were delivered every 1s if the animal remained in the poke. Each afternoon, $\approx 4$ hours after training, mice were given access to water for 30 min.

*Conditioning phase:* During phase two, for six days, mice were allowed to drink from the centre poke, X only or Y only on alternate days in 30 min. sessions (Figure 3.4B). When the sessions finished, after drinking X, all mice received an injection of 0.15M LiCl (40ml/kg). After drinking Y, mice received a 0.9% saline injection.

*Test:* For the test session, A and B were presented simultaneously for 30 min (Figure 3.4C). Mice had a choice of drinking either from the left or from the right pokehock. A and B location was counterbalanced. Consumption was measured by calculating the duration of nosepokes.

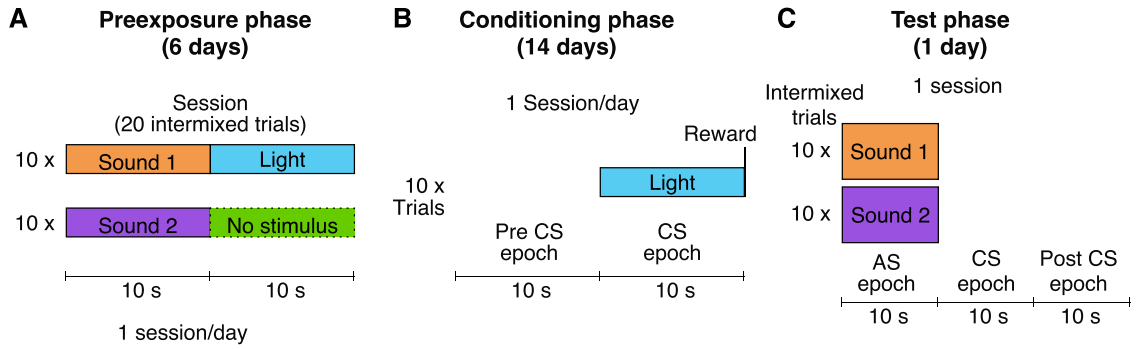



**Figure 3.1: Appetitive SPC - Protocol I.** (A) The preexposure phase consisted of presentations of sound cues with a 10 s duration. Sound 1 was followed by 10 s of a flashing light (5 Hz). Sound 2 was presented alone, and no other stimulus followed. Sound cues were counterbalanced and were either a pure tone of 3 Hz or white noise. (B) Conditioning to the flashing light (5 Hz for 10 s) with presentation of reward (40 µL sucrose solution) happening at light onset. Analysed epochs included 10 s prior to the start of the light (Pre CS epoch) and 10 s during the light presentation (CS epoch). (C) Single session test. Intermixed presentations of 10 s sound cues. Analysed epochs involved 10 s during auditory stimulus presentation (AS epoch), 10 s afterwards which corresponded to the 10 s period when light would be expected and 10 s after this period (Post CS epoch). AS - auditory stimulus; CS - conditioned stimulus. (repeated from page 44)

## 3.3 Results

### 3.3.1 Developing an appetitive sensory preconditioning task in mice

The initial aim was to establish for mice a similar sensory preconditioning protocol as to the one found for rats in the literature (Brogden, 1939; Robinson et al., 2014). Initially, I developed an appetitive sensory preconditioning paradigm. Here, a within subjects design is employed. As explained before, the paradigm is composed of three phases. On the first phase, preexposure (Figure 3.1A), mice received 20 intermixed trials. On half of the trials, the animal was given the opportunity to associate an auditory stimulus (Tone or WN) with a light stimulus. On the other half of the trials, a control (Unpaired) AS was delivered without any visual stimulus following it. This

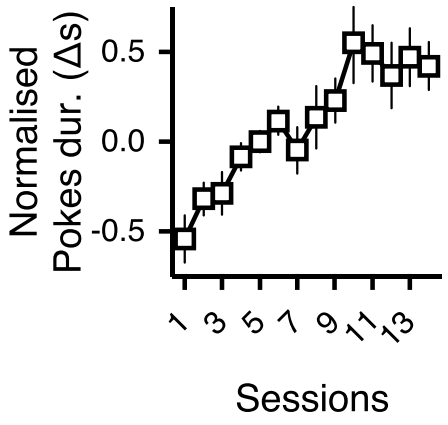



**Figure 3.6: Appetitive SPC - Conditioning Phase.** Normalised duration of pokes in the magazine (CS epoch responses minus responses during the Pre CS epoch) during the 14 sessions of the conditioning phase averaged over all mice (N: 16). Conditioning phase where a flashing light at 5 Hz was followed by a reward delivery (40 μL) at the offset. Analysed periods were 10 s before the light (Pre CS epoch) and the 10 s during the light presentation (CS epoch). CS - conditioned stimulus. Bars represent standard error of the mean (SEM).

procedure was repeated over 6 days. During the conditioning phase (Figure 3.1B), the light stimulus was now presented alone, paired with a single reward delivery at light offset. This was repeated until asymptotic behaviour was observed. The final phase was a choice test (3.1C) where the two auditory stimuli (AS) were delivered intermixed for 20 trials. Below, I present the analysis of responses as the duration of magazine entry during each phase, which is understood as the best measure for value change in sensory cues (McDannald et al., 2011).

#### 3.3.1.1 Mice undergo conditioning to a light stimulus and respond differently to preconditioned stimuli

*Preexposure phase:* For the first phase of the experiment (Figure 3.1A), there is no measurement of how the animal responds to the stimuli presented. But its result will be expressed by a preconditioned response in the test Phase.

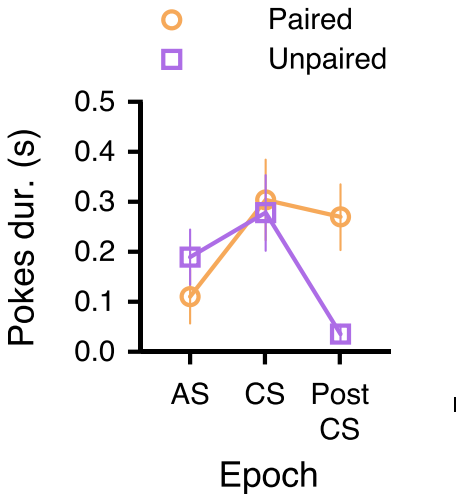



**Figure 3.7: Appetitive SPC - Test.** Single session test. Two sound cues (pure tone of 3 Hz and white noise, WN) were presented in 20 intermixed trials. Analysed epochs are: 10 s during the sounds presentation (AS epoch), 10 s where the light would be expected (CS epoch) and 10 s afterwards when animals might look for reward (Post CS epoch). Mean total duration of poke is compared between the analysed epochs (AS, CS and Post CS) for the cue that was previously paired with light (Paired, orange) and the cue by no stimulus (Unpaired, purple). AS - auditory stimulus; CS - conditioned stimulus. Bars represent SEM (N: 16).

*Conditioning phase:* To evaluate conditioned responses to light (Figure 3.1B), normalised duration of poke responses were calculated. The averages for each day are shown in Figure 3.6. An increase over days of training can be observed with values stabilising apparently at around 9 days. This means that animals started to poke more during the light period as they were expecting the reward to come. This increase in response was significant ($F(13, 195) = 6.957$, $p < 0.001$) as shown by statistical analysis. Asymptotic behaviour was assessed using Helmert contrasts, which indicate that increase in responses was not significant from day 8 onwards (Day 8 vs later: $F(1, 15) = 3.915$, $p = 0.067$). This analysis show that mice start responding more during the CS epoch, indicating conditioning to light was successful.

*Test Phase:* Mice went through a day of test where the two auditory stimuli (Paired sound or Unpaired sound) were presented by themselves on intermixed trials

(Figure 3.1C). Poke duration responses during the auditory stimulus (AS) epoch, the period when Light would be expected (CS epoch) and the 10 s post-CS epoch were analysed (Figure 3.7).

The results for the test day are shown on Figure 3.7. Responses to the Paired and Unpaired stimulus were compared during the AS epoch CS epoch and the post-CS epoch. An increase in response to both cues is observed from the AS epoch to the CS epoch. Then, while responses after the unpaired sound decreased, responses to the paired sound remained high. An ANOVA revealed a significant epoch and pairing interaction ($F(2, 30) = 4.866$, $p = 0.015$) suggesting that the response to the Paired or Unpaired stimulus is different over the three analysed periods. A simple effects analysis of the epoch and pairing interaction revealed that response to the stimuli was significantly different on the post-CS epoch ($F(1, 15) = 13.860$, $p = 0.002$) but not on the preceding periods (All $F < 0.893$, $p > 0.36$). A strong preconditioning effect would be expressed by a strong discrimination of the two stimuli on both the CS and post-CS epochs. Because this effect was not observed, I decided to change a few protocol details in order to get a better preconditioning effect.

#### 3.3.1.2 Reward delivery throughout the CS epoch leads to faster conditioning to light stimulus

In order to improve sensory preconditioning effects we changed the original protocol to:

1. Decrease the duration of the conditioning phase:
   (a) By having less days to reach criteria;
   (b) By making the learning stronger;
2. Increase opportunity to see conditioned approach behaviours;

Thus, we changed the following details on the original protocol:

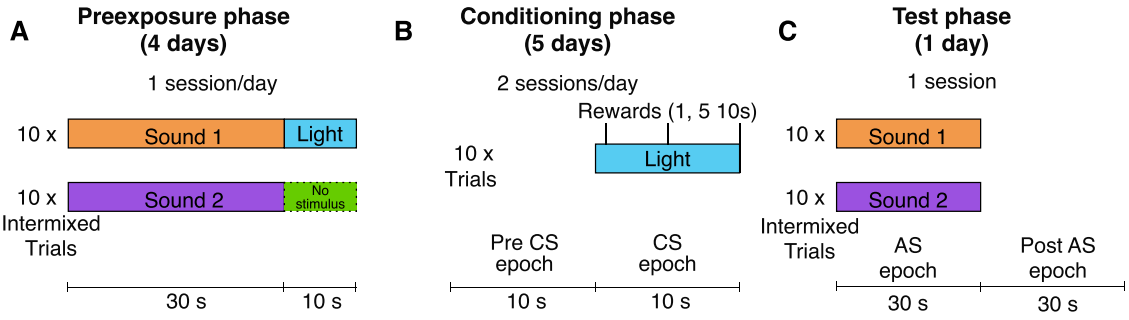



**Figure 3.2: Appetitive SPC - Changed protocol.** (A) Preexposure phase changed protocol. Sound cues had a 30 s duration. One of them was followed by 10 s of a flashing light (5 Hz). No other stimulus followed the other one (counterbalanced). (B) Changed protocol for conditioning to the flashing light (5 Hz for 10 s) where sucrose solution delivery was done 1 s, 5 s and 10 s after light onset (40 µL each). Analysed epochs included 10 s prior to the start of the light (Pre CS epoch) and 10 s during the light presentation (CS epoch). (C) Single session test. Intermixed presentations of 30 s sound cues (pure tone of 3 Hz and white noise, WN). Analysed epochs involved 30 s during sounds (AS epoch) and 30 s afterwards (Post AS epoch), which included the 10 s period when light would be expected. AS - auditory stimulus; CS - conditioned stimulus. (repeated from page 46)

1. (a) Increased the number of sessions per day on the conditioning phase (since increasing the number of trials would only lead to faster satiety);

   (b) Increased number of rewards (three), now delivered throughout the Light Stimulus;

2. Increased the duration of the Auditory stimuli from 10 s to 30 s;

3. Increased the ITI duration from an average of 2min to a 4min average;

With these changes, animals were submitted to the protocol as shown in Figure 3.2. Head entries durations were used as the conditioned and preconditioned responses.

*Conditioning:* As seen in the previous experiment, animals showed conditioning responses to the light that increased almost 400% over repeated pairings of the flashing light with three reward deliveries throughout the duration of the cue (Figure 3.9). This increase was significant as showed by a main effect of Session ($F(9, 135) = 21.045$,

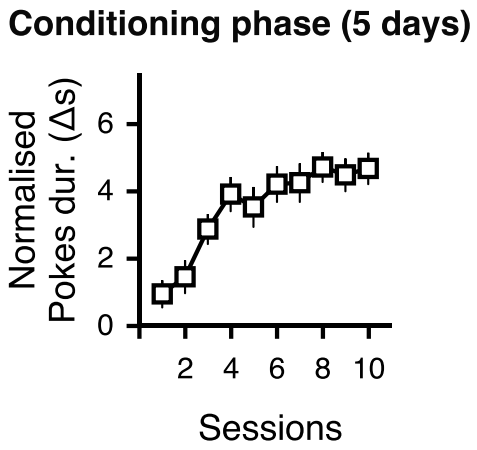



**Figure 3.9: Conditioning to light.** Normalised duration of pokes in the magazine (poke duration in the CS epoch minus duration in the Pre CS epoch) for each conditioning session (N: 16). Bars represent SEM.

$p < 0.001$). This time, asymptotic behaviour seemed to start around session 6, which was confirmed by post hoc tests on the last sessions (Session 6 vs later sessions: $F(1, 15) = 1.917$, $p = 0.186$).

*Test:* Figure 3.10 shows mean duration of pokes over 10 s bins in two epochs of the test (AS and PostAS). Although, responses to the two cues looked similar during the sound period (AS epoch), the paired cue appeared to lead to increased responses in the PostAS epoch. A full analysis including the 30 s AS epoch and a 30 s PostAS epoch split into 10 s bins was used to investigate the expected preconditioning effect. The three way interaction between epoch, bins and pairing was not observed in the ANOVA ($F(2, 30) = 1.494$, $p = 0.241$), but the analysis revealed an interaction between pairing and epoch ($F(1, 15) = 7.117$, $p = 0.018$) and a main effect of epoch ($F(1, 15) = 6.689$, $p = 0.021$). When looking at the simple effects of the interaction, the interaction was driven by the difference in response to the paired stimulus between the two epochs ($F(1, 15) = 9.985$, $p = 0.006$). To observe a sensory preconditioning effect, the expectation would be to see a significant difference between the response to

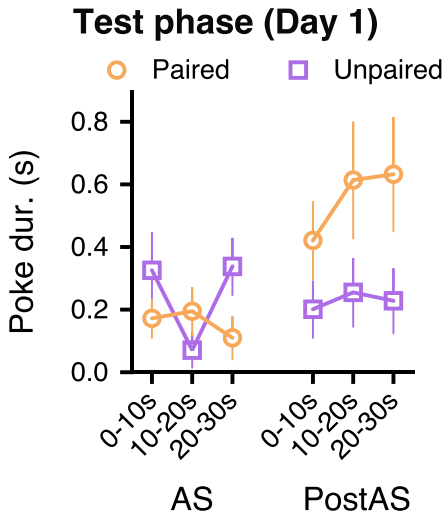



**Figure 3.10: Test of preconditioning effect.** Total poke duration averaged over mice (N: 16) in response to Paired (orange) or Unpaired (purple) sound cues during 10 s bins in the AS and Post AS epochs. AS - auditory stimulus. Bars represent SEM.

the two cues during the PostAS epoch, but differences just approached significance ($F(1, 15) = 4.335$, $p = 0.055$). This indicates that although animals had increase magazine responses after the Paired sound cue was presented, there was not a robust difference between the responses to the two auditory stimuli.

#### 3.3.1.3 No differences observed when mice are reconditioned to preconditioning stimuli

*Conditioning to preconditioning cues:* In order to better understand why no differences were detected at the test phase between the auditory cues I used a reconditioning test, which involved conditioning, this time, to the two auditory stimuli. Mice were presented with intermixed trials of paired and unpaired AS followed by a single delivery of reward at the end of each auditory cue. If the animals had been able to associate the paired AS with the light cue, I would have expected conditioning to the paired cue to happen faster than conditioning to the unpaired cue.

Figure 3.11 shows normalised poke duration responses for the two cues. Mice were

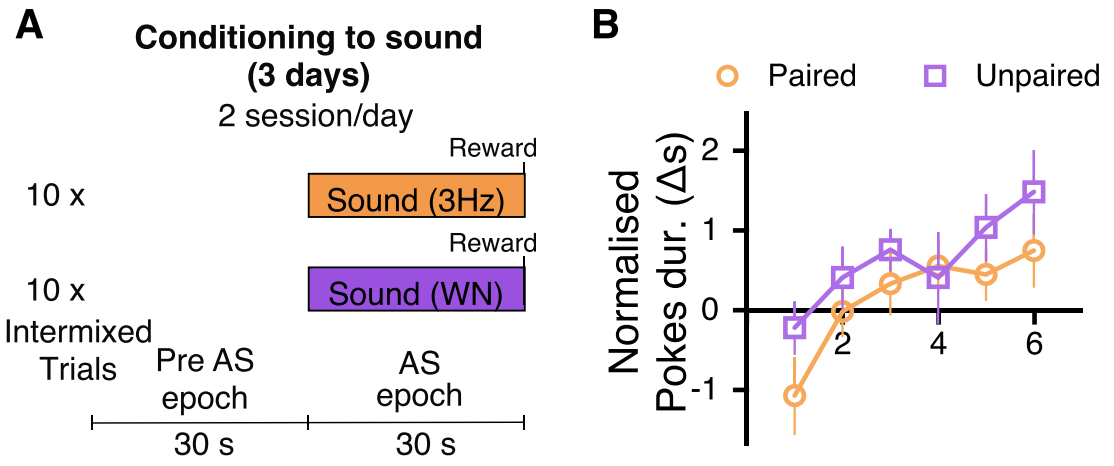



**Figure 3.11: Conditioning to preconditioning cues.** (A) Protocol for each session where trials of 30 s of either the pure tone (3 Hz) or the WN were presented. Both cues were followed by delivery of sucrose solution at the offset. Analysed epochs included 30 s before delivery of sound (Post AS epoch) and 30 s during the sound (AS epoch). AS - auditory stimulus; CS - conditioned stimulus. (B) Mean normalised responses to both sound cues and SEM over sessions (N: 16).

conditioned to both sounds as an increase in conditioning response with time to both paired and unpaired cues can be observed. An ANOVA showed that no interaction between session and pairing or main effect of pairing were observed (All $F < 3.032$, $p > 0.102$) but a main effect of session was present ($F(5, 75) = 4.982$, $p = 0.001$), indicating conditioning was successful at similar rates for both cues.

### 3.3.2 Aversive sensory preconditioning: a robust task for probing associative memory in mice?

Although a trend was observed on the above protocols for appetitive sensory preconditioning, results were not so robust. Thus, I decided it would be important to have another possibility to test sensory-sensory associations. An aversive version of a Sensory Preconditioning task based on outcome devaluation (Rescorla and Cunningham, 1978) was chosen as an alternative test of neutral sensory associations.

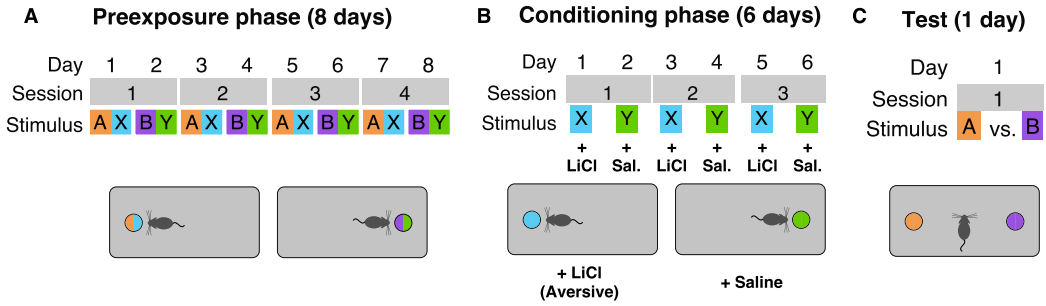



**Figure 3.3: Aversive SPC protocol.** (A) Preexposure phase protocol. Stimuli were presented on alternate days such that Session 1 corresponds to Day 1 (presentation of AX) and 2 (presentation of BY) and so on. (B) Conditioning phase protocol when mice were presented with pure solutions X and Y on alternate days. X presentations were followed by injections of lithium chloride (LiCl). Y was followed by saline (Sal.). (C) Single session choice test. Both pure solutions A and B were presented at the same time. For all phases, solutions part of the same session were presented on opposite sides of a housing cage. Liquid solution identities were counterbalanced. (repeated from page 48)

#### 3.3.2.1 Mice undergoing aversive sensory preconditioning using LiCl devaluation show preference for the unpaired solution

First, we set out to adapt the protocol described in the literature (Rescorla and Cunningham, 1978; Blundell et al., 2003), where pairs of liquid compounds are associated in the first phase and then one of the compounds is devalued in the conditioning phase. The chosen protocol is summarised on Figure 3.3. On the Preconditioning phase, two pairs of compounds (AX and BY) were presented to the animals on alternate days on opposite sides of a holding cage. Compound identities and concentrations are presented on Section 3.2.3, but concentrations were set so none of the flavours was preferred over the others. This was repeated for 8 days (4 presentations of each pair). Then, during conditioning, X and Y, only were presented on alternate days. After a X had been consumed, animals would get a lithium chloride (LiCl) injection and after session with Y, they would get a saline injection. Finally, on the test day, mice had a choice test where A and B were presented simultaneously

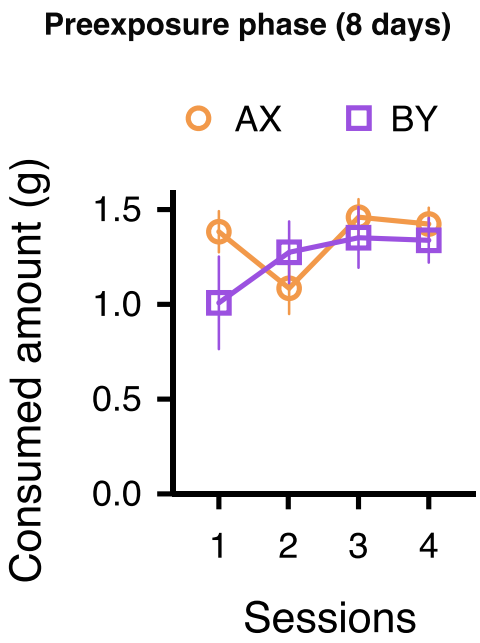



**Figure 3.13: Responses during the preexposure phase.** Total consumed amount in grams of compounds AX (orange) and BY (purple) over sessions of preexposure. Values are averages over mice (N: 8) with SEM.

on opposite sides of the cage. Again, I used a within subjects design and expected all animals to show the preconditioning effect at the test day by drinking more of compound B than A.

*Preexposure phase:* In this experiment, it is possible to quantify responses during the preexposure phase, when animals are presented with two different pairs of compounds (AX or BY) on alternate days. Average consumed amounts are expressed as difference in weight of the food cup before and after each session. Consumption during the 8 days of preexposure is presented on Figure 3.13. Both compounds AX and BY looked to be consumed at similar rates. But, when comparing the consumed amount between alternate days, an interaction between compounds and counterbalancing was revealed ($F(3, 4) = 842.766$, $p < 0.001$) and also a main effect of compound ($F(1, 4) = 118.629$, $p < 0.001$). This would indicate that there is a difference in consumption of the different compounds depending on their identity. At the same time, a session and compound

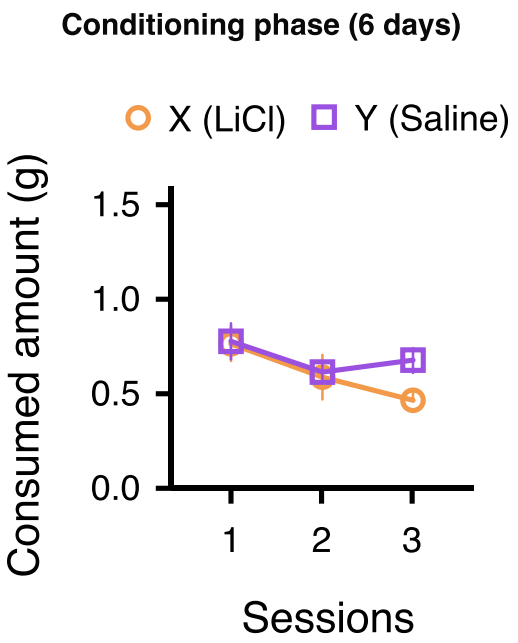



**Figure 3.14:** Averaged consumption of pure solutions X or Y over sessions (N: 8). X presentation was paired with LiCl injections and Y with saline. Bars are SEM.

interaction could be observed ($F(3, 12) = 3.673$, $p = 0.044$). This would indicate that consumption of compounds is changing over sessions. More importantly, when looking at the simple effects of that interaction, there was no significant difference on the last sessions between AX and BY consumption, which means that all compounds are consumed at similar levels by the time animals move to the next phase ($F(1, 4) = 1.019$, $p = 0.370$).

*Conditioning phase:* One session of conditioning was composed of two days. On odd days one simple flavoured solution (X) was paired with a lithium chloride injection after the 30 min session. Then, on even days, simple solution Y session was followed by a saline injection. Figure 3.14 presents consumption of X and Y over these repeated pairings for 3 sessions. It is possible to observe that initially both solutions decrease in consumption on day 2 but then only X keeps decreasing. This time no counterbalancing interaction was detected so it was removed from the analysis. When analysing consumption of X and Y over days, no significant interaction between

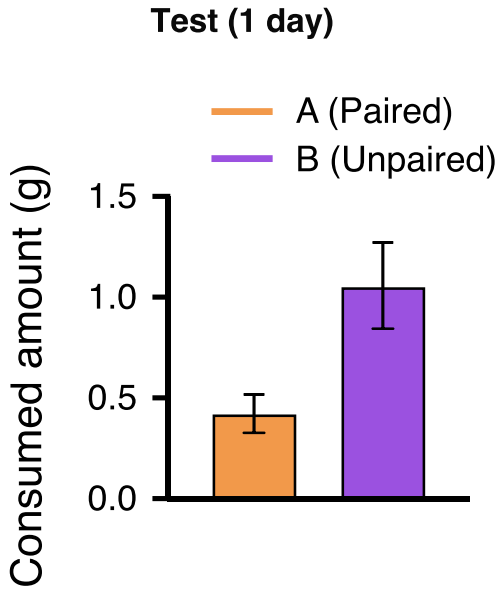



**Figure 3.15: Consumption during test for aversive SPC.** Mean consumption with SEM for choice test between A, which had been paired with X in the preexposure phase (Paired), and B previously paired with Y (Unpaired). Bars represent SEM (N: 8).

sessions and flavours was detected ($F(2, 14) = 2.709$, $p = 0.101$). There was a linear decrease ($F(1, 7) = 5.648$, $p = 0.049$) in consumption for both compounds over days which is also indicated by a main effect of session ($F(2, 14) = 5.148$, $p = 0.021$). This analysis indicated that although a decrease in consumption was observed, this decrease was not significantly different between the different compounds. It should be considered though that another pairing was made on the last session which is not quantified but could strengthen the conditioning effect before the test phase.

*Test phase:* The test was composed of a single session where mice would be able to choose whether to drink flavour A or B, which were positioned on opposite sides of the cage. Consumption of these flavours is reproduced on Figure 3.15. When comparing consumption of A and B, we observe that consumption of B is greater than consumption of A. This is confirmed by a main effect of flavour ($F(1, 7) = 6.413$, $p = 0.039$) indicating a significant difference between how much the animal drank of

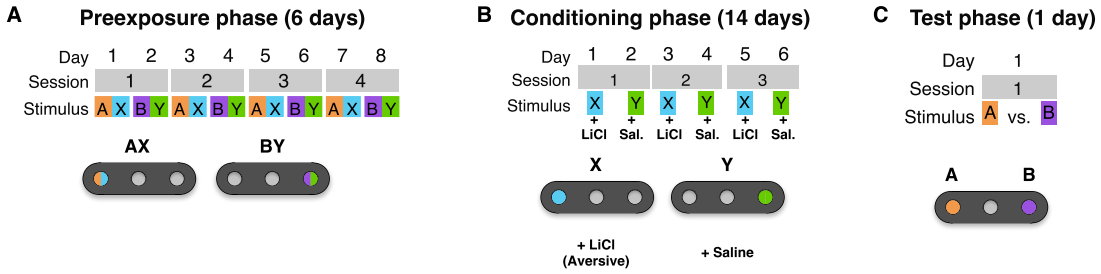



**Figure 3.4: Aversive preconditioning in operant boxes.** Aversive sensory preconditioning protocol adapted to operant boxes. The boxes had three nosepokes. Solutions were delivered from the side pokes when animals poked with ITI of 1 s. For all phases, solutions part of the same session were presented on opposite sides and identities were counterbalanced. (A) Preexposure phase with AX and BY being delivered on alternate days. (B) Conditioning phase protocol. LiCl injection were applied after sessions where X was presented, and saline injections were used after Y sessions. (C) Single session choice test between A and B. (repeated from page 49)

A and B. Thus, confirming the expected preconditioning effect, since A was previously paired with X, which was devalued in the conditioning phase.

#### 3.3.2.2 An operant box version of aversive sensory preconditioning

In order to be able to use optogenetic manipulation while testing animals in the aversive sensory preconditioning task it was necessary to transfer the established protocol described on section 3.2.3 to an operant box setting. This would allow the triggering of LED illumination for opsin activation when the animal poked to drink. For this pilot testing, seven wildtype C57Bl/6 male mice were used. The adapted protocol is described on Section 3.2.4 and Figure 3.4 displays a schematic view of it.

In summary, the protocol followed the previously used one for aversive sensory preconditioning. The main difference was that instead of having food cups to deliver liquids, animals were put in a operant box with three nosepokes that were able to deliver liquids. Liquid compounds were presented on the side pokes on alternate days. Once more, their identities were counterbalanced as explained in Section 3.2.3. In the test day, mice had a choice test where A and B were presented simultaneously in the

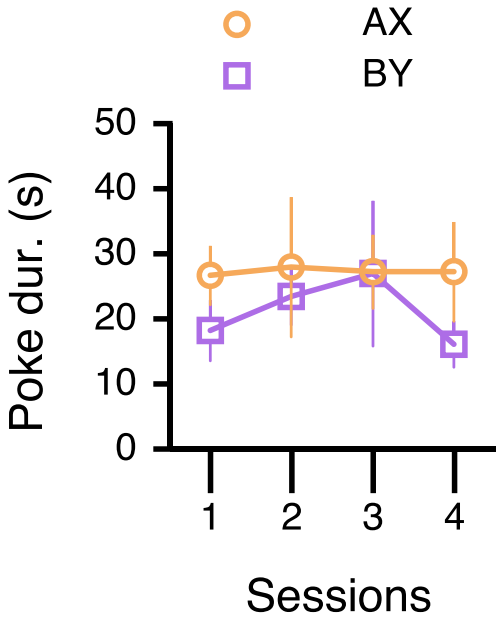



**Figure 3.17: Responses during preexposure.** Mean duration of poke with SEM is presented over the course of four sessions of preexposure (N: 7). Each session was composed of two days with AX and BY being presented on separate days.

side pokes. Duration of pokes could be measured based on infrared beam break and was used as measurement of consumption on all phases of the task.

*Preexposure phase:* During the preexposure phase, animals were presented with two different pairs of compounds (AX or BY) on alternate days in opposite side pokes (Figure 3.4A). Average poke duration as consumption during the 8 days of preexposure (sessions 1 to 4) is presented in Figure 3.17. Both mixtures, AX and BY, were consumed at similar levels at a mostly constant rate over sessions (poke durations around 20 to 30 s). BY consumption levels varied more but this was not significant as demonstrated by the following statistical analysis. By comparing the consumed amount between alternate days, no significant interaction or main effect were observed (All $F < 1.435$, $p > 0.266$). This indicates that there was no difference in consumption of the different compounds over sessions.

*Conditioning Phase:* Conditioning followed the preexposure phase. Now, X and Y

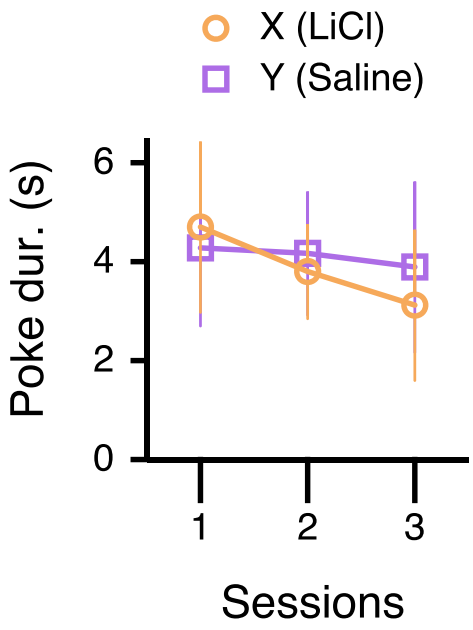



**Figure 3.18: Conditioning phase.** Duration of pokes for consumption of X, which was devalued by repeated pairings with LiCl, and for Y, that was paired with saline injections (N: 7).

were presented alone on alternate days (Figure 3.4B). X sessions were followed by a lithium chloride injection and Y sessions by a saline injection. After repeated pairings for three sessions the experiment moved to the next phase. Consumption of X and Y over sessions during conditioning was measured as duration of pokes and results are presented in the graph in Figure 3.18. Consumption of X was expected to decrease over repeated pairings. A decreasing trend is observed for duration of pokes for X and not for Y, but a large variability is also present. No significant interaction or main effects were detected (All $F < 0.169$, $p > 0.809$). Although only a trend was observed and no difference in consumption of the two compounds was observed, one should consider that another pairing of X and LiCl was made on the last session, the effects of which is not quantified. The effect of that last pairing will only be observable in the test phase.

*Test phase:* During the test, mice were given the choice between two simple

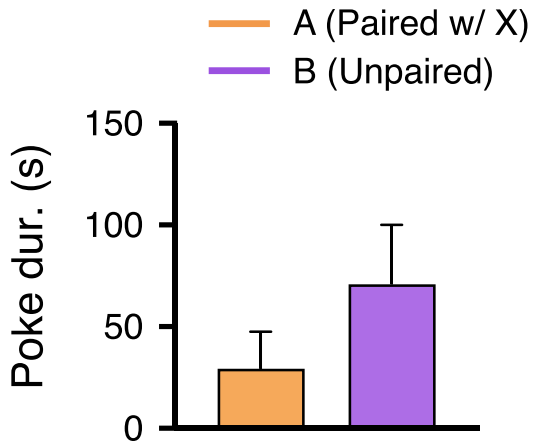



**Figure 3.19: Consumption of A vs B at test.** Mean duration of pokes during test is compared between flavours A (orange) and B (purple) (N: 7). Bars indicate SEM.

flavoured stimuli, A and B, in a single 30 min session. A and B were delivered in the side pokes at the same time (Figure 3.4C). Poke duration for each of these stimuli is presented in Figure 3.19. Consumption of A, previously paired with the devalued X, is decreased in comparison with consumption of B, initially paired with Y. Statistical analysis, though, showed no effect of compound in poke duration ($F(1,6) = 1.022$, $p = 0.351$). Thus, indicating that the preconditioning effect was absent, despite the trend observed.

## 3.4 Discussion

### 3.4.1 Results Overview

The principal aim of this chapter was to identify and establish an associative memory task in mice that could be used in combination with optogenetics to probe stimulus-

stimulus associations in the RSC. The paradigm chosen was sensory preconditioning (Brogden, 1939) because it comprises a preexposure phase where two neutral sensory stimuli are presented either sequentially or concurrently without any unconditioned stimulus.

The first attempt was to establish an appetitive version of this paradigm with stimuli from different sensory modalities. Mice were exposed to a pair of sound and light cues and to a control sound during the first phase. Then, results demonstrate that mice were successfully conditioned to the light on the second phase. Finally, when presented with the two sound cues mice expressed a stronger response to the light-paired cue but only during the post-CS epoch. A differential response during the period when light would be presented (post-AS epoch) would mean a stronger preconditioned association, but this was not observed.

By trying to decrease the duration of the conditioning phase and to increase the possibility of observing conditioned behaviour, changes were made to the appetitive SPC protocol and a new cohort of mice was tested. Although once more conditioned behaviour was observed during the second phase, preconditioned responses to the paired versus unpaired stimulus only approached significance and subsequent conditioning to the AS did not revealed a differential response to either stimulus.

Since only marginal effects were observed in the appetitive version of SPC, then a within subjects version of an aversive SPC task was established to test sensory-sensory associations with stimuli in the same sensory modality. In the preexposure phase of this task, mice received pairs of liquid solutions, AX and BY, that were repeatedly paired over four sessions of each solution. Statistical analysis of the responses during this phase indicated that some innate preference was biasing the consumption of the pairs, but any preference was gone by the last session. Then, on the second phase, X was devalued with lithium chloride while Y was not. A general decrease in consumption of both liquids was observed, although no significant differences between

X and Y consumption could be concluded. However, devaluation was reflected on the final test when A and B were presented at the same time in the same session and mice showed a significant preference for B over A.

Finally, in order to be able to use optogenetic manipulation of RSC activity in freely-moving behaviour, the aversive sensory preconditioning task needed to be adapted into an operant box setting. Mice received LED implants and a pilot experiment was run to adjust parameters in this setup. Although no significant results were achieved, results displayed a trend towards the expected outcomes for the different phases.

Both versions of SPC had never been established in mice before their presentation here. Although appetitive SPC has been successfully used with rats (Rescorla, 1980; Blaisdell et al., 2009; Robinson et al., 2014), here, the aversive sensory preconditioning paradigm seems to be more robust.

### 3.4.2 Appetitive Sensory Preconditioning

Two different protocols were tested to establish an appetitive version of a sensory preconditioning task in mice (Rizley and Rescorla, 1972; Robinson et al., 2014). Although the results from the initial protocol indicated that mice responded differently to paired and unpaired cues, the observed difference is only present 10 s after the presentation of the AS. A previous study has shown a similar response pattern with rats (Blaisdell et al., 2009), but other studies focused their analysis on both CS and post-CS periods (Robinson et al., 2011, 2014). Here, it is considered that since responding during the light epoch was present during the conditioning phase, one could expect to see the preconditioning effect just after the AS epoch (CS epoch). Instead, in the results presented here, there is an increase in responding to both cues during that time. Not showing a differential effect during this phase could indicate a generalisation between sounds, although the preconditioning effect seems to be present at the later epoch.

There are a number of reasons that might explain the lack of differentiation during the CS epoch at test. Despite the fact that the flashing light was successfully paired with reward delivery, asymptotic behaviour was only detected after 8 sessions of conditioning to light. This could indicate that the light-reward pairing was not so strong. Moreover, the longer the conditioning phase means the initial pairing in the preconditioning phase is further apart from the test, this possibly weakening the association. Also, having more light-reward pairings means that sound-light associations could have been weakened.

The second protocol showed an improvement during the conditioning phase with animals now reaching asymptote around the $6^{\text{th}}$ session. Interestingly, this change did not lead to a robust statistically significant sensory preconditioning response. Animals responded differently on the AS and post-AS epochs at test. However, that was driven mostly by a difference in the two epochs for the paired stimulus only. The following reconditioning test confirmed the idea that no differences in value acquired by the two cues could be detected. One other possibility to explain the reconditioning results is that, since that phase happened after a test under extinction, the previously formed associations could have been erased. In any case, if a preconditioning association was formed, it was most probably not robust enough.

One interesting observation relates to how increasing the duration of the AS did not seem to help obtain a stronger SPC effect. This contrast with previous observations that indicated that a longer AS could improve SPC (Hoffeld et al., 1958). But one should consider the fact that they use overlapping cues and other aspects of the protocol differ.

### 3.4.3 Aversive Sensory Preconditioning

Because only a marginal preconditioning effect was observed using an appetitive version of sensory preconditioning, it would be important to have another possible test for

neutral associations. The test chosen was an aversive sensory preconditioning paradigm based on previous experiments in rats (Lavin, 1976; Rescorla and Cunningham, 1978; Blundell et al., 2003). The main difference, besides the reinforcement type, is that in this task flavoured solutions were use and presented concomitantly.

When mice were presented with the different pairs of compounds in the preexposure phase, some innate preferences were revealed. A preference for the pair Sucrose-Quinine over Saline-HCl appeared on the initial days, but with repeated pairings that effect dissipated, as can be observed on Figure 3.13. Also, the observed difference between AX and BY on the first session could be explained by neophobia to BY, since in the previous day AX was the presented mixed compound. This could be avoided in the future if the AX and BY order of presentation was counterbalanced.

Following that, in the conditioning phase, one would expect to see a preference for Y over X emerge with time. Although no significant results indicated this was happening, the test demonstrated that animals have developed a preference since it showed a significant preference for the unpaired compound B over A.

Another observation that should be made is that even though only eight animals were tested on the aversive SPC a significant response was observed, indicating an increased power of this test when comparing with the appetitive version. All of this highlighted that the aversive version was probably the more robust task to probe the function of the retrosplenial cortex in mice.

When piloting the aversive SPC task in operant boxes, no significant results were achieved, but one possible explanation is that adapting the task to operant boxes led to a change in the power required to detect differences in the aversive SPC. Also, the fact that animals were tethered could have caused differences in the power since it might have affected the consumption rates. This indicates that a pre-training phase added initially might be important to have animals comfortably drinking in this setup.

### 3.4.4 Testing associative memory

The main objective here was to establish an associative memory task to test the importance of retrosplenial cortex for the formation of neutral stimuli associations. The two versions of the SPC paradigm establish here are able to probe this type of associations since they separate stimulus-stimulus associations from stimulus-response associations. Different versions of this task has been used to test neutral stimuli associations in rats while studying the functional role of several structures (Wheeler et al., 2013; Robinson et al., 2014; Talk et al., 2016).

One could raise the question if second-order conditioning could not be an alternative test for the objectives of this thesis. But it has been shown consistently in the literature that this other form of higher-order conditioning leads to stimulus-response associations rather than neutral stimuli associations (Rizley and Rescorla, 1972; Rescorla and Cunningham, 1978; Todd et al., 2016).

Another observation regarding the tasks established here is that they might test slightly different stimulus-stimulus associations. While the appetitive version used here tests two cues from different modalities that happen sequentially, the aversive SPC probe sensory stimuli which are both flavour and are delivered in conjunction. Previous studies show that simultaneous associations could be stronger in this paradigm than successive ones (Rescorla, 1980), which could mean that previous studies on the role of the RSC in this task (Robinson et al., 2011, 2014) will lead to different conclusions. This should be taken into consideration when analysing the results presented in Chapter 4.

# Chapter 4

# RSC and Associative Memory

## 4.1 Introduction

Episodic memories are composed of several sensory elements: scents, touch, images and spatial context (Davachi, 2006). Thus, our ability to form associations between sensory stimuli, objects and contexts is extremely important to understand the environment and survive in it (Bar, 2007). The region where associative memories are formed would require not only a constant input of information regarding the surroundings, but also sensory information from several regions of the brain. Experiments show that the associative memory network might be far more complex. Evidence comes, for example, from overlapping detected between areas recruited for associative processing and what is called the brain's default mode network (Raichle et al., 2001; Bar et al., 2007).

A region such as the hippocampus, which is implicated in both episodic nonspatial memory and spatial memory (Battaglia et al., 2011; Burgess et al., 2002), would seem like an appropriate place for associations to take place. Although neurotoxic lesions have been implicated in deficits in sensory preconditioning, the same study showed that hippocampal activity was more related to later phases of the task than with the initial

stimulus-stimulus association (Talk et al., 2002). At the same time, another study reported no deficits in SPC due to hippocampal excitotoxic lesions (Ward-Robinson et al., 2001). This was recently corroborated by a study on humans which showed that the hippocampus is more concerned with the spread of value during the reward phase of SPC (Wimmer and Shohamy, 2012). Moreover, a study by Iordanova et al. demonstrated that the hippocampus is not necessary for simple associations between components of an episodes (Iordanova et al., 2011). Thus, another region might be implicated in this specific task of biding together neutral stimuli.

The retrosplenial cortex (RSC) is mostly known for its role in spatial memory and navigation. With regards to its anatomy, the retrosplenial cortex is targeted by several cortical and thalamic regions and also strongly connects with the parahippocampal region and the hippocampal formation (Sugar et al., 2011). Its different areas will project to all subdivisions in the parahippocampal region and to the subiculum in the hippocampus (Wyss and Van Groen, 1992; Van Groen and Wyss, 2003). Its reciprocal connections to the anterior thalamus are strong (Wright et al., 2010) and it also receives sensory information from visual and somatosensory cortices (Makino and Komiyama, 2015). Consequently, it provides major polymodal inputs to parahippocampal areas (Burwell and Amaral, 1998). All of these structures connected to RSC have been implicated in episodic memory with each region contributing in different ways to that (Davachi, 2006; Eichenbaum et al., 2007). In addition, the RSC connects reciprocally with prefrontal areas (Vann et al., 2009), thus connecting hippocampal formation with cortical regions related to executive functions. This privileged position makes the RSC an important region to study in relation to both navigation and episodic memory.

Most studies focused on the RSC importance for navigation, revealing that around 8% of the RSC cells encode head-direction signals (Cho and Sharp, 2001) and that RSC lesions impair navigation in humans (Maguire, 2001) and radial arm and Morris water maze performance in rats (Vann et al., 2000). One idea is that RSC's importance

for navigation would be linked to context encoding (Bar, 2007; Bar et al., 2007). While studies show that RSC neuron activity responds to context, and specifically to patterns during learning (Keene and Bucci, 2008b,c), most animal lesion studies are conflicting. It seems that RSC is important for acquisition and recall of contextual fear conditioning and not tone-cued conditioning (Keene and Bucci, 2008c). On the other hand, one study showed that RSC-lesioned rats were able to dissociate contexts and associate an outcome with a specific context and its associated cues, but they were unable to use acquired contextual information to disambiguate between two conflicting response cues (Nelson et al., 2014). Besides the usual questions regarding lesion extent and location, what could explain the observed differences would be a more specific role of the RSC in encoding associations between cues in a space, as supported by experiments showing RSC encoding of landmarks (Auger et al., 2012).

A few studies started looking into RSC importance for encoding cues rather than context or space. In humans, a fMRI experiment proposed that RSC is active when the processing of permanent and reliable features is necessary (Auger and Maguire, 2018). Moreover, the same study showed that RSC representations are not necessarily related to space and extend to other non-spatial associations. Nelson et al. showed that damage to the RSC prevents animals from responding selectively to a cue that was found previously in a certain context (Nelson et al., 2014). Moreover, other lesion studies showed normal object recognition but impaired object–location associations (Ennaceur et al., 1997). Indicating that RSC's involvement in contextual learning could be explained by an involvement in coding associations between sensory stimuli.

In a series of experiments, a group proposed that lesions in the RSC would disrupt sensory-sensory learning (Keene and Bucci, 2008b; Robinson et al., 2011). They showed that RSC-lesioned rats could not acquire learning when two sensory cues were presented simultaneously (Keene and Bucci, 2008b) or sequentially (Robinson et al., 2011). In addition, in a recent study using chemogenetic methods in a SPC task,

Robinson et al. temporarily inhibited the RSC excitatory neurons during the initial phase of SPC and indicated that RSC is indeed necessary for creating associations between two neutral stimuli (Robinson et al., 2014). A more recent study showed that increasing input activity from the RSC to the visual cortex is required for sensory associative learning (Makino and Komiyama, 2015).

These studies led to the hypothesis that RSC's role is to aid in the formation of associative memories by binding together stimuli that are not initially behaviourally relevant to the subject. Another aim was to dissociate the role of the RSC in spatial and non-spatial associative memories. In order to address these aims, I used optogenetic manipulation of neuronal activity with adaptations of the sensory associative task developed in Chapter 3. Affording high temporal precision of manipulation required to directly target the time of stimulus delivery, RSC activity disruption during acquisition of a sensory preconditioning paradigm prevented associations from being formed. Moreover, by designing stimuli that have or not a spatial component, enabled the clarification of whether RSC's role in association formation is due to its importance in spatial cognition.

Here, I used a red-shifted opsin, Jaws, which is a chloride pump able to hyperpolarise a cell by pumping chloride inward and is activated by red light (Chuong et al., 2014). In all optogenetic experiments we expressed Jaws in excitatory cells through a CaMKII ($Ca^{2+}$/calmodulin-dependent protein kinase II) promoter. Thus, I set out first to confirm Jaws inhibition of neuronal activity when using a red light source. For that, we perform electrophysiological recordings in the RSC of anaesthetised mice while using a red laser at different light intensities to test for inhibition of my target region.

Finally, one of the purposes of developing a sensory associative task for mice was to also study how the brain represents these sensory-sensory associations and how learning affects these neural representations. Responsive neurons to task events and sensory stimulus have been described in the RSC. In a T-Maze task, Vedder *et al*

discovered neurons that responded to a light stimulus that cued the reward location (Vedder et al., 2017). They also describe how the number of responsive neurons increased with training. In addition to that, another study reported increased activity in RSC V1-projecting axons after learning in response to a visual stimulus (Makino and Komiyama, 2015). Although these studies have described conjunctive coding in the RSC, so far none of them has tried to understand RSC responses to simple sensory stimuli and associations between them.

To achieve that, a way of recording neuronal activity was to combine calcium sensors with two-photon excitation laser scanning microscopy (Denk et al., 1990; Svoboda et al., 1997). The position of the RSC in rodents makes this region really accessible for two-photon calcium imaging. These two techniques put together are able to record *in vivo* fluctuation in calcium concentration as a readout of neural activity while a head-fixed animal performs behavioural tasks.

Two-photon imaging allows the rapid scanning of the neural tissue with a high spatial resolution by combining two-photon excitation trough a pulsed laser light of high wavelength (around 900 nm) and the tight focusing of laser scanning microscopy. This confines photobleaching and heat-related damage to the localised focal plane and avoids out-of-focus contamination (Denk et al., 1990). Calcium sensors respond to intracellular calcium concentration synthetic and genetically encoded fluorescent sensors allow the detection of changes in this concentration as an indirect measure of neuronal activity (Chen et al., 2013).

The development of sensory preconditioning task and the confirmation of RSC involvement in sensory-sensory associations had the objective of studying this associations in a mechanistic way. In the final part of this chapter, a simple passive sensory presentation of different flavoured solutions is developed under two-photon calcium imaging to address the question of whether the RSC is able to represent neutral sensory stimuli and how it encodes sensory-sensory associations. By using mice

expressing the calcium sensing protein GCaMP6s (Chen et al., 2013), it is possible to record neural activity in the RSC while the animal is performing that task. Here, a preliminary analysis of these experiments is described.

## 4.2 Methods

### 4.2.1 Electrophysiology recordings

Injections of rAAV9/CaMKII-Jaws-KGC-GFP-ER2 were carried out as described on Chapter 2. In order to obtain a good level of opsin expression, electrophysiological recordings were performed at least three weeks after surgeries were completed. For experimental controls, either naive C57Bl/6 mice or control virus injected animals were used (rAAV5/CaMKII-EYFP).

Anaesthesia was induced using Urethane (10% in saline) at 1 mg/kg body weight. Once depth of anaesthesia was ascertained by the lack of tail-pinch and leg-withdrawal reflexes, mice were placed in a stereotaxic frame. A craniotomy (larger than 2 mm in diameter) was made dorsally of the RSC region using a dental drill in a similar way as described on section 2.1.2. In some cases, mice were used for recordings after being tested behaviourally. In these cases, prior to the craniotomy, after ensuring a suitable depth of anaesthesia the head-cap was removed by drilling carefully around it, melting the cement with acetone (Sigma-Aldrich), adding saline to loosen the implant and then pulling it carefully off the skull.

Then, a silicon probe, either a single shank (A1x32-Poly2-5mm-50s-177; Neuronexus) or a four-shank (A4x8-5mm-100-200-177; Neuronexus) probe was lowered next to the sagittal sinus around the centre of the injection sites. The probe was slowly lowered to 800 μm and recordings started after 10 minutes. Recordings were made with a laser intensity of 65, 25 and 6 mW at the output, that came to 50, 20, and 5 mW at the end of the fibre optic cable. The recordings used the same stimulation

regime as the one used for behavioural tests.

For the data analysis, multiunit activity was detected by filtering the signal with a 500 Hz highpass butterworth filter. Any threshold crossing higher than 5 SD of baseline was considered as a spike. The peristimulus time histogram (PSTH) was plotted as z-scores (normalised by mean firing rate) on 50 ms bins. The change in neural activity was calculated by normalising the activity to baseline and then comparing the difference in firing rate between the stimulus period and the pre-stimulus period and dividing that by the firing rate in the stimulus period.

### 4.2.2 Aversive Sensory Preconditioning - Operant Boxes - Protocol II

The protocol used for optogenetic manipulation of RSC during aversive sensory preconditioning had a few modifications from the initially tested protocol in Chapter 3 (Figure 3.4). The protocol presented here was used for both the spatial and non-spatial versions of the aversive SPC task. Below, changes from the previous protocol (Section 3.2.4) are highlighted and the procedure is summarised.

*Subjects:* 16 C57Bl/6 mice received injections of virus for expressing the Jaws opsin and other 16 C57Bl/6 mice were injected with control virus. Both groups also received LED implantation as described in Section 2.1.2. Animals were run into two cohorts each containing eight subjects of each group. Animals where the LED headstage was not functioning or fell off were again removed. Thus, the final count of subjects for each experiment was for the Spatial version: 15 Controls and 13 Jaws; and for the Non-Spatial version: 14 Controls and 16 Jaws.

*Pretraining phase:* The first change to the protocol was the inclusion of a pretraining phase. Water restricted mice were tethered and put in the operant box for 30 minutes or until they had consumed 30 water rewards delivered from one of the three pokes, which was lit to indicate where they could find reward. In this phase, the reward was

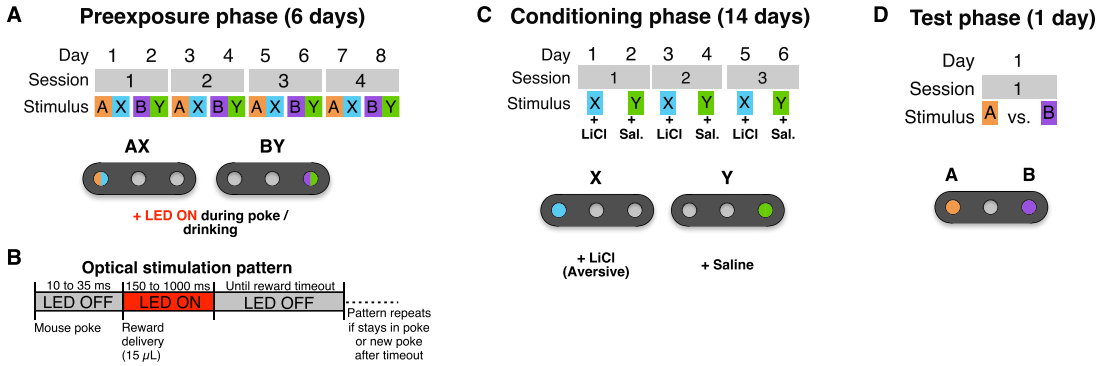



**Figure 4.1: Optogenetic silencing during Spatial aversive SPC.** Aversive sensory preconditioning protocol in operant boxes with optogenetic silencing during the preexposure phase. For all phases, solutions part of the same session were presented on opposite sides and identities were counterbalanced. (A) Preexposure phase with AX and BY being delivered on alternate days. Silencing of RSC was done in this phase. (B) Optogenetic stimulation pattern. Periods of non activation are in grey and light on periods in red. The pattern repeats after a timeout of 2 s if the animal is still in the poke. (C) Conditioning phase protocol. LiCl injection were applied after sessions where X was presented, and saline injections were used after Y sessions. No stimulation present. (D) Single session choice test between A and B. No stimulation present.

drinking water (15 μL). They had up to three pretraining sessions one with each poke to be used in the tasks.

This time, unlike the previous aversive SPC, in all phases, there was a timeout of 2 s before the next reward was delivered if the animal was still in the poke or tried to poke again (Figures 4.1B and 4.2B). This allowed the animal to fully consume the reward delivered before getting a new one.

*Preexposure phase:* The first phase was composed of eight days with exposure to flavoured solutions AX or BY on alternate days. For the Spatial Sensory Preconditioning AX would be delivered through one of the side pokes and BY, the next day, on the opposite side poke (Figure 4.1A). Side of presentation of solutions was counterbalanced between animals, but constant over days. For the Non-Spatial version of the task, both were delivered on the centre poke on alternate days (Figure 4.2A). Pokes were lit up to indicate that reward was available. Subjects had to poke to get a 15 $\mu$L delivery

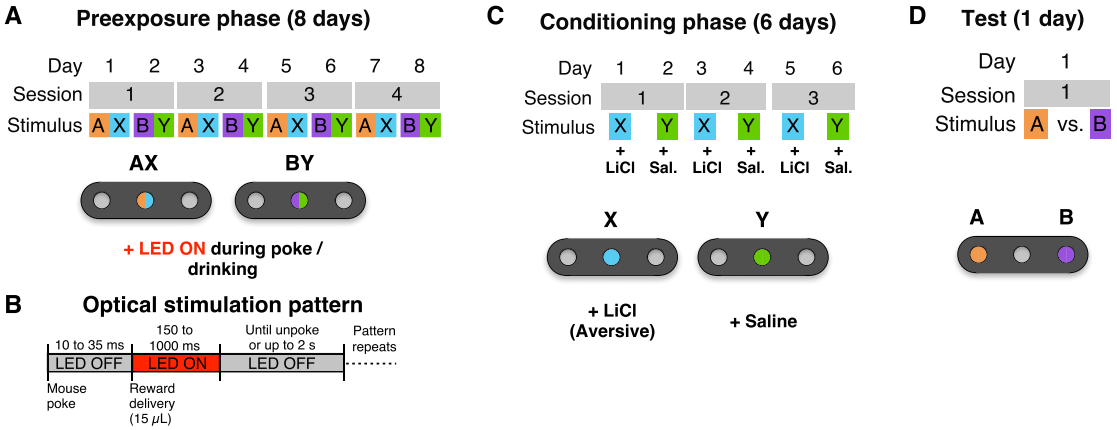



**Figure 4.2: Non-spatial Aversive SPC.** Aversive sensory preconditioning protocol with less spatial confounds. Optogenetic silencing happened during the preexposure phase. (A) Preexposure phase with AX and BY being delivered on alternate days. Silencing of RSC in all experimental subjects was done in this phase. All solutions were delivered from the central poke. (B) Optogenetic stimulation pattern. Periods of non activation are in grey and light on periods in red. The pattern repeats after a timeout of 2 s if the animal is still in the poke. (C) Conditioning phase protocol. LiCl injection were applied after sessions where X was presented, and saline injections were used after Y sessions. No stimulation present. All solutions were delivered from the central poke. (D) Single session choice test between A and B which were delivered from the side pokes. Side of presentation was counterbalanced. No stimulation present.

of the mixed compounds. Poking triggered the reward after a pseudorandom interval (10 to 35 ms) and the optogenetic stimulation by turning on the implanted LED (at 50 mW). Stimulation would last for a pseudorandom duration (150 ms to 1 s). Rewards were delivered every 2 s if the animal remained in the poke. Every time a reward was delivered, stimulation would be triggered as described above. Each afternoon, $\approx 4$ hours after training, mice were given access to water for 30 minutes.

*Conditioning phase:* For six days, mice were allowed to drink X only or Y only on alternate days in 30 minutes sessions. When the sessions finished, after drinking X, all mice received an injection of 0.15M LiCl (40ml/kg). After drinking Y, mice received a 0.9% saline injection. For the Spatial version X and Y were delivered from the side pokes, respectively where AX and BY had been delivered (Figure 4.1B). For the Non-Spatial SPC, both X and Y were delivered from the centre poke on alternate days (Figure 4.2B).

*Test:* For the test session, A and B were presented simultaneously for 30 min (Figure 4.1C and 4.2C). Mice had a choice of drinking either from the left or from the right pokes. A and B location was counterbalanced for the Non-Spatial version (Figure 4.1C) of the task and A and B followed the presentation pattern for AX and BY in the preexposure phase.

For all phases, consumption was measured by calculating the duration of nosepokes. Counterbalancing factors (stimulus identity, side of presentation) were included in the statistical analysis when any interaction was observed.

### 4.2.3 Two-photon calcium imaging

*Subjects:* Four transgenic mice expressing GCaMP6s (Chen et al., 2013) in excitatory cells went through chronic window implantation, handling and water regulation as described in Sections 2.1.2 and 2.1.3.2.

*Pretraining:* Water regulated mice were head fixed to the running wheel for

habituation and pretraining, as shown in Figure 4.17A. They received three days of pretraining where 50 drinking water rewards (10 $\mu$L) were made available at variable intervals from the spout placed in front of the mouse's mouth. The objective was to have all mice constantly seeking rewards at $\approx$ 10 s intervals. On the first day, mice were allowed to drink rewards with an average inter-trial interval of 2 s (ranging from 1 to 3 s). After two or three sessions, mice received a final session of 5 s ITI (ranging from 4 to 6 s) before being returned to their home cage. On the second day of pretraining, mice started with 5 s ITIs and after two sessions the average ITI was increased to 10 s (ranging from 8 to 12 s) on the final session. Finally, the next day, mice received only two sessions of 50 rewards delivered at 10 s average ITI.

*Behavioural protocol:* The protocol used involved the passive presentation of stimuli to the mouse. All the mouse was required to do is to lick when the 10 $\mu$L reward was delivered from the spout. The same compounds and concentrations from previous aversive sensory preconditioning experiments were used. Y was always Quinine and X was always hydrochloric acid. A and B identities were counterbalanced (Sucrose or Saline) between animals. The full passive stimulation protocol was composed of five days. On the first day, compounds A, X and Y were presented as pure solutions to the mouse in three sessions, one of each, with the order of presentation partially counterbalanced. Each session had 30 reward deliveries with an average ITI of 10 s. On the $2^{\text{nd}}$ to $4^{\text{th}}$ days, mice received one session of delivery of a mixture of AX and another of delivery of Y alone. The order of the sessions was counterbalanced. This time 50 rewards of each were delivered at the 10 s ITI. On the final session, all four compounds were presented alone one in each session of four sessions, following the same delivery scheme as day 1.

*Calcium imaging protocol:* For the last pretraining day and the passive stimulation days 1 to 5, two-photon imaging was acquired from the retrosplenial cortex (part of granular and dysgranular areas) at $\approx$ 30 Hz for the duration of the reward deliveries.

On the first imaging session, the RSC region was located on the posterior part of the window and next to the sinus. The z-coordinate was zeroed at the surface so the depth of imaging could be calculated. Imaging areas were chosen at around 250-330 nm from the surface. Short 300 frame videos were acquired to aid in localising areas in future sessions. Before each imaging session, the cranial window was cleaned with double distilled water and water gel was applied before lowering the objective to the window. Once the imaging location was detected using the short videos initially acquired, behavioural programs and imaging were started.

*Analysis:* Preprocessing of imaging videos was done as described in Section 2.2.2.1. Once behavioural data was aligned with the fluorescence data, trials were defined as starting when the first lick was detected right after a reward delivery was made. If the first lick happened after 4 s from reward delivery the trial was not considered. Trials were aligned and data was processed either at single trial level or trial averaged.

## 4.3 Results

### 4.3.1 Optogenetic manipulation of RSC

In order to test if retrosplenial cortex activity could be manipulated optogenetically, Dr. Liad Baruchin and I performed electrophysiological recordings *in vivo* in anaesthetised animals, that I had injected with the red-shifted opsin, Jaws, to be expressed in excitatory cells of the RSC (Figure 4.3A). Activation of this opsin would lead to inactivation of RSC's excitatory cells. Control animals used for electrophysiology included injected with rAAV5/CaMKII-EYFP or naive C57Bl/6 animals.

The effect of laser activation on RSC neurons expressing the opsin Jaws was immediate and very strong. Figure 4.3B shows the peristimulus time histogram for the normalised firing rate (normalisation by the mean firing rate) for recorded RSC cells in both Control and Jaws animals ($N_{Control}$: 2; $N_{Jaws}$: 2). By comparing the three bins

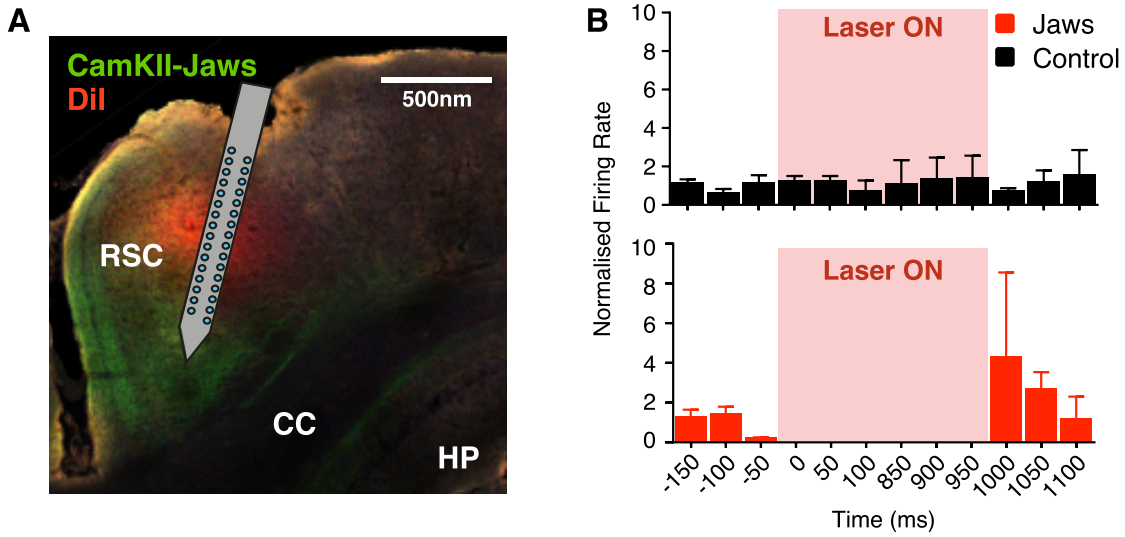



**Figure 4.3: Optogenetic inactivation of the RSC.** (A) Coronal slice of the right RSC where it is possible to see the expression of the opsin Jaws (green) and the DiI labelling (red) at the place where the recording probe was inserted. (B) Normalised firing rate (z-score) for Control (black) ($N_{Control}$: 2 mice) and Jaws (red) animals ($N_{Jaws}$: 2 mice) before, during (pink box, Laser ON) and after light stimulation. Bars represent standard error of the mean(SEM). RSC - retrosplenial cortex; CC - corpus callosum; HP - hippocampus

pre stimulus with the three bins post stimulus start (Bins x Light condition x Group), a significant interaction between light condition and group arises ($F(1,2) = 4096$, $p < 0.001$) and a main effect of light ($F(1,2) = 2774$, $p < 0.001$) and of group ($F(1,2) = 4096$, $p < 0.001$). When looking at the simple effects of the interaction, while no difference between Jaws and control is observed in the period when the light is off, a significant difference appears when the laser is turned on ($F(1,2) = 4096$, $p < 0.001$). Now, a comparison of the pre and post stimulation periods revealed a rebound of activity that returned to baseline levels after around 100 ms. This observation was not confirmed by statistical analysis as no main effect or interaction was detected (All $F < 1.359$, $p > 0.354$).

In addition, we analysed the effect of laser activation at different probe depths on both experimental groups. As can be seen on the raster plots, the laser activation suppressed RSC firing in the Jaws mouse, whereas the same was not as marked in

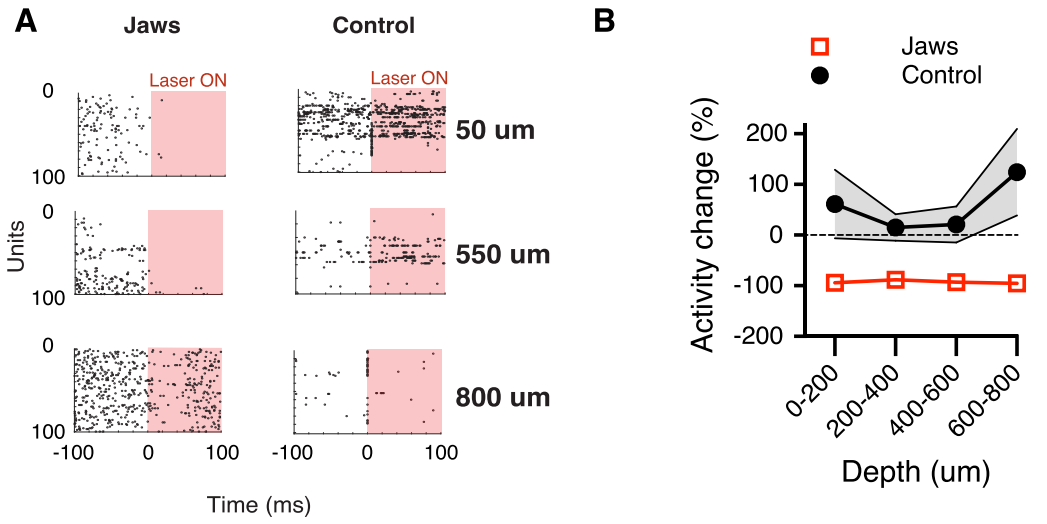



**Figure 4.4:** (A) Raster plots from one Jaws animal and one control showing spikes (black dots) over time before and after optogenetic stimulation (pink , Laser ON) at different depths into the brain. (B) Percent change in activity when comparing spike rate during the stimulus with spike rate before the stimulus for both Control (black) and Jaws (red) groups ($N_{Control}$: 2; $N_{Jaws}$: 3). SEM is expressed by shadings around traces.

the Control (Figure 4.4A). Figure 4.4B quantifies what is seen in the raster plot over more animals ($N_{Control}$: 2; $N_{Jaws}$: 3). The activity change in percentage is shown per different depths. The activity in Jaws animals showed almost 100% decrease for the different depths, while the control animals showed no change to increased activity. Although, statistical analysis did not reveal a group and depth interaction ($F(3,9) = 0.383$, $p = 0.768$), a main effect of group was detected ($F(1,3) = 61.47$, $p = 0.004$). Indicating that the light decreases activity in the Jaws animals more than in controls independent of depth.

To account for possible *dura mater* and bone regrowth, which might affect tissue illumination, we also tested weaker light intensities at the different depths for the Jaws mice (N: 2). We observe that inhibition increases around 100 µm which correlates to the start of cell layers (Figure 4.5). There seems to be a bit of variance, possibly when there is a change from one cell layer to the other. Depth had a significant effect

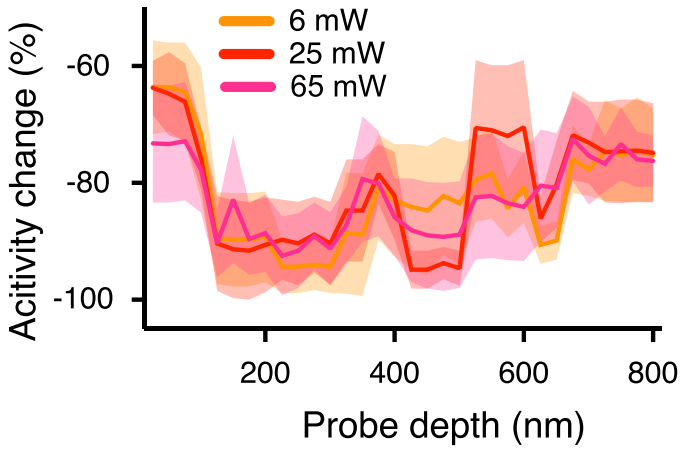



**Figure 4.5: Variation of inactivation power with laser intensity.** Percent change in neuronal activity in the RSC by depth in the probe for different laser light intensities.

on activity levels ($F(31, 93) = 2.252$, $p = 0.001$) with deeper regions exhibiting less percent change in activity. For different light intensities, on the other hand, there was no interaction between depth and intensity ($F(62, 93) = 0.196$, $p = 1.000$) and no main effect of light was observed ($F(2, 3) = 0.122$, $p = 0.889$).

### 4.3.2 Histological Analysis

Histological procedures were performed after behavioural testing was completed and are described in Section 2.1.5. For all behavioural optogenetic experiments a total of four cohorts were used (two cohorts for each experiment). Cohorts 1 and 2 were used for the first aversive SPC protocol (Section 4.3.3) and for the T-maze experiment (Chapter 5). Cohorts 3 and 4 were used on the second SPC task (Section 4.3.4) and the spatial reference memory task (Chapter 5). In this section, histological analysis is presented in advance of the behavioural results in order to be taken into consideration for interpretation of data.

Slices were collected every 0.3 mm in the range from around 1.0 to -3.8 mm

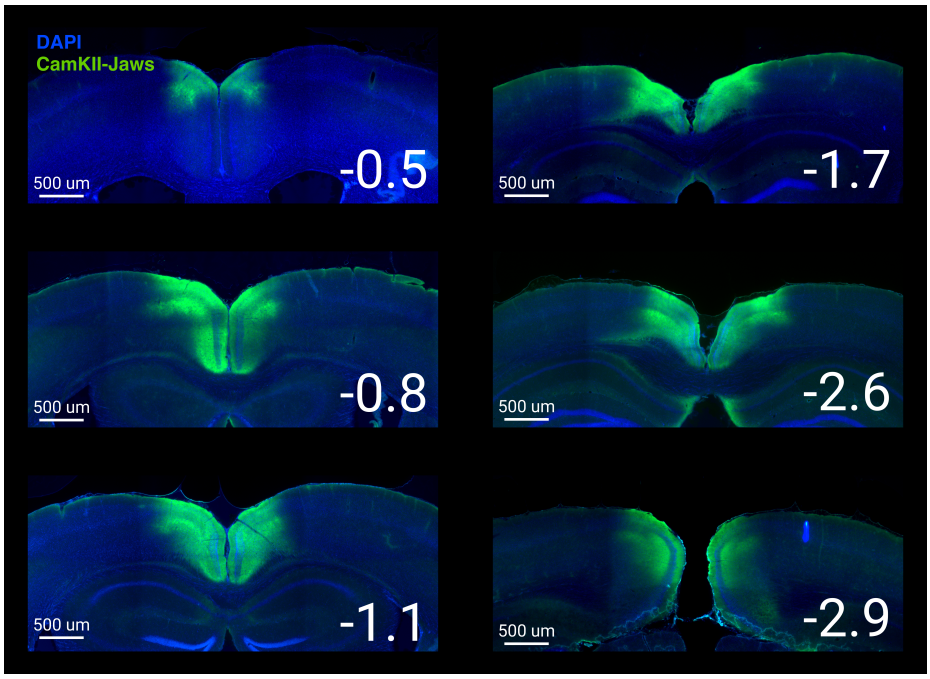
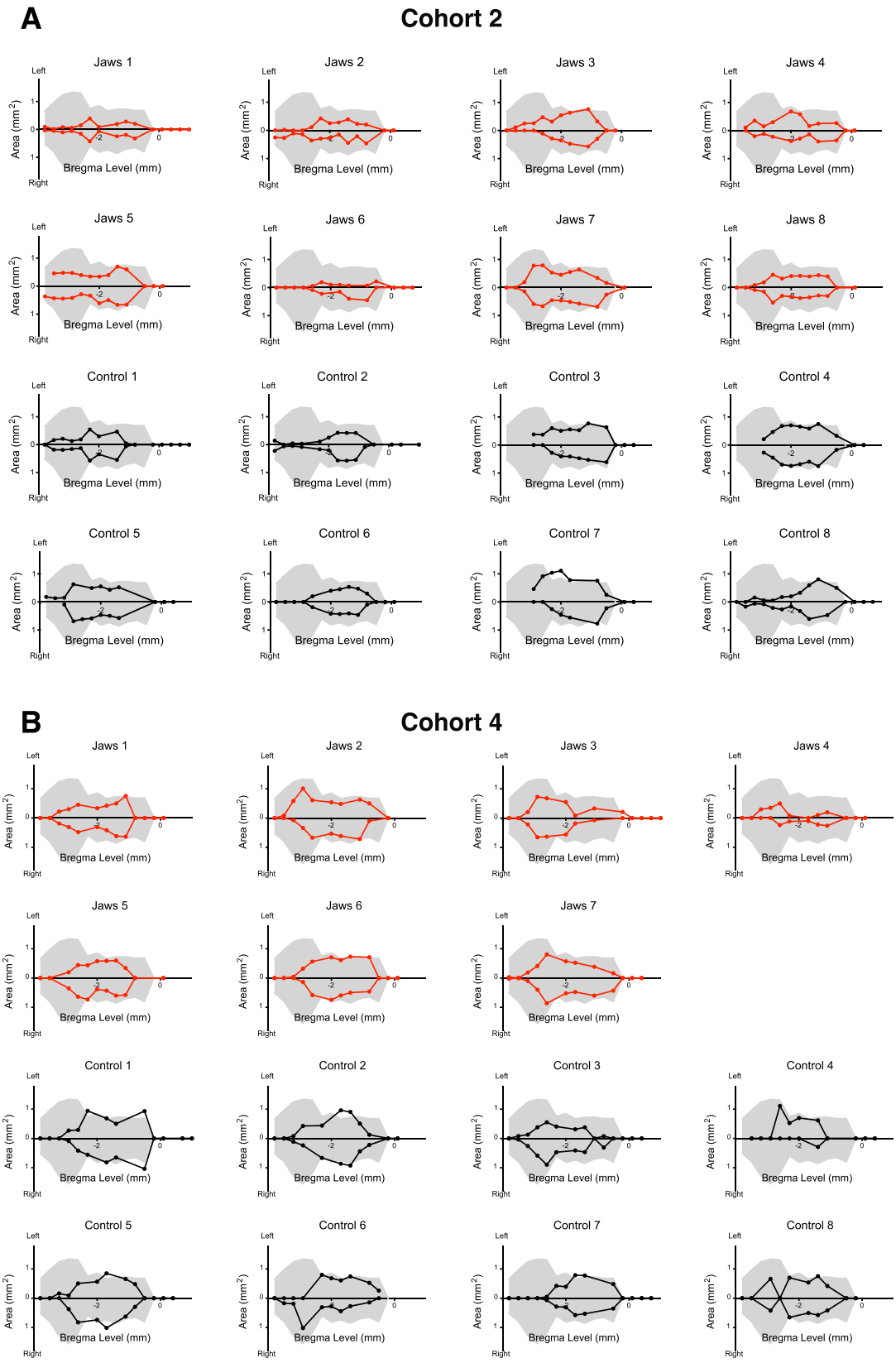



**Figure 4.6: Jaws expression in the RSC.** Coronal slices of a mouse injected with the opsin Jaws (green). DAPI (blue) is used to stain the cell nuclei. Bregma levels (μm), indicating anterior-posterior level, are shown in white on the lower right corner.

in distance from bregma. Cell nuclei were stained with the fluorescent dye, DAPI (4',6-Diamidine-2'-phenylindole dihydrochloride). No additional staining procedure was performed. Figure 4.6 displays a series of slices from one of the subjects that had been injected with the opsin Jaws. It is important to call attention to the fact that, because of the KGC-ER2 sequences, the virus construct targets the expression of Jaws to membranes and the opsin is localised mostly in processes and not in soma. KGC is an endoplasmic reticulum forward transport sequence and ER2 is a Golgi export sequence, both from the potassium channel Kir2.1 (Ma et al., 2001; Hofherr et al., 2005). This causes difficulty in identifying cell bodies in the brain slices. The RSC starts at around -0.7 mm from bregma and goes as far as -4.5 mm (Paxinos and Franklin, 2001). Hence, we observe mostly filled processes that expanded most of the area of the RSC from about -0.8 to -2.9. The slice at -0.5 μm from bregma would not contain RSC, but processes are visible in the area which can be identified as M2


A
Cohort 2
Jaws 1
Jaws 2
Jaws 3
Jaws 4
Jaws 5
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Control 1
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Control 6
Control 7
Control 8
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Area (mm²)
Bregma Level (mm)
B
Cohort 4
Jaws 1
Jaws 2
Jaws 3
Jaws 4
Jaws 5
Jaws 6
Jaws 7
Control 1
Control 2
Control 3
Control 4
Control 5
Control 6
Control 7
Control 8
Left
Right
Area (mm²)
Bregma Level (mm)

**Figure 4.7: (Previous page) Area of viral expression in individual animals.** (A-B) Expression spread displayed as area calculated over different bregma levels for both viral constructs, Jaws (red) and Control (black). Shaded grey indicates total RSC area.

(Paxinos and Franklin, 2001).

Figures 4.7A and B display the area of expression in the retrosplenial cortex along the anterior posterior axis for each animal in two different cohorts (Cohorts 2 and 4) used in separate experiments. Left hemisphere expression is plotted on the positive y-axis and right side expression on the negative y-axis. Animals are separated in groups and these cohorts are considered to be representative of the other two cohort not shown here.

Virus injections were made in two sites at each hemisphere (see Section 2.1.2). In the RSC expression plots (Figure 4.7), this is perceived as two peaks in both hemispheres which are visible in some of the animals. Which means that area of expression is maximum near the injection sites and decreases depending on the distance to the sites. The craniotomy where the LED was implanted is centred around -2.05 mm from bregma and had a 1.8 mm diameter. Most animals, from both groups, show a larger infected area around -0.8 to -3.5 mm from bregma. Which means the centre of the expression area is roughly aligned with where the LED is centred.

Traced areas were also separated into RSC areas of expression and extra-RSC areas. Figure 4.8 shows the added expression of both hemispheres in regions outside the RSC in relation to AP axis (bregma level) for one of the cohorts used. Other cohorts showed a similar pattern. A larger spread to areas outside the RSC is observed for injections of the opsin Jaws than for the injection of the control virus (rAAV5/CaMKII-EYFP). These areas concentrate mostly in the anterior part of the brain. The spread to other regions than the RSC can also be observed in the example in Figure 4.6 at -0.5 μm from bregma. This extension affected mostly neighbouring regions secondary motor

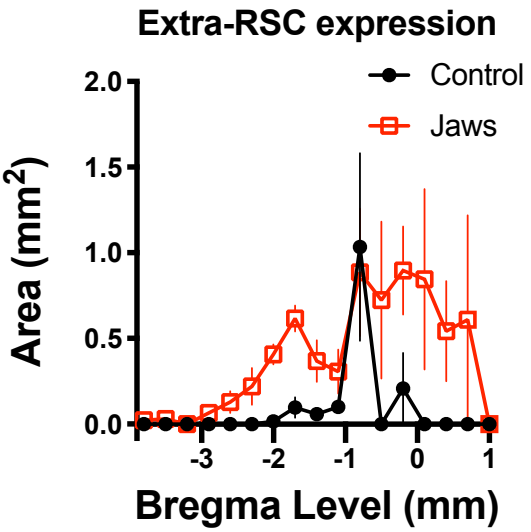



**Figure 4.8: Viral expression outside the RSC.** Averaged area of viral expression for both Jaws (red) and Control (black) constructs by distance to bregma ($N_{Control}$: 6; $N_{Jaws}$: 8). Bars express SEM.

cortex and anterior cingulate cortex.

### 4.3.3 Optogenetic silencing of RSC affects associations in aversive sensory preconditioning

As our electrophysiological experiments (Section 4.3.1) confirmed that RSC activity is disrupted by Jaws activation, we set out to test the effect of RSC optogenetic disruption in neutral sensory associations.

We adapted a sensory aversive preconditioning paradigm described in Blundell et al. (2003). We developed an operant box setup which allowed us to trigger Jaws activation whenever the animals were drinking, i.e. receiving the sensory stimuli. We measured the amount of time the mice spent inside the nose pokes during the three phases of the task. The used protocol is portrayed in Figure 4.1.

*Preexposure:* we compared the fluid consumption of the compounds by measuring the amount of time mice spent inside pokes. Figure 4.10 shows the average duration of pokes for AX and BY pairs in each group ($N_{Control}$: 15; $N_{Jaws}$: 13). It is possible

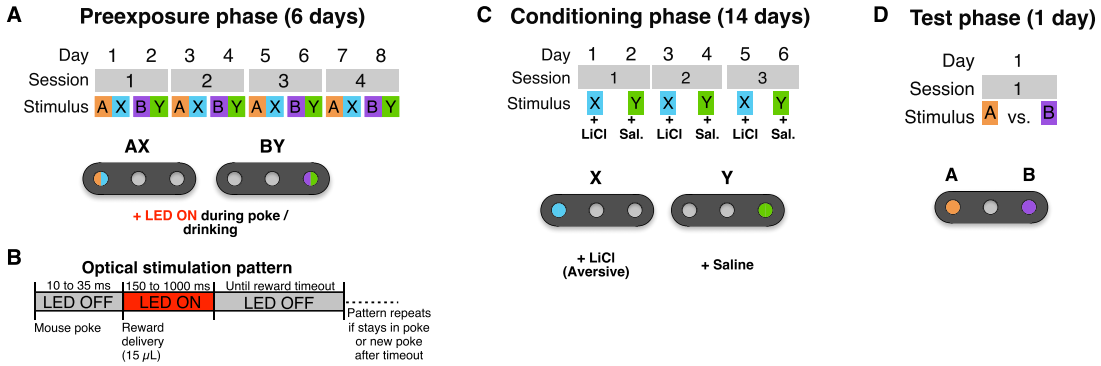



**Figure 4.1: Timeline of optogenetic silencing during aversive SPC.** Aversive sensory preconditioning protocol in operant boxes with optogenetic silencing during the preexposure phase. For all phases, solutions part of the same session were presented on opposite sides and identities were counterbalanced. (A) Preexposure phase with AX and BY being delivered on alternate days. Silencing of RSC was done in this phase. (B) Optogenetic stimulation pattern. Periods of non activation are in grey and light on periods in red. The pattern repeats after a timeout of 2 s if the animal is still in the poke. (C) Conditioning phase protocol. LiCl injection were applied after sessions where X was presented, and saline injections were used after Y sessions. No stimulation present. (D) Single session choice test between A and B. No stimulation present. (repeated from page 78)

to observe that both groups consumed similar amounts of the compounds AX and BY. Poke durations in each day was between 100 and 200 s. There was an interaction between group, session and flavour ($F(3, 60) = 2.982$, $p = 0.038$). This was due to a transient preference for BY in the Control group shown by analysis of simple effects of the interaction (Control, Day 3: $F(1, 20) = 4.969$, $p = 0.037$). This does not appeared to be a flavour preference. It was probably due to mice being run at a slightly later time which increased thirst on that day. Importantly, there were no differences for flavours on days 1, 2, or 4 (Control: All $F < 4.969$, $p > 0.083$; Jaws: All $F < 3.175$, $p > 0.09$). Furthermore, a two-way ANOVA on data from session 4 revealed no interaction between group and compound ($F(1, 20) = 0.044$, $p = 0.836$) indicating that by the last session there was no difference in both group's drinking behaviour.

*Conditioning:* Both Jaws and Control groups successfully acquired conditioned

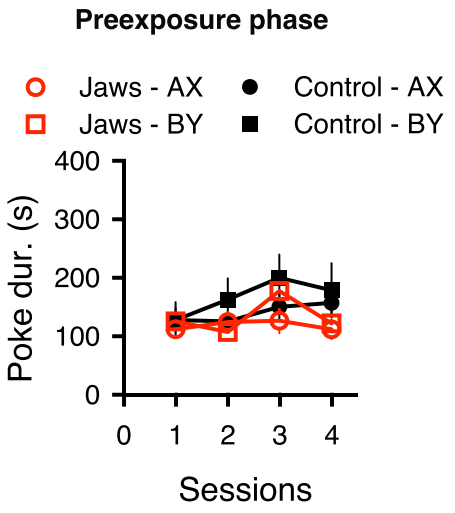



**Figure 4.10:** Mean duration of poke with SEM is presented over the course of four sessions of preexposure for both experimental groups ($N_{Control}$: 15, black traces; $N_{Jaws}$: 13, red traces). Each session was composed of two days with AX (circles) and BY (squares) being presented on separate days. There was optogenetic stimulation whenever the animal poked to drink, for both compounds.

flavour aversion to X. This was shown as a significant reduction in consumption of X over repeated pairings compared to Y, this did not look different between groups (Figure 4.11). Statistical analysis revealed a significant interaction between flavour and session ($F(2, 48) = 7.848$, $p = 0.001$) and no significant main effect of group ($F(1, 24) = 0.887$, $p = 0.356$) or any interaction with group (All $F < 0.827$, $p > 0.444$), suggesting a robust acquisition of taste aversion to X by both groups. A simple effects analysis of flavour and session interaction revealed that the taste aversion (consumption of X lower than Y) was significant on session 2 (Control: $F(1, 24) = 36.078$, $p < 0.001$; Jaws: $F(1, 24) = 9.959$, $p = 0.004$) and 3 (Control: $F(1, 24) = 24.547$, $p < 0.001$; Jaws: $F(1, 24) = 15.356$, $p = 0.001$) but not session 1 (Control: $F(1, 24) = 1.539$, $p = 0.227$; Jaws: $F(1, 24) = 2.927$, $p = 0.1$) for both Control and Jaws animals.

*Test:* In this paradigm, mice would demonstrate sensory preconditioning if they showed a preference for B, which had been paired in the preexposure phase with Y, the flavour that was associated with saline in the conditioning phase. One would expect

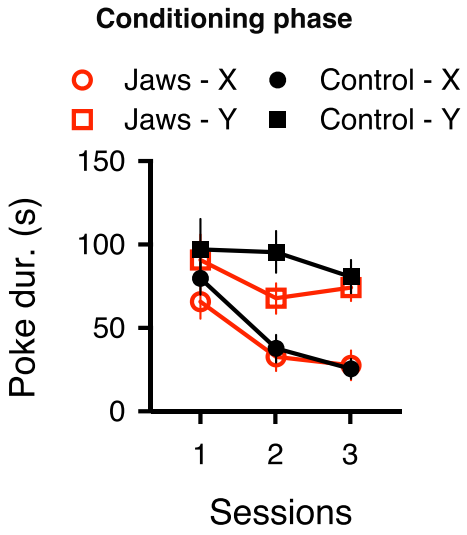



**Figure 4.11: Responses during conditioning phase.** Duration of pokes as consumption of X (circles), which was devalued by repeated pairings with LiCl, and for Y (squares), that was paired with saline injections for both experimental group ($N_{Control}$: 15, black traces; $N_{Jaws}$: 13, red traces). There was no optogenetic stimulation in this phase. Bars indicate SEM.

animals to avoid A, that was previously paired with X, which was devalued during conditioning. In a simultaneous choice test between flavours A or B, Control animals displayed a strong preference for flavour B over A, but the Jaws animals did not show preference to flavour A or B (Figure 4.12). Our analysis showed a significant interaction between flavour and group ($F(1, 24) = 4.788$, $p = 0.039$ and a main effect of pairing: $F(1, 24) = 16.301$, $p < 0.001$). Moreover, simple effects showed a significant difference in consumption of B over A for Controls ($F(1, 24) = 21.445$, $p < 0.001$) and not for Jaws ($F(1, 24) = 1.56$, $p = 0.224$). The overall total amount drank for both groups did not differ as demonstrated by the absence of a main effect of group ($F(1, 24) = 1.877$, $p = 0.183$). Thus, only Control animals showed a preconditioning effect while Jaws animals drank similar amount of both the solution that had previously been paired with a flavour they developed an aversion to and the solution that had been paired with a non-aversive flavour, despite no significant group differences observed in the previous phases.

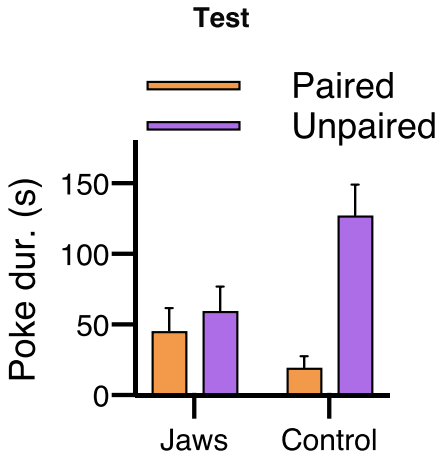



**Figure 4.12: Consumption of A (Paired) vs B (Unpaired) at test.** Mean duration of pokes during test is compared between flavours A (orange) and B (purple) for both experimental groups ($N_{Control}$: 15; $N_{Jaws}$: 13). No optogenetic stimulation was performed in this phase. Bars indicate SEM.

### 4.3.4 Aversive preconditioning: probing sensory-sensory association or sensory-spatial association?

In our first experiment, the compounds were presented in a specific poke depending whether they were AX and BY. Although location was counterbalanced between animals we cannot dissociate the importance location might have on the observed preconditioning effect or the lack of that in optogenetically manipulated animals. Thus, we modified the paradigm in a way that during the first two phases, flavour compounds were always presented in the centre poke of the operant box (Figure 4.2). Only when the animals had the single session choice test flavours A and B were presented on the side pokes.

*Preexposure:* Again there were no obvious differences between the duration of pokes for pairs AX or BY or differences between groups ($N_{Control}$: 14; $N_{Jaws}$: 16) as shown in Figure 4.14. There was a slight increase in drinking of BY by controls on session 2, but that was transient. Statistical analysis revealed no interaction between

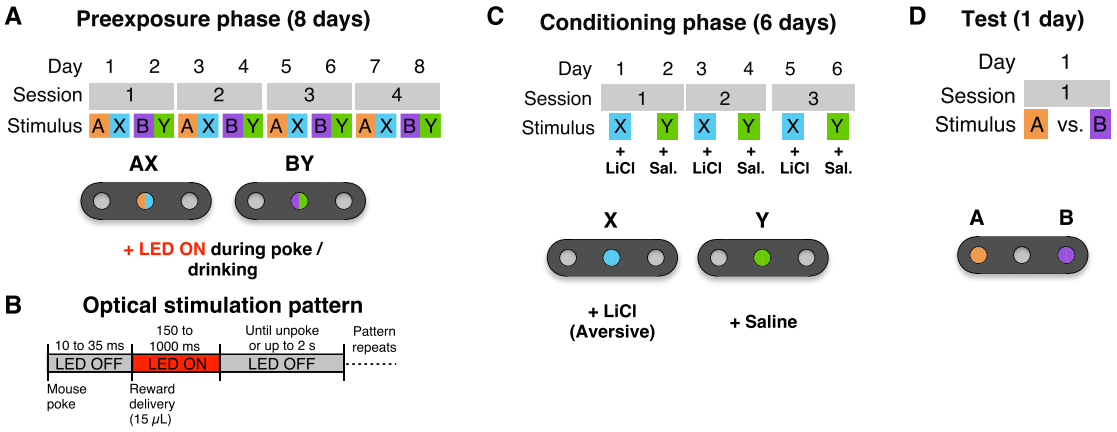



**Figure 4.2: Non-spatial Aversive SPC.** (A) Preexposure phase with AX and BY delivered on alternate days. Silencing of RSC in all experimental subjects was done in this phase. All solutions were delivered from the central poke. (B) Optogenetic stimulation pattern. Periods of non activation are in grey and light on periods in red. The pattern repeats after a timeout of 2 s if the animal is still in the poke. (C) Conditioning phase protocol. LiCl injection were applied after sessions where X was presented, and saline injections were used after Y sessions. No stimulation present. Solutions were delivered from the central poke. (D) One session choice test between A and B which were delivered from the side pokes. Side of presentation was counterbalanced. No stimulation present. (repeated from page 79)

group, session and flavour ($F(3, 66) = 2.061$, $p = 0.114$), but there was an interaction between flavour and session, between session and group and main effects of session, of flavour and group (All $F > 4.245$, $p < 0.046$). Because of these effects, analysis of simple effects of the three way interaction was run and showed that Control animals transiently preferred BY over AX on the first 2 sessions (Control - Session 1 and 2: $F > 5.281$, $p < 0.031$ and Jaws - Session 1 and 2 $F < 2.306$, $p > 0.143$). Importantly, no preference was observed on the last two sessions (Control and Jaws - Session 3 and 4: $F < .318$, $p > 0.579$). This was probably due to experimental error as some animals were still adapting to water regulation. In particular, ANOVA of flavour and group variables on the last day showed no interaction ($F(1, 22) = 0.086$, $p = 0.772$) and no main effects (Flavour: $F(1, 22) = 0.224$, $p = 0.641$; Group: $F(1, 22) = 2.954$, $p = 0.100$).

*Conditioning:* even with both flavour X and Y being delivered from the same poke

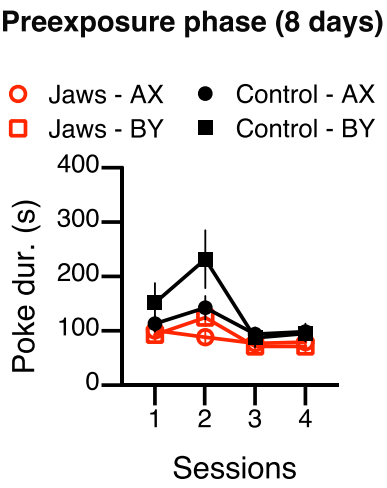



**Figure 4.14: Response during preexposure in the non-spatial aversive SPC.** Mean duration of poke with SEM is presented over the course of four sessions of preexposure for both experimental groups ($N_{Control}$: 14, black traces; $N_{Jaws}$: 16, red traces) in the non-spatial version of aversive SPC. Each session was composed of two days with AX (circles) and BY (squares) being presented from the centre poke on alternate days. There was optogenetic stimulation whenever the animal poked to drink, for both compounds.

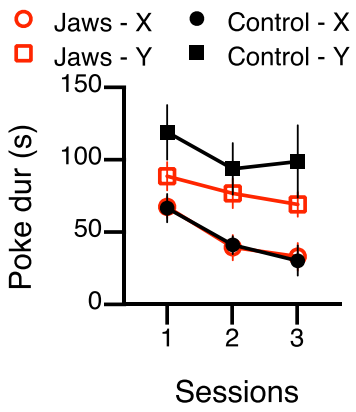



**Figure 4.15: Conditioned responses in the non-spatial aversive SPC.** Averaged duration of pokes as consumption of X (circles), which was devalued by repeated pairings with LiCl, and for Y (squares), that was paired with saline injections for both experimental group ($N_{Control}$: 14, black traces; $N_{Jaws}$: 16, red traces). Both solutions were delivered through the centre poke on alternate days. There was no optogenetic stimulation in this phase. Bars indicate SEM.

in alternate days, both groups showed conditioned flavour aversion to X. Again, it was possible to observe a reduction in consumption of X over repeated pairings compared to Y and that happened in both groups (Figure 4.15). Levels of Y drinking by controls looked a bit higher than normal. Although no interaction between flavour and session ($F(2, 52) = 0.804$, $p = 0.453$) was observed, there were main effects of session and of flavour (Flavour: $F(2, 52) = 16.524$, $p < 0.001$; Session: $F(2, 52) = 26.984$, $p < 0.001$). Simple effects of the session and flavour interaction showed a taste aversion effect from session 1 that continued till session 3 ($F > 9.921$, $p < 0.004$). This could be explained by a change of the animal's weight and a paired sample T-test comparing weights on session 1 showed a significant decrease in weight between X and Y presentations which would explain the taste aversion effect present already in session 1. There were no interactions with group and no main effect of group (Interactions: $F < 1.105$, $p > 0.339$; Main effect: $F(1, 26) = 0.042$, $p = 0.839$). Thus, highlighting that both in

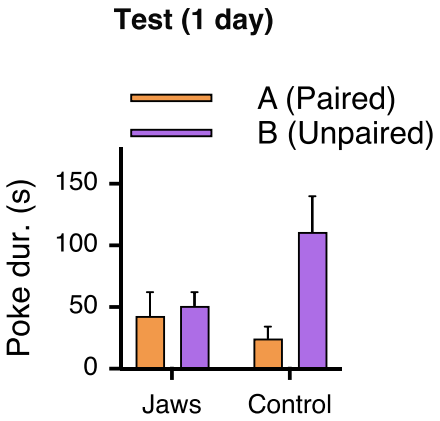



**Figure 4.16: Consumption at test for the non-spatial version of aversive SPC.** Mean duration of pokes during test is compared between flavours A (orange) and B (purple) for both experimental groups ($N_{Control}$: 14; $N_{Jaws}$: 16). No optogenetic stimulation was performed in this phase. Bars indicate SEM.

preexposure and conditioning phases there were no group differences.

*Test:* In the choice test between flavours A or B, flavours were now presented on the side pokes. As seen in the previous experiment, Control animals displayed a strong preference for flavour B over A, while Jaws group did not show preference to flavour A or B (Figure 4.16). Data analysis showed a significant interaction between flavour and group ($F(1, 26) = 4.949$, $p = 0.035$). Simple effects reveal that while the Control group displayed a significant difference in consumption of B over A ($F(1, 26) = 11.204$, $p = 0.002$), that difference did not appear in the Jaws group ($F(1, 26) = 0.104$, $p = 0.750$). A main effect of pairing was also present ($F(1, 26) = 7.098$, $p = 0.013$). Again, the absence of a main effect of group ($F(1, 24) = 1.877$, $p = 0.183$) indicates that the overall total amount drank by both groups did not differ. Thus, after developing a taste aversion to X, only Control animals showed a preconditioning effect, by drinking less of A, which had been paired with X in the preexposure phase. Even though Jaws animals also displayed taste aversion, they drank similar amount of both

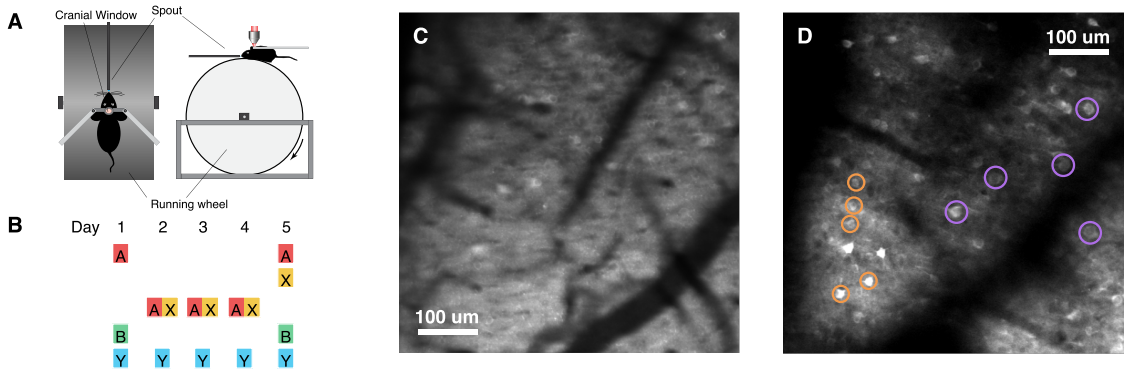



**Figure 4.17: Two-photon calcium imaging.** (A) Imaging setup with a view from the top (left) and from the side (right). The mouse is headfixed but can run on a wheel while calcium imaging is performed. (B) Schematic of the timeline for presentation of the different stimuli. (C) View from the surface of the right hemisphere of the brain. The sagittal sinus is located just at the left border of the image. (D) View from the imaging plane. A few cells are highlighted by circles. Smaller (orange) and larger cells (purple) can be observed in the field of view.

A and B.

### 4.3.5 Imaging of sensory associations in the RSC

To better understand how the retrosplenial cortex might be involved in these sensory-sensory associations it is important to answer the question of whether sensory information is encoded in RSC activity and how the association between neutral stimuli is expressed there. To do that, a passive presentation of flavoured stimuli was developed while RSC activity was recorded using two-photon calcium imaging.

Transgenic animals expressing GCaMP6s received chronic windows to allow two-photon calcium imaging of neurons in the RSC. Mice were head-fixed under the two-photon microscope but were allowed to run in a wheel during all the experiment (Figure 4.17).

The details of the task used are presented in Section 4.2.3. In summary, the task involved five days where mice passively received solutions of simple flavours or a compound mixture of two flavours (Figure 4.17B). On day 1 after pretraining, mice had

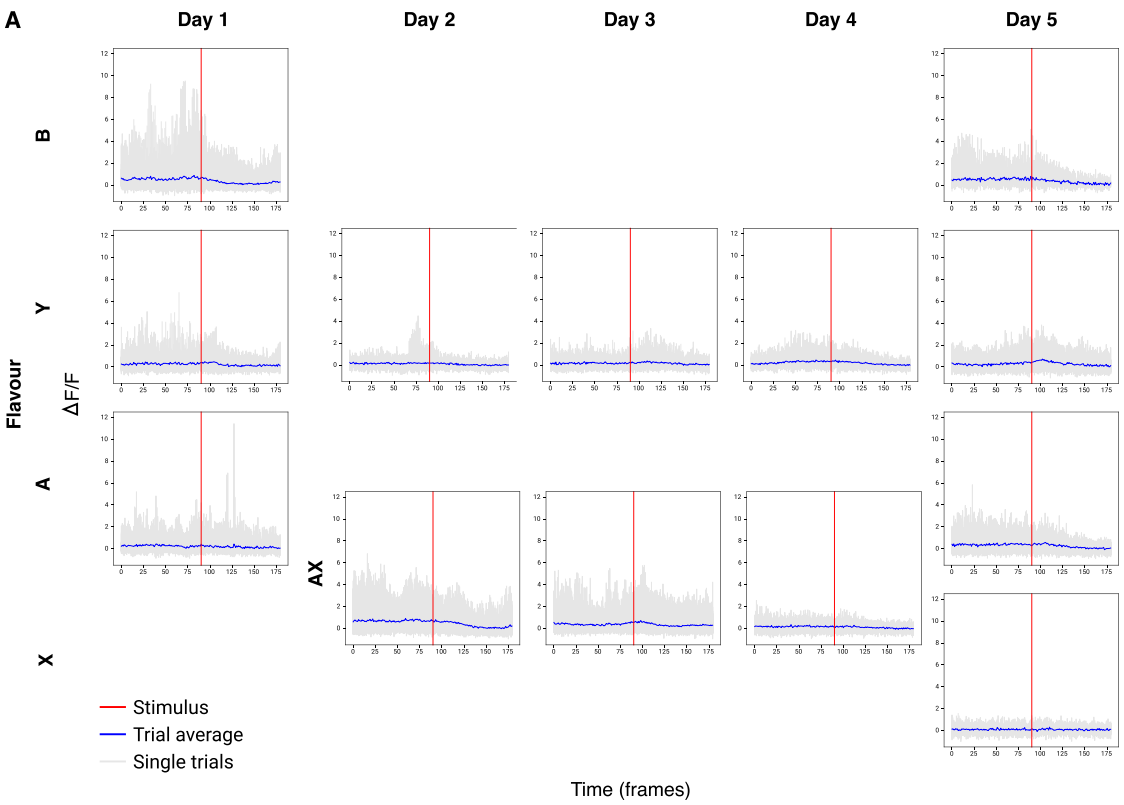
three sessions with presentations of flavours A, Y and B (order was counterbalanced). Then on the following three days they received the same two sessions. One with Y alone and one where they got the mixture AX. Finally, they received a final day with presentation of all flavours by themselves, A, B, Y and X.

Recordings were made around 250-330 μm from the surface of the brain. In a usual cortical region of the brain that would mean imaging cortical layer 2/3. Since the RSC bends in around the midline it is possible to observe that the imaging captures probably more than one layer. In Figure 4.17D it is possible to observe a group of smaller cells on the left, which were probably layer 2/3 of the RSC. But a group of larger cells on the right was also present. These could be cells from deeper layers of the retrosplenial cortex.

Neuronal activity was extracted from chosen ROIs in recorded videos and aligned with behavioural output. Initially running trials were separated from non-running trials as it has been observed that activity is influenced by locomotion (Niell and Stryker, 2010). Only running trials were analysed and they were defined as trials where the average speed of the mouse exceeded a certain threshold. In order to evaluate changes through days it is important to be able to compare the same cells over days (Trachtenberg et al., 2002). Thus, the cells that could be tracked in the different imaging sessions over days were selected and only they are presented in this analysis. The number of tracked cells over the five days varied from 20 to 40 cells per animal.

The analysis presented in this part is mostly a preliminary qualitative analysis of the responses of neurons in the RSC to the different stimuli. Trials were aligned to the first lick after stimulus presentation and the change in fluorescence is used as a readout of neuronal activity. It should be highlighted that although this is considered to be the stimulus start, it is hard to predict when exactly the mouse experienced the flavour.

Figure 4.18 shows the single trial and averaged trial activity of two different neurons


A
Day 1
Day 2
Day 3
Day 4
Day 5
Flavour
B
Y
A
X
AX
ΔF/F
Stimulus
Trial average
Single trials
Time (frames)

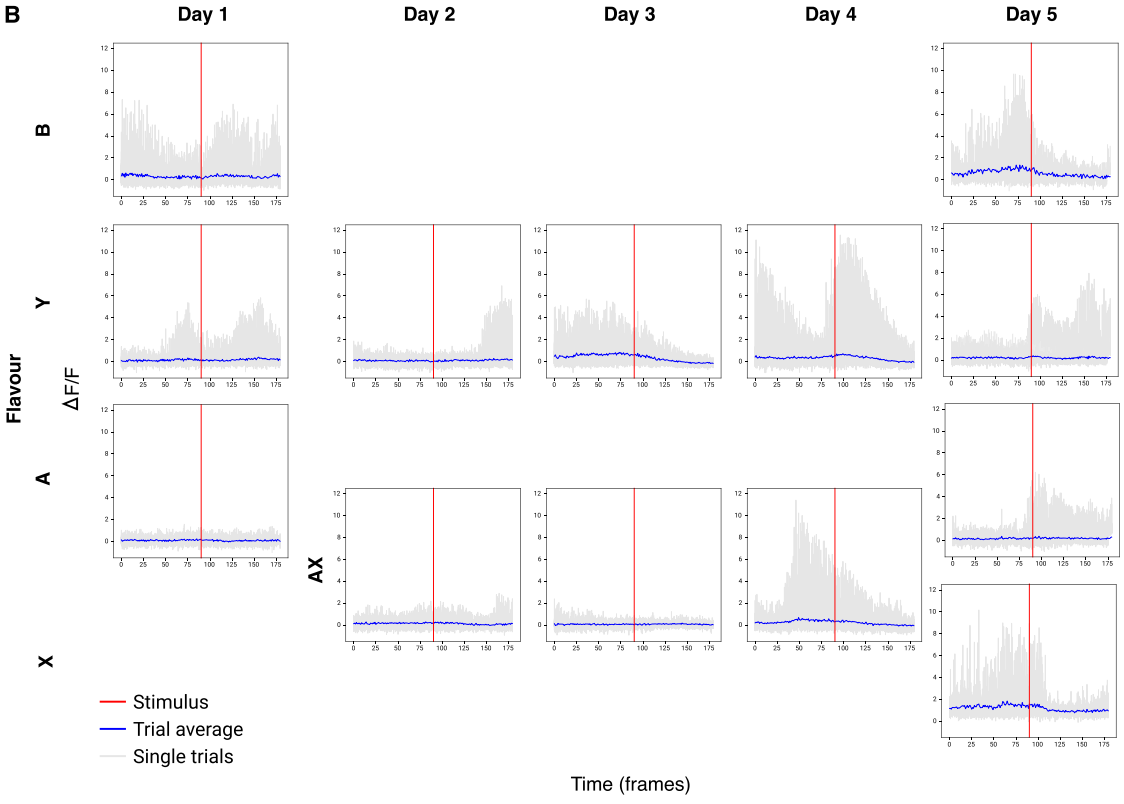

B
Day 1
Day 2
Day 3
Day 4
Day 5
Flavour
B
Y
A
X
AX
ΔF/F
Stimulus
Trial average
Single trials
Time (frames)

**Figure 4.18: (Previous page) Single cell trial averaged activity.** (A-B) Normalised changes in fluorescent for two units tracked over the different days and sessions. Single trial values are shown in grey and the averaged trace in blue. The red line indicates the when the animal licks to receive the specified flavour.

to the different stimuli over days. To some flavours on specific days the neuron's responses seemed to be locked to stimulus start. The neuron presented in Figure 4.18A showed a slight decrease in response to flavour B at stimulus onset only on day 1. On day 5 this decrease was not so marked. Increases in activity in response to stimuli could also be observed for another neuron (Figure 4.18B) on day 5 to flavours A and Y. Other cells showed decreases or increases in response to the stimuli but this was not reliably observed over different sessions and days as represented by cells in Figure 4.18.

Regarding the presentation of compound stimulus AX, responses at the single neuron level were varied. As shown in Figure 4.18 some neurons increased activity around the stimulus presentation over subsequent days (Figure 4.18B) while others responded more in the initial days (Figure 4.18A). For flavour Y, on the other hand, the same pattern of increased or decreased activity was not observed.

One important question that needs to be asked is whether the RSC represents AX as a sum of the representation of A and X. Although in our design AX is never presented with A and X on the same day it would be possible to ask whether responses to A and X on the last day add up to the last response of the neuron to AX. A quick look at the responses of the two neurons seemed to indicate that (Figure 4.18A-B). But a quantitative analysis is crucial to respond this question.

Since single cells did not respond specifically to a certain stimulus, it could mean that the RSC is encoding stimulus identity at the population level. Hence, Figure 4.19 shows the averaged trial activity for each tracked neuron on the first and last days for flavours Y, B and A. Although a proper quantitative analysis is necessary to

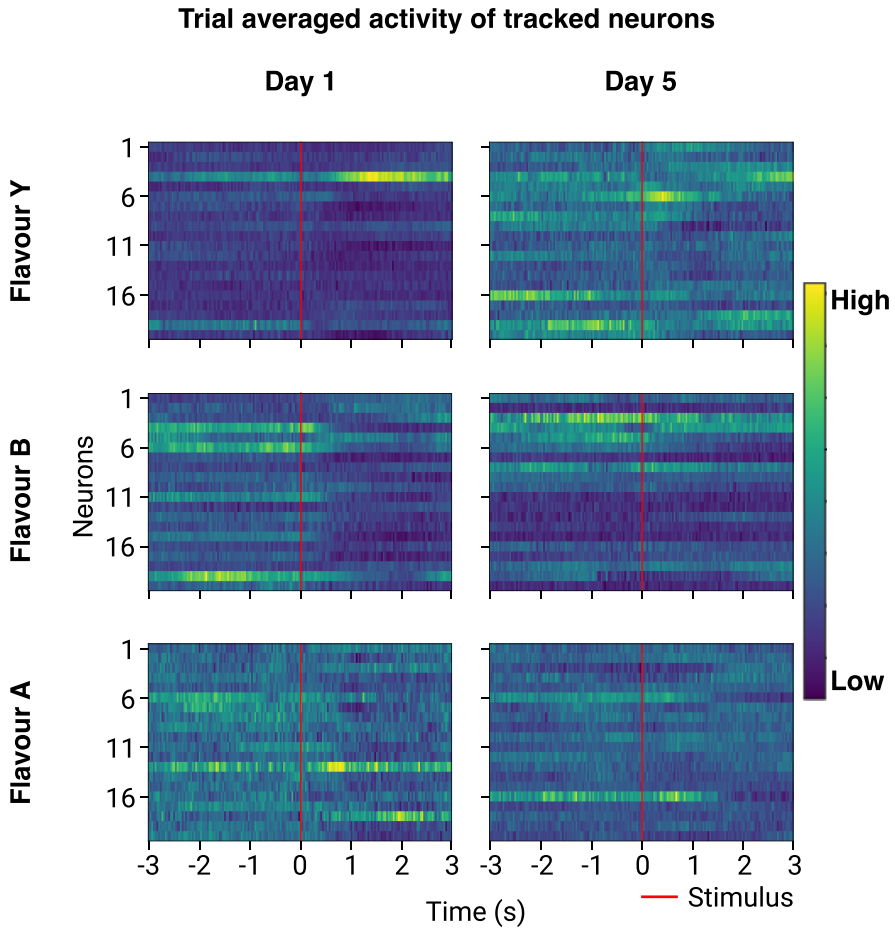



**Figure 4.19: Population responses to flavoured stimuli.** Trial averaged change in fluorescence for tracked neurons in the 1$^{st}$ and 5$^{th}$ days for presentation of different flavoured stimuli. Stimulus start is marked by the red line.

affirm this for sure, it is possible to express a few qualitative impressions regarding the population activity. One initial observation is that change in activity does not correlate to stimulus onset. A slight delay ($\approx$ 0.5 s) after stimulus start could be detected. Although it looked as responses on day 1 changed more after the stimulus than responses on day 5.

Another question that could be asked regarding the data is whether the representation of A changed after it was paired with X. A more detailed analysis needs to be done before answering that, but we can observe in Figure 4.18 that both A and B displayed changes between first and last days. At the population level, the general activity pattern seemed more conserved for flavours A and B, which were only presented on days 1 and 5 (Figure 4.19). A had also been presented as a compound with X, but this pairing possibly did not change the population representation of A. Meanwhile, the activity of the population to flavour Y, presented alone in each day, seemed to have changed more between days 1 to 5.

## 4.4 Discussion

### 4.4.1 Results Overview

In this chapter, I set out to clarify the involvement of the RSC in forming neutral sensory associations. First, I combined optogenetic inhibition with an aversive sensory associative task to precisely disrupt RSC activity during the preexposure phase of the associative learning. During the preexposure phase, mice received pairs of compounds on alternate days while the implanted LED was activated when they drank the different compounds. No effect of optogenetic stimulation was observed in this phase and by the end of this phase, animals were drinking similar amounts of each compound.

On the next phase, the value of compound X was devalued by repeated pairings with LiCl injections while compound Y consumption remained mostly the same.

On this phase, no optogenetic stimulation was used and no effect of the previous stimulation could be observed as both experimental groups acquired the taste aversion equally.

On the test day, when presented with a choice between compounds A and B, mice were expected to prefer flavour B which had been paired with Y, which was not devalued on the conditioning phase. This behaviour is observed in the Control animals, but Jaws mice display no preference for any compound. Silencing the RSC during the preconditioning phase, prevented a preconditioning response from being expressed. Thus, indicating that RSC is involved in forming neutral stimuli associations.

Then, by changing a few task parameters to create a non-spatial version of the task, it was possible to investigate if the RSC's involvement is strictly spatial. The same protocol was run again, this time with all compounds delivered from the same central poke. Once again, during the preexposure phase, no effect of optogenetic stimulation was observed and by the last sessions animals were drinking similar amounts of all mixed compounds.

The conditioning phase showed that both groups were able to develop an aversion for X over Y, even if both compounds were being delivered from the same poke. Then, on the test day, compounds were delivered from the side pokes and were available at the same time. While Controls preferred compound B over A, Jaws animals did not show any preference, thus, lacking the sensory preconditioning effect. This result confirmed that a role of the RSC in binding together neutral stimuli and that this is not strictly limited to the spatial domain.

In addition, we also showed that optogenetic stimulation of Jaws-expressing cells lead to silencing of the neuronal network. We demonstrated that silencing effects when the animal expresses the opsin Jaws and that even at 800 nm deep into the tissue it is possible to observe decrease in firing activity.

After confirming the involvement of the RSC in the association of two neutral

stimuli, the next step was to try to investigate the mechanism by which this happens. By combining two-photon calcium imaging with a passive sensory presentation of flavoured solution it was possible to begin a qualitative analysis of RSC responses. A few neuronal responses to the stimuli were observed. They sometimes developed only after a certain delay and were not consistent through days. A quantitative analysis will be necessary to further understand the collected data and possibly identify if sensory stimuli are indeed represented in the RSC.

### 4.4.2 A lack of sensory-spatial associations

Our initial version of the associative task showed that disrupting RSC activity during the preexposure phase affected preference during testing of whether associations had been formed in the final phase. A similar result had been previously observed by Robinson et al. (2014) using chemogenetics. On their study, they state that RSC activity is necessary for formation of associations between a tone followed by a flashing light in an appetitive sensory preconditioning Robinson et al. (2014). Optogenetics offers advantages over chemogenetics techniques as it allows us to timely control neuronal activity. Hence, one can highlight the fact that in the task used here RSC activity was precisely disrupted when animals were receiving the stimuli. That was sufficient to disrupt the preconditioning effect, even though the animals had normal RSC activity when they were not receiving stimuli. In addition, for the task presented here it is required an association that could be formed more easily, since it depends on one single sensory modality and both stimuli are presented concomitantly. Moreover, our design allows for a preference test in the end, which is more sensitive than comparing absolute response levels to stimuli. Thus, although our design would be less prone to disruption, the timely optogenetic stimulation caused the associations to not be formed.

Now, in this first version of our task, flavours were delivered from spatially separate

locations. RSC lesioned rats responded less to displaced objects in an object-in-place task, thus indicating RSC involvement in object-location associations (Vann and Aggleton, 2002). Moreover, problems with using landmarks to solve spatial task were also described in humans with similar lesions (Maguire, 2001). Thus, the effect observed could be due to RSC's importance for object-location associations. The spatial component had always been present in previous associative task designs (Robinson et al., 2014; Blundell et al., 2003). For the appetitive sensory preconditioning task used by Robinson *et al*, one can argue that cues still retained a spatial orientation between each other (light had one place in space, sound another) (Robinson et al., 2014). Therefore, it would be important to be able to test association independently of space. In order to address that I developed a new version of the aversive sensory preconditioning paradigm where flavoured compounds were delivered from the central spout during the associative phases.

### 4.4.3 A role in stimulus-stimulus associations

When the animals were tested on the non-spatial version of a sensory preconditioning task, no group differences were observed in the first two phases of the task, but the Jaws group again did not show the sensory preconditioning effect. The fact that despite no spatial component was present, the Jaws animals were still unable to show aversion to compound A over B indicates that RSC involvement in sensory associations is not solely dependent on space. This goes in line with the effect described by previous work (Auger and Maguire, 2018), where non-spatial associations were disrupted by RSC lesion in humans.

By using the same taste aversion procedure in sensory preconditioning Ward-Robinson and collaborators have shown that changing the context between the different phases of the experiment can lead to a lack of preconditioning (Ward-Robinson et al., 1998). Although this is a possible explanation for previous experiments (Robinson

et al., 2014), here, it is unlikely that the animals consider the contexts differently since they experience the context even when not under optogenetic stimulation. Nevertheless, one possibility that might explain the effect observed is that the Jaws animals are unable to link the association made with the context in which they experience it in the first phase and therefore do not express the preconditioning effect at test.

### 4.4.4 RSC response to neutral sensory stimuli

By developing a passive sensory stimulation task with flavoured solutions and combining that with two-photon calcium imaging, the objective was to try to identify sensory responses in the RSC as observed in previous physiological studies (Vedder et al., 2017; Miller et al., 2019) that observed response to a light stimulus and to reward location respectively. In primary sensory areas, it is common to find cells that respond always to the same stimulus such as the visual cortex receptive fields and single neuron responses to bars of different orientations (Hubel and Wiesel, 1962). The preliminary analysis presented here was unable to find such marked responses in the RSC. One possible explanation to this lack of stimulus locked responses is that the sample used here is just not big enough (around 20-40 cells). Vedder *et al* are able to record over 200 neurons (Vedder et al., 2017). Nevertheless, they are not interested in tracking the same neurons over sessions, which is here the cause of the reduced sample. Another possibility, that should be taken into consideration for a quantitative analysis, is that the time chosen as the stimulus start is not the actual time when the animals experienced the stimuli. In a passive stimulation study with odours, the authors considered a window of around 100 ms to account for delays in odours getting to olfactory receptor neurons (Shen et al., 2013). Moreover, since the RSC is not a primary sensory area and there are no connections from the gustatory areas described, it probably encodes flavoured stimuli in a more complex way. Possibly at a populational level rather than at the single neuron level and taking into consideration

other information provided by many of its other inputs (see Introduction).

Another prediction that this experiment tried to test was whether pairing A with X would change how the pure solution A is represented in the RSC. At a first look, it seemed that at the single neuron level there is a change in response, but the same change was observed in B, which was presented as a control for single presentations on days 1 and 5. At the same time, when looking over the whole population changes in the representation of A were more diluted.

### 4.4.5 Methodological considerations

#### Optogenetic stimulation

With the electrophysiological studies of RSC response to Jaws stimulation we tried to provide supporting evidence of inhibition of activity in this region. The optogenetic inhibition is pronounced but one marked feature can be observed on Figure 4.3B: an activity rebound. This post-illumination rebound activity has been observed before (Chuong et al., 2014) and careful interpretation of the results here presented should be taken. Although, I would not be able to say for certain that inhibition is the main effect here, I am definitely disrupting normal RSC activity. Chuong and collaborators propose ramping of the light stimulation as a way of dampening the rebound but with the hardware used in these experiments this solution was not possible.

#### Bone regrowth

When brains were histologically processed (Section 4.3.2) we did observe some bone regrowth under the LED implant on some of the mice. A very thin and translucent bone membrane formed. This could potentially dampen the light excitation and prevent optogenetic stimulation, but it is unlikely that this bone regrowth should affect inhibition of the RSC since our experiment did not show a remarkable difference when decreasing almost 10 times the light intensity.

#### Opsin expression

Another source of behavioural variability is how much of the retrosplenial area could be affected by optogenetic stimulation. This will be determined by two factors in our experiment:

1. The extent of the area that is illuminated;

2. The extent of the opsin expression.

One advantage of the opsin used here, Jaws, is that it responds to red light, which is less absorbed by haemoglobin than light of other wavelengths and hence can penetrate deeper into tissue (Chuong et al., 2014). The electrophysiological experiments demonstrate that inhibition can happen as far as 800 µm into the tissue, thus probably hitting deeper layers of the RSC.

Now, in the anterior-posterior axis, the mouse RSC extends from around -0.7 mm to -4.5 mm from bregma (Paxinos and Franklin, 2001). Light is delivered through an LED implanted on top of a 1.8mm diameter craniotomy centred around -2.05 mm from Bregma. One should consider that the cone of light coming from the LED will go over the range of the craniotomy, so it is possible that inhibition was not confined to the -2.95 mm to -1.15 mm range.

This might be relevant when taking into account the extra-RSC expression. This was mostly localised anterior to the RSC which probably means that LED light did not reach these areas. Areas lateral to the RSC might have been affected though. Nevertheless, even when trying to remove the animals with larger extra-RSC expression from our analysis it did not change conclusions presented in this and the next chapter. Moreover, it should be taken into consideration that expression was restricted to the AP range of -0.8 to -3.5, so most probably the caudal part of the RSC was not affected by inhibition.

### 4.4.6 Conclusions

In this chapter, I presented the use of a new aversive sensory preconditioning task in mice to study the effect of optogenetic silencing in the RSC. It supports the idea that RSC is involved in the formation of stimulus-stimulus associations. The comparison between a version of the task possibly dependent on spatial cues with another version where spatial information is not relevant leads to the conclusion that the role the RSC plays in this task is not due to a possible function in spatial encoding.

These results lead us to ask several questions. How does the RSC forms these neutral associations? Is it possible to find a representation of the associations in the RSC? Even though, conjunctive coding has been observed in the RSC (Vedder et al., 2017) does it mean that AX is one single representations? Or are A and X represented separately and eventually co-activated? With these questions in mind the two-photon calcium imaging experiments were designed and although further analysis is still needed, the data collected will help clarify these questions and help understand the role of the retrosplenial cortex in sensory-sensory associations.

# Chapter 5

# RSC and Spatial Memory

## 5.1 Introduction

In order to navigate the world, we rely on contextual representations or maps (Tolman, 1948) that we build in our mind. One type of representation is to use an allocentric frame, where we store information about the landmarks and cues of the environment and the relationship between them.

A number of observations on patients with damage to the retrosplenial cortex that displayed topographical disorientation (Takahashi et al., 1997; Katayama et al., 1999; Maguire, 2001) highlights a role for the RSC in allocentric representation. Patients were unable to place landmarks in a map of a known environment, even when they recognised the landmarks. The same patient's ability to use egocentric representations seemed intact (Takahashi et al., 1997). Thus, patients consistently show inability to use landmarks to localise oneself.

Problems with the use of allocentric cues for navigation were also observed in animals with lesions in the retrosplenial cortex. The Morris water maze task requires that animals swim from different starting locations to a platform, which is sometimes hidden below the surface of water or not, depending on the task conditions. The

animals can use cues around the maze to localise themselves and find the target (Morris et al., 1982). Several studies showed that animals with retrosplenial lesion had impairments in learning this task (Sutherland et al., 1988; Whishaw et al., 2001; Vann and Aggleton, 2002). Aspiration lesions of the posterior cingulate cortex in rats led to subjects taking longer to learn to find the platform in the water maze task (Sutherland et al., 1988; Whishaw et al., 2001). Neurotoxic lesions also show small but significant impairments in learning.

When rats are tested on a working memory version of the radial maze, they have to collect food from different arms and remember the previously visited arms in order to avoid them. Vann and Aggleton (2002) used this task to test for use of egocentric cues when there is damage to the RSC. They showed that RSC-lesioned rats could learn the task although initially making more errors than controls, but when the maze was rotated, they could not use allocentric references properly to solve the task (Vann and Aggleton, 2002). Later on, they expanded this finding by showing that the dysgranular RSC could be the main area responsible for this effect (Vann and Aggleton, 2005).

How would allocentric space be represented in the retrosplenial cortex? When Chen et al described for the first time the existence of head-direction cells in the RSC (Chen et al., 1994) that served as further evidence towards the RSC being able to hold some kind of spatial representation of the environment. Interestingly, RSC head-direction cells are influenced by landmarks and local cues (Jacob et al., 2017).

Other studies started relating landmark processing and their permanence (Auger et al., 2012) to the RSC's importance in allocentric representations. Lesion studies in monkeys have also highlighted the importance of this region to landmark processing as bilaterally lesioned monkeys present retrograde amnesia in an object-in-place scene discrimination task and have impaired new learning in the same task (Buckley and Mitchell, 2016).

The use of c-Fos as a marker for recruitment of networks has proven useful in defining the involvement of the RSC in spatial memory. First, Fos labelling post spatial training showed the recruitment of both rostral and caudal RSC (Vann et al., 2000). Then, Czajkowski et al used chronic imaging of Fos expression after sessions of training in the water maze task to observe reliable recruitment of a group of RSC cells through learning. Interestingly, when they tested them on a cued-platform version of the task, they observed less recruitment of RSC (Czajkowski et al., 2014). Thus highlighting a role of distal cues for RSC involvement.

Recently, recording studies in rodents are starting to show us exactly how the RSC encodes space and aids in navigation and spatial learning. Vedder et al. (2017) observed that RSC uses conjunctive coding when rats learned a cued T-maze task. While some RSC cells encoded a light stimulus that served as a beacon to reward in the T-maze, others would code for the reward location depending on the side of the reward and others had firing timely locked to the reward itself (Vedder et al., 2017). In a later study, on a continuous T-maze, RSC spatial encoding of the task was shown to be variable and develop with learning (Miller et al., 2019).

The studies described so far indicate that the retrosplenial cortex involvement in spatial memory and navigation may be related to its capacity of encoding landmarks or distal cues, their relationship and their location in the environment and thus supporting an allocentric representation of the space. However, the involvement of the RSC with other types of spatial representation is not yet discarded.

In a very elegant study, Alexander and Nitz (2015) have contrasted different frames of reference while recording activity in the retrosplenial cortex. Rats were allowed to traverse two W-shaped routes placed in different positions of a larger environment. The routes forced the rats to perform either left-right-left turns or right-left-right turns to reach reward. This allowed them to record RSC ensembles of cells that represents the rat position in allocentric and route-centric references while also coding for left or

right turning behaviour (Alexander and Nitz, 2015). Hence, the retrosplenial cortex seems to be able to integrate information from different frames of reference, which contrast with a few experimental observations where the use of egocentric or idiothetic cues are not affected by RSC lesions (Zheng et al., 2003).

Rodent lesion studies using a working memory paradigm, the T-maze Alternation, have led to contrasting results. Aggleton et al had shown that T-maze alternation is dependent on both the hippocampus and the anterior thalamic nuclei (Aggleton et al., 1986, 1996). Both regions are extremely well connected with the RSC. However, the same group reported that retrosplenial lesions were unable to cause a large deficit in spatial working memory (Neave et al., 1994; Pothuizen et al., 2008). Later, they went on to show that RSC inactivation with muscimol, a GABA A receptor agonist, led to striking deficits in T-maze Alternation (Nelson et al., 2015). This emphasises how compensation mechanisms may be responsible for different lesion studies outcomes. Several studies have tried to understand the reason behind the different outcomes (Vann and Aggleton, 2002, 2004). One of the causes might lie in the lesion method used (aspiration, electrolytic, cytotoxic). Another one could be the extent of the lesions since initially lesions would spare the caudal RSC, which potentially led to conflicting results (Vann and Aggleton, 2004).

In one of the above mentioned studies, they aimed to understand the reason why RSC lesions could not cause deficits in spatial alternation (Pothuizen et al., 2008) by testing the implication of different frames of reference. Using a combination of two cross-mazes with one of the arms blocked (thus making it essentially a T-maze), they tested the use of direction information while removing different sources of spatial reference. The crucial experiment was when two mazes (one for the sample run and another for the choice run) were placed next to each other in the dark so animals could not use allocentric cues or intra-maze cues. With this task design, retrosplenial lesions prevented rats from performing as well as controls. Previous reports of RSC

inactivation had already shown a deficit in spatial tasks when animals were switched from light to dark conditions (Cooper and Mizumori, 1999), possibly because of the lack of allocentric information. But in the Pothuizen et al. experiment, when the task was run in light conditions but allocentric cues were ambiguous, lesioned rats still were able to solve the task. Thus, animals were unable to use direction information to solve the task.

Another group of experiments revealed that it was not just a shift in light conditions that could bring about a retrosplenial deficit. Deficits in a spatial hoarding task run in light conditions could arise if the animal had to face a different situation (a probe test from a different start location, for example) (Cooper et al., 2001). This outcome when added together with the lesion studies presented earlier showing allocentric deficits and problem with directionality plus the observation of conjunctive coding of different frames of reference (Alexander and Nitz, 2015) leads to another theory of how the retrosplenial cortex function could be important for spatial memory.

Lately, the proposed role of the RSC in spatial memory in mediating between spatial reference frames (Byrne et al., 2007; Vann et al., 2009) has gained weight among researchers. This theory combines a role for head-direction cells in translating from a more allocentric representation coming from the hippocampus to a more egocentric one from parietal areas. Hence, the RSC would be involved in mediating the switch and lesion and inactivation deficits in this region would only appear when there is need for that switch.

Finally, as highlighted here, most of previous studies relied on a lesion or complete inactivation of the retrosplenial cortex in different spatial task to probe its contribution to spatial representations and memory. Conflicting results are only now being elucidated and it would be of great interest to see how chemogenetic and optogenetic inactivation of RSC would affect spatial memory and navigation.

Therefore, I propose to investigate how inactivating the retrosplenial cortex during

different spatial memory tasks can affect performance in order to build up on the literature thus far.

Mice were tested on two spatial tasks. The first one, the T-maze alternation, a working memory task, where silencing of RSC activity is done after animals learn the alternation. It has been described that muscimol inactivation (Nelson et al., 2015) leads to a drop in performance after learning. The expectation would be to see a deficit when animals are under RSC optogenetic inactivation. Possibly, by making use of the timed aspect of optogenetics, one could later ask the question whether the RSC is important in a specific part of the task.

The other task is a spatial reference memory task, a Y-maze task using a cross-shape maze. In this task, silencing was done throughout the training phase. This task resembles a task like the water maze, so we could expect impairments in learning. Since deficits in lesion studies had been observed when cues are put in conflict, a reversal training phase was added to the reference memory task. So, even if no learning impairments were observed on the normal training maybe they should show up on reversal learning.

## 5.2 Methods

### 5.2.1 Spatial Tasks

Animals used and surgical procedure are described in detail on sections 2.1.1 and 2.1.2. In summary, C57Bl/6 mice received injections of either the control virus (rAAV5/CaMKII-EYFP) or the opsin Jaws (CaMKII-Jaws-KGC-GFP-ER2) and then had a red LED implanted on top of the RSC region. It should be highlighted that the same animals used for the spatial version of the aversive SPC (Section 4.3.3) were used in the T-maze alternation paradigm. Animals that had been through the non-spatial version of the aversive SPC (Section 4.3.4) were then run in the cross-maze spatial

reference memory test. They had an interval of a few days before they were started in these paradigms. Behaviour apparatus are described on section 2.1.3.3.

#### 5.2.1.1 Rewarded T-maze Alternation

All mice were water-regulated as described on 2.1.3.2.

*Habituation phase:* For this phase, the maze was kept in a different room than the training and testing room. On day 1, mice that were housed together got to explore the maze in a group for 5 minutes. Water was available at food cups in all three arms of the maze. On the next days, mice explored the maze alone for 5 minutes each day.

After the 2$^{nd}$ day of habituation, the food cup was removed from the starting arm. Once mice were habituated (taking less than 30 s to go to one of the arms for reward), a forced choice session was given. On this session, each mouse received intermixed forced trials (6 of each side) with an inter-trial interval (ITI) of approx. 5 minutes. Training started on the next day.

*Training phase - no stimulation:* Subjects received training on a T-maze alternation task for 3 days moving freely, without the LED tether connected. They received 8 intermixed trials and for each trial mice were run one after the other in groups of 4 or 5, thus amounting to an ITI of approx. 5-6 minutes.

Each trial consisted of one Forced Run followed by a choice run, with an interval of on average 5s in between. On a sample run, mice were forced to turn to one specific side (counterbalanced and no more than 2 repeated trials to the same side were permitted), where they would get a drop of water (30 µL) as reward. On a choice run, animals could choose which side to turn to. Once they had chosen a side, they were blocked inside it. If they turned to the opposite side of the sample run, then they would get a reward. If not, then no reward was given. Animals were removed from the maze after 15 seconds of exploration in the chosen arm or after they consumed the reward.

Finally, mice received a $4^{\text{th}}$ and final day of training where the LED tether was plugged in but no stimulation was given. On this day, each mouse was run through all trials before the next mouse was trained. The ITI was of approx. 1 minute.

*Test:* Since behavioural performance was not affected by the plugged cable, animals were subjected to a test under LED stimulation (at 50 mW). The same protocol as the one for the 4th day of training was followed, but LED stimulation was present through both Forced and choice runs. The pattern for LED stimulation followed the design shown in Figure 5.1D. Stimulation started before the mouse was taken from the resting box. A button press triggered the optogenetic stimulation by turning on the implanted LED (at 50 mW). Stimulation would last for a pseudorandom duration (5, 10 or 15 s). Then, the LED turned off for another pseudorandom interval (0.5 to 2 s) and then this pattern would repeat until mouse was put back in the box and stimulation was turned off.

#### 5.2.1.2 Rewarded Cross-Maze

For this experiment, mice were food-regulated as described on section 2.1.3.1.

*Habituation phase:* Habituation consisted of 4 days. Again, the maze was kept in a different room than the training and testing room. On day 1, mice that were housed together got to explore the maze in a group for 5 min. Diluted condensed milk (Nestle, 1:1) was available at food cups on all four arms of the maze. On the following day, mice explored the maze alone for 5 min. Finally, the $3^{rd}$ and $4^{th}$ days, consisted of daily 5 min explorations, this time with the LED thread connected so the animals would get used to being tethered. Once mice were habituated (taking less than 30s to go to one of the arms for reward), training started.

*Training phase - with stimulation:* Training days were composed of 12 trials where the subject would leave from one of the longer arms (N or S) in a pseudo-random order. The opposite arm would be closed off. No more than 3 consecutive starts from

the same arm were permitted. Each subject was assigned one specific side arm (E or W) where it would always get a reward (the chosen arm was counterbalanced) and the number of correct choices and the latency to make a choice were counted.

At each trial, optogenetic stimulation would start before the animal is put in the starting arm ($\approx$ 10 s). Once the animal chose one of the arms, it is locked in the arm and has to consume the reward or explore empty food well before being removed. After each trial, the animal would be removed to a closed off box, the LED would be turned off and the maze would be wiped from intra-maze cues. The ITIs were of approx. 1 min. This phase lasted for 6 days.

*Non-baited test:* After the training phase, animals went through a non-baited test. On this test, the animal has to choose the designated arm, but no reward was present. The same testing schedule as for the normal training days was used.

*Reversal phase:* Two days after normal training was completed, half of the animals went through a reversal learning training. First, one day of retraining with designated arms as the same as at the previous phase was done. Then, each individual mouse had the designated arm reversed (if the animal would get a reward on arm E during training, now rewards would only be found on arm W). The same stimulation patterns were used and the same protocol as during the Training phase. Reversal learning lasted 9 days. After the last day of reversal, animals were tested on the maze without any optogenetic stimulation.

*New Context phase:* A group of the animals (5 Controls and 5 Jaws) went through learning in a New Context without any LED activation. The Cross Maze was moved into a different room with different objects as cues. The designated reward arm was inverted once more. Then, mice were trained on this new context for 7 days.

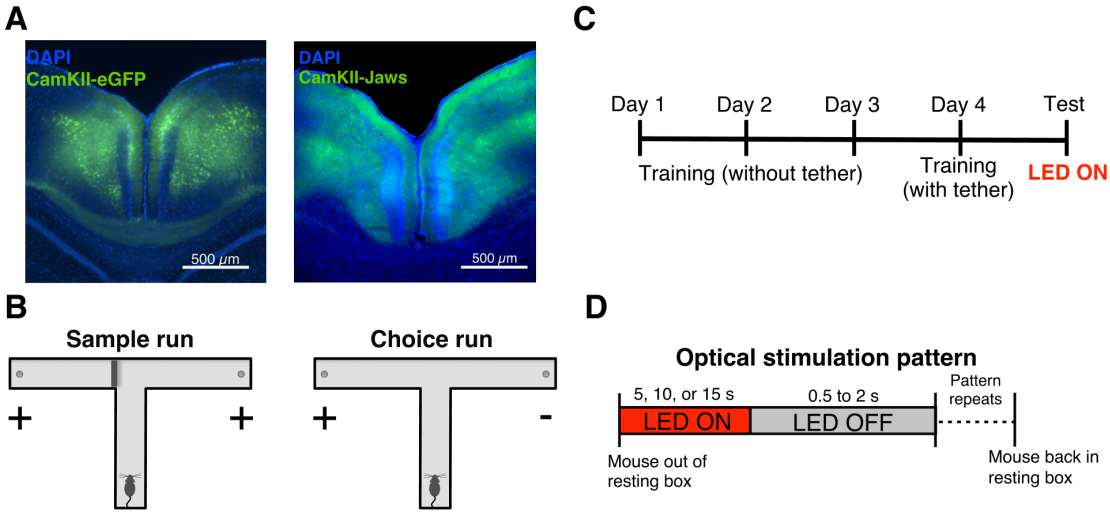



**Figure 5.1: Rewarded T-maze Alternation.** (A) Mice were injected in the RSC with either the control virus (CaMKII-eGFP, left, green) or Jaws (CaMKII-Jaws, right, green). (B) Schematic representation of the rewarded T-maze alternation with the sample and choice runs. Presence of reward is indicated by + sign. (C) Timeline of training in the T-maze alternation task. LED ON indicates the use of optogenetic stimulation. (D) Optogenetic stimulation pattern. Red periods indicate the LED light was on (LED ON) and grey indicates no stimulation (LED OFF). Pattern repeats until turned off when mouse is back in the resting box.

## 5.3 Results

### 5.3.1 Optogenetic disruption of RSC after training does not affect T-maze Alternation

Since the retrosplenial cortex main function is thought to involve spatial navigation, I set out to establish if silencing the RSC would affect different navigational tasks. The rewarded T-maze Alternation task is thought to involve spatial working memory. The animals have to remember the side they were forced to enter and got a reward on the sample run in order to alternate and run to the opposite side on the choice run to be rewarded. In this case since the interval between the Sample and choice run is small (5-10 s) then working memory is recruited and RSC seems to be involved in solving this task (Nelson et al., 2015; Miller et al., 2019). The percentage of corrected

choices on the choice run can then be calculated as a percentage. Figure 5.1A shows a schematic drawing of the maze on the two different runs and Figure 5.1B depicts a timeline for the training done for this task. Optogenetic stimulation was used only on the final Test day by activating the LED implant on both runs. In order to avoid the rebound artefact described on Section 4.3.1 a pseudo-random pulsed pattern of stimulation was used (Figure 5.1C).

#### 5.3.1.1 Experimental and Control groups learn to alternate

Figure 5.2A shows percentage of correct trials for the different training sessions for both experimental groups ($N_{Control}$: 18; $N_{Jaws}$: 11). We compared learning between the two groups during all the training days and test. The analysis included the first three days of training, when mice had the LED cable unplugged and no stimulation was delivered; the $4^{th}$ day, when mice received training with the tether plugged but still no LED stimulation was given; and the $5^{th}$ day, when mice were tested while receiving LED stimulation during both Sample and choice runs.

Both experimental groups displayed an ascending learning curve with increased percentage of correct trials over days. This was confirmed by statistical analysis. An ANOVA revealed no interaction between groups and days of training ($F(4, 108) = 1.016$, $p = 0.402$) but a main effect of day ($F(4, 108) = 4.047$, $p = 0.004$) indicated that, although group differences over days were not significant, a change over days could be observed. This change had a linear trend ($F(1, 27) = 8.859$, $p = 0.006$). Even though the interaction was not detected, a main effect of group ($F(1, 27) = 4.546$, $p = 0.042$) is present as well indicating that Control and Jaws animals are generally different (Jaws are lower most of the time).

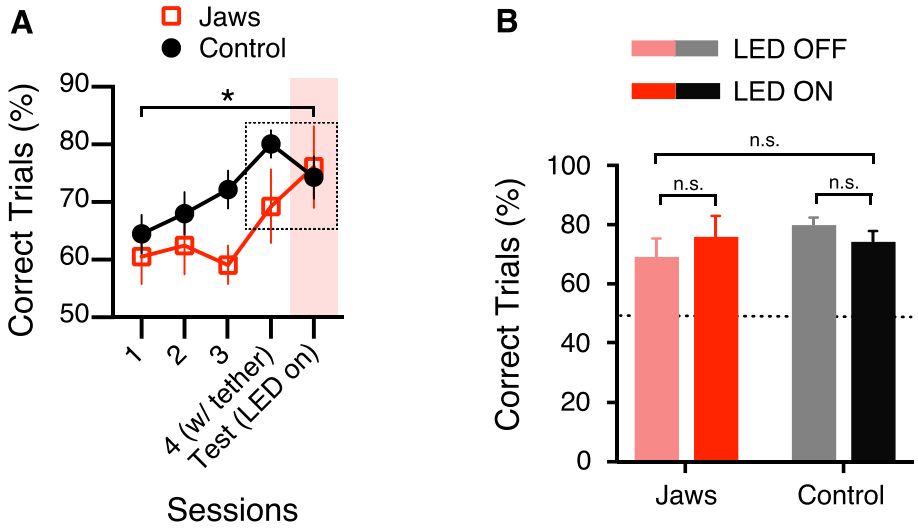



**Figure 5.2: Performance in the T-maze.** (A) Learning plotted as average percentage of corrected trials over training sessions for both Jaws ($N_{Jaws}$: 11, red squares) and Control groups ($N_{Control}$: 18, black circles). Optogenetic stimulation was active in the last session, Test(LED on). Bars represent SEM. *p=0.004. (B) Last two days, when animals had the LED wire connected, replotted for better comparison of Jaws and Control groups with or without optogenetic silencing of the RSC.

#### 5.3.1.2 RSC activity disruption does not affect performance on Rewarded T-maze Alternation

No obvious differences can be observed when focusing on the Test day when both groups received optogenetic stimulation during the tasks. Now, in order to compare only the sessions when the animals were tethered (Figure 5.2B), I ran another analysis on only the last training session (LED OFF) and the test session, when stimulation was on (LED ON). There were no interactions or main effects observed (All $F < 2.601$, $p > 0.118$), showing that both groups behaved similarly with or without LED stimulation.

#### 5.3.1.3 Comparison of time to Reward for Sample and choice runs

In order to evaluate possible changes in motivation or whether optogenetics affected motor ability in any of the groups, the latency to reach reward was calculated for the Sample and choice runs. The results are summarised in Figure 5.3, which shows the

average of the median time that animals ($N_{Control}$: 10; $N_{Jaws}$: 6) took to get from the start of the maze to one of the reward cups.

The analysis of the sample run data over the different sessions did not reveal any significant difference between groups. No Group and Session interaction or simple effects were detected (All $F < 1.303$, $p > 0.273$). The apparent difference between groups, observed in Figure 5.3, was driven by two mice that when removed from this or previous analysis did not alter the initial results. Identical analysis on choice run latencies yielded the same result. No interaction or simple effects were observed (All $F < 1.433$, $p > 0.251$), thus demonstrating that both groups, Control and Jaws, run at similar speeds to get to the reward and that speed did not vary over sessions.

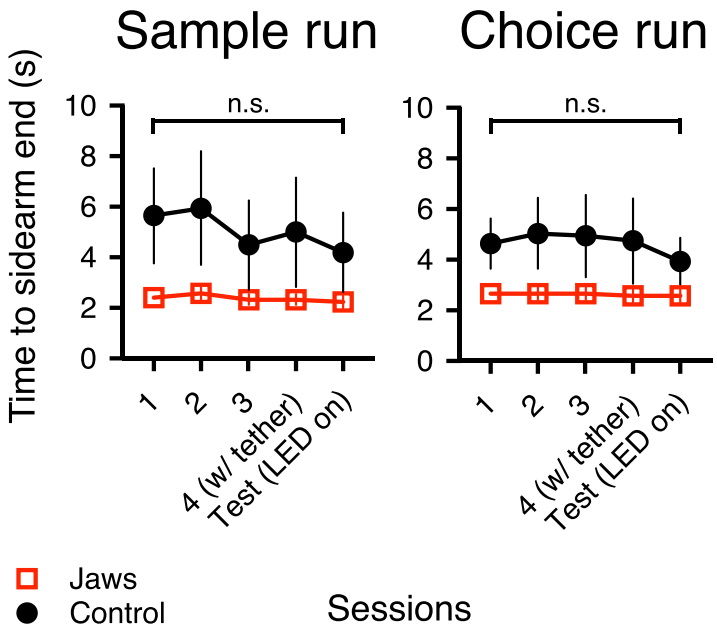



**Figure 5.3: Time to sidearm end for Sample and choice runs.** Median time taken by animals to reach the end of the sidearms for both experimental groups ($N_{Control}$: 10, black circles; $N_{Jaws}$: 6, red squares) over training sessions. Bars indicate SEM.

### 5.3.2 Optogenetic disruption of RSC during learning of a spatial reference task

Although the rewarded T-maze alternation is considered a spatial task since it involves navigation, it is a working memory task that can be solved in different ways. Dudchenko showed that animals can solve the T-maze alternation task without using extramaze cues (Dudchenko, 2001). Another study showed that knocking out GluA1, AMPA receptor subunit, which is important for non-associative learning, leads to impairments in the T-maze alternation task (Sanderson et al., 2010). Thus indicating that it is possible for animals to solve the T-maze without associative memory.

Thus, I set out to test whether the retrosplenial cortex role might be in aiding spatial reference memory task, which is long-term memory that requires encoding of distal and local cues to solve. Thus, two groups of mice ($N_{Control}$: 10; $N_{Jaws}$: 12) received LED stimulation while being trained on a Cross-Maze in a task that resembles a Y-Maze Spatial Memory task.

In this experiment (Figure 5.4A), mice left from either the South (S) or North (N) arms and the reward (+) would be located always on the same side arm (counterbalanced). A pseudo-random pulsed stimulation (Figure 5.4B) was on during the whole trial in order to inactivate the RSC during learning. The percentage of correct choices was calculated.

#### 5.3.2.1 Optogenetic disruption of RSC does not affect spatial reference learning

Learning on the 6 days of training is represented as a percentage of correct trials in Figure 5.4C. As can be observed, both groups increased the percentage of correct trials as the number of training sections increased, but differences between groups were not present. This is confirmed by our analysis which showed a main effect of sessions ($F(5, 100) = 23.004$, $p < 0.001$) but no other interaction or main effect (All

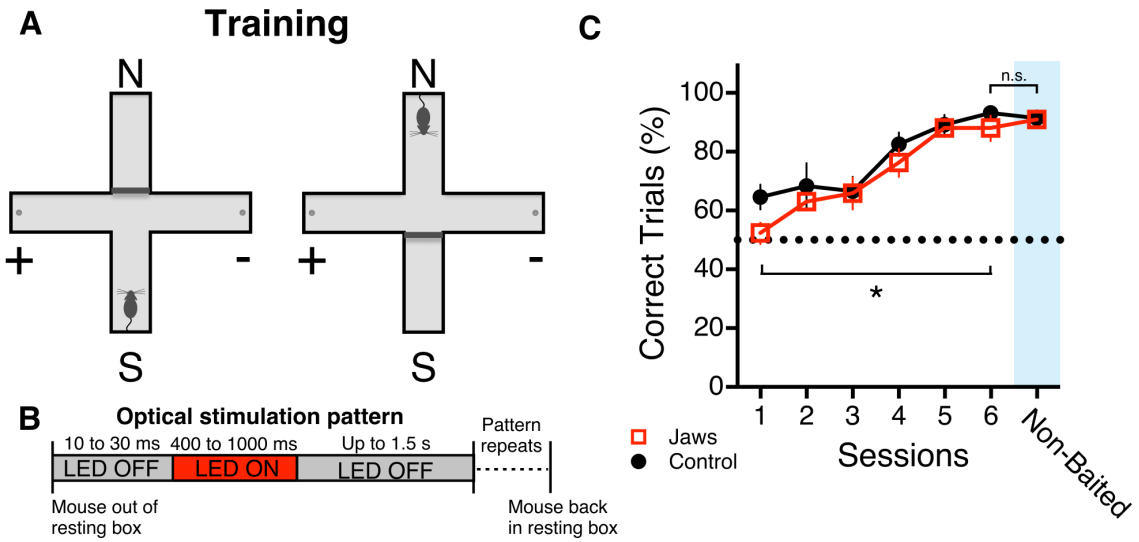



**Figure 5.4: Spatial reference memory task.** (A) Cross maze schematic drawing for a start on the south arm (left) or north arm (right). + indicates position of reward. (B) Pulsed optogenetic stimulation pattern. Grey period indicates no stimulation and red indicates activation of the LED. Pattern repeats until stimulation is turned off. Optogenetic silencing of the RSC was present during the whole training phase. (C) Mean percentage of corrected trials for both groups, Jaws ($N_{Jaws}$: 12, red squares) and Controls ($N_{Control}$: 10, black circles). SEM is shown as bars. Dotted line indicates chance level (50%). Final training session, Non-baited, means reward was added only after the animal had chosen one arm. $^*p < 0.001$

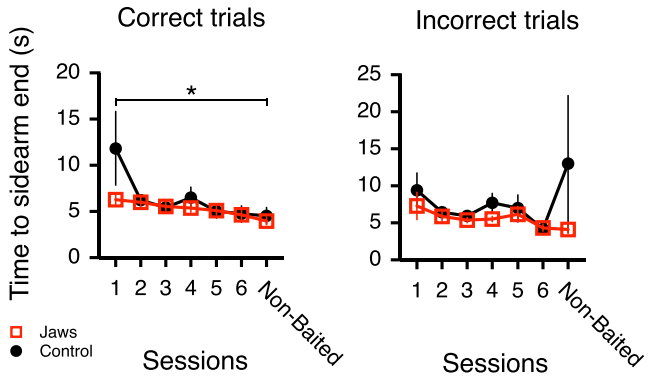



**Figure 5.5: Time to sidearm end for correct and incorrect trials.** Median time taken by animals to reach the end of the sidearms for both experimental groups ($N_{Control}$: 10, black circles; $N_{Jaws}$: 12, red squares) over training sessions separated by whether the animal had a correct (left) or incorrect choice (right). $^*p = 0.001$, bars indicate SEM.

$F < 1.955$, $p > 0.177$).

At the end of training, animals received a test where no reward was present until they had chosen a arm (Non-Baited) in order to assess if they had been using odour as a guide to reward. They were expected to perform at the same level as the last day of training and no group differences were expected. This is observed in Figure 5.4C. The statistical analysis showed no interaction or main effects of session and group were found (All $F < 0.749$, $p > 0.397$).

Once more, we looked at the time taken to go from the start point to reward location at the end of the side arms to check for any effect on locomotion and whether learning also involved a decrease in latency to reward. A slight decrease in latency is observed for both groups in both correct and incorrect trials (Figure 5.5). An analysis of correct trials revealed that there was a main effect of Session ($F(6, 120) = 3.935$, $p = 0.001$). This effect had linear trend ($F(1, 20) = 8.349$, $p = 0.009$). Apart from that, no other effects or interactions (All $F < 0.123$, $p > 0.839$) were detected.

#### 5.3.2.2 Reversal learning is not dependent on RSC activity

The retrosplenial cortex has been implicated in disambiguation of conflicting cues. Since inactivating RSC during normal learning of a reference memory task did not cause any impairments, I decided to test part of the animals ($N_{Control}$: 6; $N_{Jaws}$: 8) in a reversal learning paradigm while inactivating the RSC. On the Cross-maze reversal, the animal had to learn that reward would be found in the opposite arm to the one previously learned (Figure 5.6A). Thus, conflicting spatial cues and reward location. Again, mice received optogenetic stimulation throughout training with the same pattern described on Figure 5.4B.

Before starting reversal training, since animals had gone through a non-baited test under extinction, I retrained the animals for a single day with the reward location unchanged. When running an analysis of this retraining session with the final session of the normal training and the non-Baited Session no significant differences arise (All $F < 1.077$, $p > 0.357$).

After the retraining session, reversal learning started and reward location was inverted. While receiving LED stimulation, both groups had to train for 8 days in order to reach levels comparable with the end of the normal training phase.

Both groups successfully learned the new reward location. But they initially both dropped below chance levels before learning the new location. This is demonstrated by a linear increase in the percentage of correct trials at each session (Figure 5.6B). Analysis showed a main effect of session ($F(7, 84) = 42.771$, $p < 0.001$) but no interaction of session and group ($F(7, 84) = 0.688$, $p = 0.682$) and no main effect of group ($F(1, 12) = 1.067$, $p = 0.322$). Thus suggesting that both groups acquired similarly.

Finally, mice were tested once more, this time without any LED stimulation. No differences from the last day of reversal can be observed (Figure 5.6B). This is supported by the statistical analysis which showed no interaction between session and

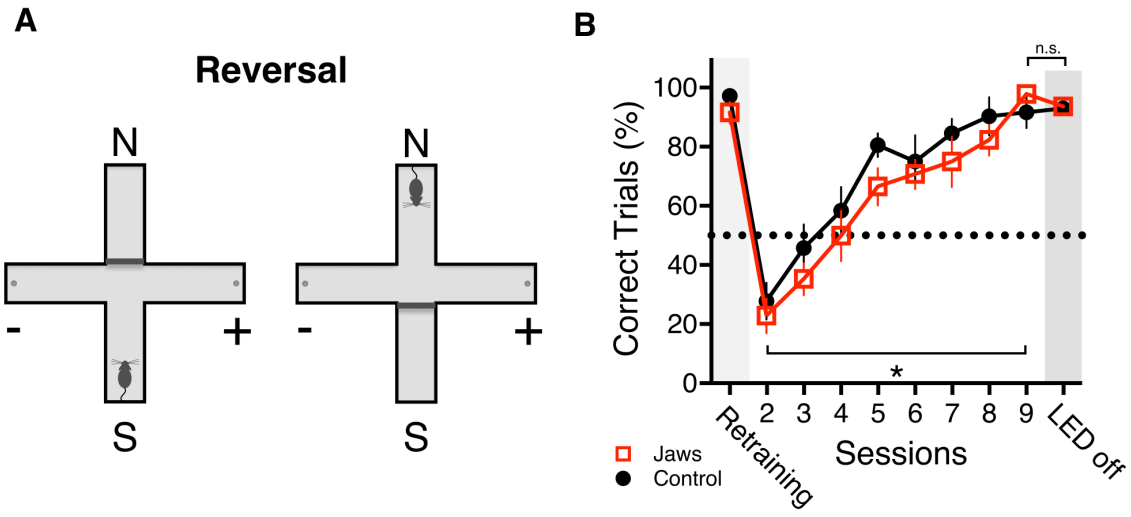



**Figure 5.6: Reversal learning in the cross maze.** Schematic representation of the reversal learning in the cross maze, where reward is now located in the opposite arm compared to training. (B) Averaged percentage of correct trials and SEM (bars) for both experimental groups ($N_{Control}$: 6, black circles; $N_{Jaws}$: 8, red squares) during retraining (normal reward location), reversal training and LED off session. Optogenetic stimulation was active during all sessions except the LED off session. $^*p < 0.001$

groups or any main effects (All $F < 1.233$, $p > 0.289$).

#### 5.3.2.3 Training without RSC inactivation is not affected

Since in this task optogenetic stimulation had started from the beginning and was present at every day of training, the question remained if it could have affected both groups by somehow entraining the network or by taking out how the RSC modified the way the brain responds in that task. Thus, by changing the maze to another room, giving the task another contest, the mice ($N_{Control}$: 5; $N_{Jaws}$: 5) were again trained on learning the location of reward. This time with no optogenetic stimulation (Figure 5.7A). Once more reward location was shifted to avoid the use of intra-maze cues only.

The results are summarised on Figure 5.7B. The last day with the LED off on the Old Context is added for comparison. It is interesting to note that while Controls do not go below chance, Jaws present a harsh drop on the first day. This is not

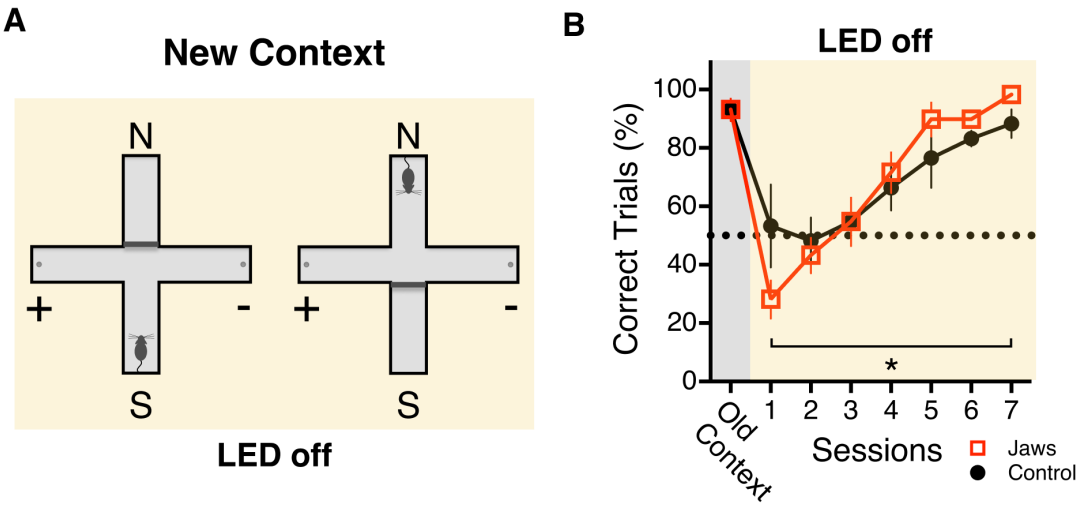



**Figure 5.7: Performance in the cross maze without optogenetic stimulation.** (A) Schematic representation of the cross maze task in a new context. (B) Mean percentage of correct trials over 7 sessions training in a new context for Jaws ($N_{Jaws}$: 5, red squares) and Control ($N_{Control}$: 5, black circles) animals. Old context performance is plotted for comparison (grey area). Bars represent SEM. $^*p < 0.001$.

significant, though. analysis of variance shows that while there is a main effect of Session ($F(6, 48) = 25.109$, $p < 0.001$) no interactions or simple effects were detected (All $F < 2.38$, $p > 0.096$). Hence, indicating a similar learning curve for both groups.

## 5.4 Discussion

### 5.4.1 Results Overview

In this Chapter, I used optogenetic manipulation of neuronal activity to inactivate the retrosplenial cortex to probe this region's importance on two spatial memory tasks with different requirements.

In the T-maze Alternation task, a working memory task, both experimental groups were able to successfully acquire learning in the absence of any optogenetic stimulation. When they were tested under optogenetic stimulation, no deficit was observed on the opsin injected group.

Similarly, when optogenetic stimulation was used throughout learning of a reference memory task, the cross maze, both groups showed almost identical performances. Now, when reversal learning started still under optogenetic stimulation, both Control and Jaws groups dropped to equivalent levels, below 50%. With training both groups were able to reacquire the new reward location successfully. Finally, when the maze was moved to a different room with different cues in the room and no optogenetic stimulation was used during training, again both groups exhibited comparable performances and learned the task in a similar time frame as the initial training.

### 5.4.2 No role for RSC in T-maze Alternation?

The results indicate that retrosplenial cortex activity is not necessary for mice to learn alternation in the T-maze. This correlates with RSC lesion studies where no deficits are detected on standard T-maze alternation (Neave et al., 1994; Pothuizen et al., 2010). But our histological studies after behavioural tests show that lesions were not present and hence not the cause of the results observed here.

On the other hand, I do observe that Jaws animal's percent correct levels are generally lower than Control animals, which can indicate that there is a difference that was not detected by our experiment. Some methodological considerations that could explain this are addressed later on this Chapter.

Another possibility is that this is driven by the drop observed on day 3. But as mentioned in the results no interaction between group and training days can be observed, thus, this drop is probably due to cohort specific noise and does not correlate with any changes in the latency to turn, so it is probably not caused by arousal.

One could also point out that the level of percent correct choices observed for Controls is generally lower when compared to other T-maze alternation studies (Reisel et al., 2002). But similar levels were found when a pilot experiment on the protocol used here was run with naive Control animals. One possible reason for that is that,

here, for T-maze experiments, water was used as reward.

The RSC is thought to be involved mainly with allocentric navigation but it has been shown that animals can rely on different types of cues to solve alternation (Dudchenko, 2001). Thus, despite the fact that RSC inactivation could potentially be disrupting the use of allocentric cues and landmarks, mice could be using other types of cues to solve this task.

Potuzhien *et al* have shown that retrosplenial lesions disrupt T-maze alternation when only egocentric and directional cues are available (Pothuizen et al., 2008) but another study from the same group showed that inactivation with muscimol is sufficient to disrupt standard T-maze alternation (Nelson et al., 2015), thus conflicting with the results presented here.

There are many reasons why that study might observe a deficit while I do not. Some of those reasons are contemplated on Section 5.4.4. But it is important to highlight that besides the use of different animal models, the two studies use slightly different behavioural protocols. Nelson *et al.* rats were given 7 training sessions before inactivation, which means that rats were probably already experts on the task. Here, inactivation happened before mice reached asymptote. This could be a crucial difference since a recent study showed that in a continuous T-maze task, the RSC only developed proper representations of the goal locations when mice reached asymptotic performance and lesions showed selectivity for the asymptotic phase (Miller et al., 2019).

An alternative reason that no deficit is observed with optogenetic inactivation of the RSC has to do with another possible function of this region. Many studies only observe decreased performance in tasks where the RSC has to disambiguate between different spatial reference frames (Byrne et al., 2007). Here, this was not required. But this is later addressed in a reference memory task, the cross-maze.

### 5.4.3 Cross-Maze

Many lesion and inactivation studies had used the Morris water maze task to interrogate the role of the RSC in learning and in long-term memory (Vann and Aggleton, 2002; Whishaw et al., 2001; Sutherland et al., 1988). Thus, I trained mice on a spatial reference task while inactivating the RSC optogenetically. Inactivation was done throughout learning and the expectation would be to see similar results as the ones found with lesion studies for reference memory in the water maze but no impairment in acquisition of the reference memory was observed. This is demonstrated by both an increase in correct choices and by a decrease in the time taken to reach the reward in both experimental groups (Figures 5.4 and 5.5).

One possible caveat in some lesion studies that leads them to display deficits in reference memory tasks is that some of them could have hit extra-RSC regions which are also involved in spatial reference memory, such as the cingulate bundle. One study compared RSC lesions to lesions in other areas in the water maze task and found no impairment (Warburton et al., 1998). Besides, the impairment observed in other studies is a lot weaker than the ones caused by fornix or hippocampus lesions (Vann and Aggleton, 2002).

On the other hand, there is evidence from Fos experiments that RSC forms a spatial representation during reference memory tasks (Czajkowski et al., 2014; Milczarek et al., 2018). However, the recruitment of RSC was decreased when the platform was cued in the water maze task (Czajkowski et al., 2014). Since, here, there are visual cues just over each of the baited arms, maybe then RSC is not as important as when having to combine cues to find a certain location.

In other reference memory tasks (Cooper et al., 2001), RSC inactivation had caused deficits both in light and dark conditions, but especially when rats were confronted with a novel situation. Many other examples exist of retrosplenial deficits arising only when cues are put in conflict or allocentric cues are removed (Cooper and Mizumori,

1999; Cooper et al., 2001). Thus, on the reversal learning phase, the location of the reward was inverted for each mouse in an attempt to conflict value of spatial cues. Again, no deficits were observed and both experimental groups relearned the new location at equivalent rates.

One possibility is that since animals had already learned the task under optogenetic stimulation, RSC's role might have been taken over by another region and it was not recruited when cues were put in conflict. The observed drop on the first day of learning in a new context (Figure 5.7) could also be related to different states that the RSC is in when either under optogenetic stimulation or not. Although no significant change was detected between Control and Jaws groups and learning showed no disruption, it could be an indication of state-dependence. Other possible methodological problems are addressed on the next Section.

### 5.4.4 Methodological Considerations

Optogenetic method, stimulation patterns, viral expression, and protocol timings are several details that can affect the outcome of behavioural functional studies, leading to contrasting results in the literature.

**Optogenetic stimulation**

Although, our recordings (Section 4.3.1) show a significant reduction in the activity of RSC, we also observed a short rebound effect after stimulation was finished. This means that the behavioural effect we observe later could be due not to simple inactivation of RSC but also to the short period of hyperactivity. While not changing the interpretation, our results have to be interpreted keeping in mind that we are disrupting activity in the RSC in two different ways and not only inhibiting this region.

One way to decrease this rebound excitation is to use shorter periods of inhibition. I attempted this by using a pulsed stimulation pattern (Figures 5.1 and 5.4). Moreover,

I used pseudo random periods of on and off stimulation to avoid any entrainment of the retrosplenial network.

Nevertheless, this leads to another potential caveat that could explain the lack of optogenetic inhibition effect on the tasks tested here. On both tasks, mice would take about 2 to 5s to get to the reward and then would be left until reward consumption, with a whole trial taking approximately 15-30s. That means that there were periods where no inhibition was happening when the mouse was in the maze. If that means RSC had sufficient time to somehow encode the spatial representation is a question that remains to be investigated.

Previous lesion studies (Neave et al., 1994; Warburton et al., 1998) that also show a lack of effect on spatial memory tasks had a significant part of the caudal retrosplenial cortex spared. Thus, this could explain the results obtained in this study. On the other hand, deficits have been observed when lesioning just the rostral part of the RSC (Sutherland et al., 1988) and c-Fos experiments do indicate an engagement of rostral and caudal RSC in a radial-arm maze working memory task (Vann et al., 2000).

Although, the RSC has been consistently implicated in spatial memory and learning it is still not clear what role this structure has in this type of memory process. The proposed ideas of RSC being important for path integration (Cooper et al., 2001), for processing of allocentric space or for translating from different frames of reference (Pothuizen et al., 2008) are not yet completely disentangled.

The experiments presented in this Chapter aimed at temporarily inactivating the retrosplenial cortex on memory tasks with different requests for memory to try to clarify the existing literature. In conclusion, using a red-shifted opsin Jaws I am able to inhibit the retrosplenial cortex while animals perform freely moving spatial tasks. It is possible to say for certain that RSC activity is disrupted, despite the considerations mentioned above. Silencing the RSC in both a working memory paradigm and a reference memory one resulted in no learning deficits.

The results here do not give strong support to any of those above mentioned views of RSC function. On the other hand, the solution to a spatial task can be reached in many different ways and even without making use of distal cues and their relationship - a problem usually associated with retrosplenial involvement - and rather using response strategies that do not depend on a spatial map of the environment (Nelson et al., 2015; Sanderson et al., 2011). For these reasons, although RSC most probably support spatial memory in some way, it might just not be necessary for the tasks tested here. Furthermore, lately, a number of studies have addressed a role for the RSC in non-spatial memory. The fact that the RSC seems to be necessary for cross-modal object recognition (Hindley et al., 2014), learning of ambiguous cues (Nelson et al., 2014) and association of sensory stimuli (Robinson et al., 2011) might indicate that a spatial function is not the main concern of this region. A possible function in support of both spatial and non-spatial episodic memory is another possibility that will be addressed on the following chapters.

These are some of the future studies that might help clarify how sensory-sensory associations work. We have come far by understanding that memory is a physical process and the it happens in our brains. Further advances and even the description of my results points towards the idea that memory is not localised in one single brain structure. It also points to the complexity of representing stimuli and their associations. Understanding how memory works is still a challenge that many scientists are pursuing. This is an account of my current path towards making valid contributions to the discussion of memory and how our brain is able to form and store them. The road does not stop here.

# Chapter 6

# Discussion

"The Road goes ever on and on
Down from the door where it began.
Now far ahead the Road has gone,
And I must follow, if I can".

J.R.R. Tolkien

## 6.1 Summary of findings

The focus of a thesis should be to add to the current state-of-the-art of the field it chooses as a target of study. This thesis has tried to contribute to the study of memory. The main route chosen to understand the way we form memories and store them has been to help understand how sensory-sensory association might be formed in a specific structure of the brain: the retrosplenial cortex.

The principal aims of this thesis were to: (1) establish for mice a behavioural task that was able to probe neutral sensory associations; (2) validate the use of optogenetics to silence RSC activity; (3) observe and assess the effects of optogenetic silencing of RSC in forming sensory-sensory associations; (4) test the role of RSC when tasks do not involve a spatial component; (5) understand how neural activity in the RSC

might be encoding associations. I have laid out the observations of experiments where behavioural tasks were combined with optogenetics in order to disrupt normal RSC function.

The main findings and conclusions of these experiments are as follows:

1. Neural activity in the RSC decreased significantly immediately when Jaws injected mice received optogenetic stimulation. This inhibition did not vary significantly with depth or intensity of light. However, rebound excitation was observed once light illumination stopped. Thus, it is possible to disrupt RSC activity optogenetically with this approach.

2. When applying a pulsed stimulation regime to silence RSC during a spatial working memory task, after animals had learned the task, no significant differences were observed between controls and Jaws-expressing mice.

3. RSC optogenetic disruption did not affect learning of a spatial reference memory task even when the contingency between allocentric spatial cues and reward location was reversed. In combination with the previous observations in lesion and inactivation studies, both spatial experiments indicate that short periods of normal RSC activity might be sufficient for spatial learning.

4. A sensory preconditioning paradigm enabled the testing of learning about neutral sensory associations. The paradigms described here had never been established in mice. The use of protocols for an appetitive version with sensory stimuli from different modalities (auditory and visual), previously established in rats, was ineffective in demonstrating a robust preconditioning effect in wild-type mice.

5. An aversive sensory preconditioning task using associations in a same modality (gustatory) allowed the expression of a robust sensory preconditioning effect. Thus, the aversive SPC paradigm was shown to be a robust way of probing sensory-sensory associations in mice.

6. Closed-loop optogenetic inhibition to RSC with millisecond precision during the presentation of neutral stimuli in spatially distinct locations in the aversive SPC task led to a lack of preconditioning only in animals where the opsin Jaws had been expressed. Hence, this linked normal activity in the RSC to proper binding of neutral sensory stimuli in this spatial task.

7. The change in the location of delivery of stimuli during the aversive SPC paradigm allowed us to probe sensory-sensory associations without potential spatial confound. By showing that Jaws-expressing animals did not exhibit a differential response to stimuli at test, a role for the RSC in formation of sensory associations is demonstrated.

8. RSC activity shows a complex and sparse response pattern to flavoured stimuli as observed through two-photon calcium imaging. Further analysis is necessary to determine if sensory stimuli is represented in this area.

## 6.2 Sensory-sensory associations

Through history we have tried to understand associations and how they are formed (See Introduction). It is still unclear how these associations support the different types of memory. One of the most studied types of memory is episodic memory (Tulving, 1972), which involves many associations. By focusing on sensory-sensory associations, this thesis aimed at helping us understand how the brain encodes these associations in order to support memory.

It should be highlighted that episodic memory is thought to involve single episodes, which means single trial events. Here, the task used to test neutral sensory associations involve repetition and several trials, which means that episodic memory is not being tested *per se*. Nevertheless, the involvement of sensory-sensory associations encoded by the retrosplenial cortex in episodic memory cannot be discarded. Although, single

trial data on RSC activity is not currently available, it has been shown that the RSC encodes sensory cues in the initial day of a cued T-maze task even in the absence of reward (Vedder et al., 2017). Further exploration of this question might reveal the role of RSC in neutral sensory associations in single trial learning.

For now, the experiments presented here show two robust and reproducible results. Inactivating the RSC while forming sensory-sensory associations prevented the expression of normal sensory preconditioning. It is also demonstrated that this disruption is independent of spatial components present in the task.

Still, it is not possible to completely dissociate the possibility of optogenetics preventing the association between the pairs of stimuli and the context rather than associations between the stimulus pair (Ward-Robinson et al., 1998). The same caveat was present in Robinson's previous inactivation study (Robinson et al., 2014). One way to possibly address that issue is to use calcium imaging to try and understand what RSC activity is representing and how it is affected by optogenetic inactivation. Nevertheless, context involves not only spatial features and it is not so clear whether it is possible to fully discard its influences.

With the paradigm setup here it is possible to start addressing this question. This was done in a preliminary way with calcium imaging of sensory representations of flavoured solutions in the RSC. The idea was to try to understand how the brain encodes the association between two neutral sensory stimuli. The lack of single neuron stimulus locked responses might indicate that RSC encodes stimulus identity more sparsely at the population level. A study performed in the locust showed that response to mixtures were a simple linear combination of the responses to the simple solutions that composed the mixture (Shen et al., 2013). This was observed at the single neuron level and at the population level. This does not seem to be the case for the RSC, but the full analysis of this data and extension of this study might help understand how a higher order cortical region encodes sensory information.

The RSC has been shown to encode value signals in its population activity (Hattori et al., 2019) which one could say goes against the idea that it encodes neutral sensory associations. But another region, the orbitofrontal cortex, well known for encoding of behavioural relevance was shown to be encoding neutral sensory pairs of cues as well (Sadacca et al., 2018).

## 6.3 RSC function

The retrosplenial cortex has yielded many different studies into its anatomy and function since it was first described (Vogt, 1976; Brodmann, 1909). Its high connectivity to medial temporal lobe areas (Sugar et al., 2011) and the many descriptions of projections from sensory areas, such as the primary sensory cortices and thalamus (Vogt and Miller, 1983; Hishida et al., 2014; Shibata, 1993), have made the RSC a structure of increasing interest over the years. This is one of the reasons that the current study attempted to improve and advance the knowledge regarding RSC function.

Despite most studies looking into the role of RSC in spatial memory and spatial representations, and others looking into a role in episodic memory formation and retrieval, one single unifying theory about its function has not yet been proposed (Mitchell et al., 2018). A great range of studies have focused on the RSC involvement in spatial memory and navigation. Descriptions of topographic disorientation due to lesions in the RSC (Takahashi et al., 1997; Katayama et al., 1999) were complemented by animal lesion studies and physiological recordings demonstrating a role of this structure in many spatial memory paradigms (Sutherland et al., 1988; Nelson et al., 2015; Vann and Aggleton, 2002).

The idea that the RSC's contribution to spatial representations is due to a role underlying the processing of sensory cues and their associations has been proposed

before (Miller et al., 2014). Consequently, here, using optogenetics, I contrasted different behavioural paradigms to better understand the role of retrosplenial cortex in either spatial or non-spatial memory with a focus on sensory associations.

Here, when optogenetic inactivation of the RSC was performed during two spatial memory tasks, no effect of optogenetic manipulation could be observed. The T-maze alternation experiment especially contrasts with the previous inactivation study from Nelson *et al.* where they used the same T-maze paradigm and inactivated the RSC using muscimol (Nelson et al., 2015).

At the same time, with regards to spatial reference memory, many studies have shown deficits in water maze navigation (Whishaw et al., 2001), some have said that the amount of deficit is dependent on lesions location (Vann et al., 2003). Other studies indicate that an intact RSC is not a requirement (Warburton et al., 1998). Others claim that deficits only arise in dark conditions (Cooper and Mizumori, 1999) or when cues are put in conflict (Nelson et al., 2015). Now, in the results presented here, no differences between control and experimental groups, even when trying to conflict reward location and cues during the reversal learning phase were observed.

Although these results seem to indicate that the RSC is not involved in spatial memory, they have to be looked at carefully as methodological considerations regarding the stimulation patterns used have to be taken into account. A pulsed stimulation regime was used in order to try and limit the rebound excitation and any thermal effects. With periods of no stimulation that may have been sufficient for learning to occur despite the inhibition periods. One way to address this caveat is to use a ramped illumination, where you gradually decrease the stimulation to allow for longer periods of optogenetic stimulation without leading to great excitation rebound effects. Unfortunately, the hardware available did not allow that to be tested.

In addition, the absence of observed effects could be explained by the tasks chosen. The T-maze alternation task can be solved using different types of cues (Dudchenko,

2001; Nelson et al., 2015) or it can be solved by using familiarity in a non-associative way (Sanderson et al., 2010) The cross-maze task has different requirements regarding motivation and navigation when compared to the Morris water-maze task where deficits in spatial memory had been observed (Whishaw et al., 2001; Vann and Aggleton, 2002). On top of that, we have to consider that spatial representation might involve more complex associations than controlled sensory-sensory stimulus pairs presented in the SPC paradigm. And more importantly, it is known that context and space may involve many redundant associations, which means that numerous linked sensory stimuli could be used to recognise a certain place. Thus, it is possible that the approach used here was insufficient to disrupt formation of some of these associations and allowed the animals to learn normally.

A final point should be made regarding the region in RSC affected by optogenetic disruption. Our histological analysis (See Section 4.3.2) shows that viral expression spared part of the caudal RSC. This could indicate that the frontal part of the RSC is more involved in sensory-sensory associations. This contrasts with lesion outcomes seen for spatial memory tasks, where only extensive lesions (Vann and Aggleton, 2002, 2004) but not lesions restricted to the anterior or caudal part of the RSC lead to deficits (Vann et al., 2003).

## 6.4 In conclusion

The findings presented here have established new ways of assessing the formation of neutral sensory associations with the development of sensory preconditioning for the first time in mice. By using closed loop optogenetic silencing of activity of the RSC during such paradigm, the importance of this brain region to formation of sensory-sensory associations was confirmed. Moreover, this conclusion regarding the RSC function was shown to be very robust as demonstrated by the ability to reproduce

the effect of optogenetic silencing in SPC tasks, with or without spatial components. This, in combination with the finding that the optogenetic manipulation was unable to provoke deficits in spatial memory tasks might shed light into how sensory-sensory associations could underlie spatial memory and spatial representations.

The possibility presented here to record activity with calcium imaging while performing sensory-sensory associations will be important in order to understand how the brain encodes this associations and how it can use them to support brain functions such as spatial memory, navigation and episodic memory. Conjunctive coding has been described in the RSC (Vedder et al., 2017; Miller et al., 2019) so it will be important to determine if for neutral sensory stimuli that are associated with each other a similar coding arises. Is it one single new representation of the associations? Or is it the co-activation of two representations one for each stimulus?

The studies presented here open a series of possible new approaches to understanding sensory-sensory associations. Taking into consideration the connectivity of the RSC, one can start probing the influences of different projections in the encoding of these neutral associations. Does it depend on thalamic or hippocampal input? Combining optogenetics with calcium imaging might shed light on such questions.

Moreover, changes in the RSC have been described for early phases of Alzheimer's disease (Poirier et al., 2011; Nestor et al., 2003). The aversive sensory preconditioning task has been demonstrated as sensitive to RSC disruptions. By using an AD mouse model in this task, it might be possible to identify cognitive deficits before other symptoms appear.

These future experiments might help elucidate how sensory-sensory associations form and support memory. We have come a long way by demonstrating that memory is a physical process and that it happens in the brain. We are now accepting the fact that memories are not restricted to one single structure and that many regions probably interact to be able to hold them in the brain. Understanding memory processes is a

task that many scientists are still pursuing. I have described here my contributions to our knowledge of memory in the hope that they will push us forward. The road does not end here.

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