Role of the Posterior Parietal Cortex in short- but not long-term Memory-dependent Behaviour

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Abbreviations

2-DG 2-deoxyglucose
AAV adeno-associated virus
ACC anterior cingulate cortex
AM anteromedial thalamic nucleus
AMPAR α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
APTD anterior pretectal nucleus dorsal part
APTV anterior pretectal nucleus ventral part
AuD secondary auditory cortex dorsal area
AuV secondary auditory cortex ventral area
BDNF brain-derived neurotrophic factor
CamKII calcium calmodulin-dependent kinase II
cAMP cyclic adenosine monophosphate
Cl claustrum
cFC contextual fear conditioning
CREB cAMP response element-binding protein
CS conditioned stimulus
DS dorsal striatum
Ect ectorhinal cortex
ERK extracellular signal–regulated kinase
fMRI functional magnetic resonance imaging
FOR familiar object recognition
GPe globes pallid us external segment
GPi globus pallidus internal segment
HFS high frequency stimulation
IEG immediate early gene
IL infralimbic cortex
InG intermediate gray layer of the superior colliculus
LEC lateral entorhinal cortex
LPLR lateral posterior thalamic nucleus laterorostral part
LPMR lateral posterior thalamic nucleus mediorostral part
LPtA lateral parietal association area

LTD long term depression
LTP long term potentiation
M1/2 primary and secondary motor cortex
M2AChR type-2 muscarinic acetylcholine receptor
MEC entorhinal cortex
MPtA medial parietal association area
mRNA messenger ribonucleic acid
MTL medial temporal lobe
MWM Morris water maze
NMDAR N-methyl-D-aspartate receptor
NOR novel object recognition
OFC optimal feedback control
OFC orbitofrontal cortex
PF parafascicular thalamic nucleus
PFC prefrontal cortex
PKA cAMP-dependent protein kinase
PKC protein kinase C
Po posterior thalamic nuclear group
PPC posterior parietal cortex
Prh perirhinal cortex
PTLp posterior parietal association area
PtPD posterior parietal dorsal area
PtPR posterior parietal caudal area
PV parvalbumin
REM rapid eye movement
RMC red nucleus agnocellular part
RSC retrosplenial cortex
S1 primary somatosensory cortex
SNc substantia nigra pars compacta
SNr substantia nigra pars reticulata
SPNs spiny projection neurons
STN sub-thalamic nucleus
TANs tonically active neurons
TeA temporal association cortex

TMS transcranial magnetic stimulation

TS tail of striatum

US unconditioned stimulus

V1/M primary visual cortex, monocular part

V2L secondary visual cortex lateral area

V2ML secondary visual cortex mediolateral area

VPL ventral posterolateral thalamic nucleus

VPM ventral posteromedial thalamic nucleus

ZI zona incerta

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Preface

Memory encompasses much more than simply remembering the past. It is the toolbox that allows us to evolve on the timescale of a lifetime. Similar to centuries of evolution for a species, the ability to form and retrieve memories endows individuals with the means to be better fit for the future based on the past.

Breaking down memory to its essential purpose, increasing the chance for survival, it becomes clear that the more effectively an individual is able to shape its future behaviour based on extracting relevant information from past experiences, the better it is equipped for success.

Thus, the crucial aspect of memory must not lie in its mere existence, but in the way individuals are able to direct their focus on what is most relevant, and to integrate the latter effectively with other memories to adapt their behaviour to changing circumstances. In many situations, deviations from existent behaviour are driven by the prospect of increasing the chances for benefit while keeping the cost as low as possible.

In this thesis, I am discussing work I have carried out in order to understand how short-term as well as long-term memories shape decision-making and associated behaviour and which role the posterior parietal cortex (PPC) plays for the latter. I have chosen the PPC as a candidate because it is traditionally viewed as an associative multisensory region whose roles are diverse, including spatial navigation, decision-making, attention direction, route planning, and multisensory integration. By connecting to visual, auditory, somatosensory and motor regions to name only a few, I was curious to find out whether PPC was particularly relevant for integrating multisensory input to respond adaptively to environmental circumstances on a short-term memory scale.

By employing chemogenetic tools in combination with viral tracing techniques in diverse behavioural paradigms, I will show that PPC plays a crucial role in enabling short-term memory to affect behaviour adaptively. Additionally, I will show which roles the downstream projections of PPC to the dorsal as well as to the tail of the striatum play regarding the implementation of PPC function.

Introduction

Memory is considered an individual's ability to process information and experiences from the outside world and to subsequently transform these stimuli into chemical and physical signals that leads to encoding of the perceived information and thus allowing for its storage. Storage in turn must imply the ability of the individual to retrieve the stored information. Various kinds of memory have been described over the second half of the 20th century.

Short-term or primary memory, sometimes also referred to as working memory, was described in a paper published by George A. Miller in 1956, in which he introduced the idea of there being a limited number of items or chunks that are available for recollection immediately after exposure to information (Cowan, 2010). Short-term memory refers to the transient storage of information, which is subsequently lost or processed for retrieval in the future as part of long-term memory.

Long-term memory comprises of declarative memory as well as of procedural memory such as remembering factual knowledge or skills respectively. Declarative memory implies awareness of the information stored and the ability to retrieve information and state the latter explicitly. In contrast, procedural knowledge is automatized through repetition and does not require a conscious effort for retrieval.
Declarative memory is further divided into semantic and episodic memory. Semantic memory is the memory concerning abstract facts that are objective such as "H&M is a brand that manufactures clothes". Episodic memory in turn includes emotions and associations, the awareness of oneself as part of the remembered situation, connected with factual knowledge such as in "I really like this shirt I bought from H&M last week". The term episodic memory was coined by Tulving in 1972, who referred to semantic and episodic memory as knowing and remembering respectively (Tulving, 1972, 1983, 1984, 1985,).
The synaptic plasticity and memory hypothesis, with the general formulation that learning is thought to result in specific patterns of neural activity leading to changes in the strength of synaptic connections that form the physiological basis for memory storage, was proposed to explain mechanisms that allow for memory formation, (Martin and Morris, 2002).

Molecular basis of memory

The idea that synapses are the central point at which changes occur during memory formation was first introduced by the Spanish pathologist Santiago Ramon y Cajal in 1894 (Kandel, 2001). Around 50 years later, Donald Hebb proposed changes in synaptic plasticity being required for memory and developed a more specific model supporting Ramon y Cajal's initial prepositions (Bailey and Kandel, 1993; Mayford, Siegelbaum and Kandel, 2012). Eric Kandel (2000 Nobel prize in Physiology or Medicine) and his colleague Tauc (1964) used the gill-withdrawal reflex of the giant marine snail *Aplysia californica* and found that both short- and long- term synaptic change takes place in memory conditions (Fig.1) (Kandel, 2001; Castellucci et al., 1970; Zucker et al., 1971; Kandel, 1976; Brunelli, Castellucci and Kandel, 1976; Castellucci and Kandel, 1976; Carew al., 1972; Pinsker et al., 1973)

This study provided the first evidence that behaviour influenced by memory had a neural basis comprising of plasticity changes in synaptic connections (Mayford, Siegelbaum and Kandel, 2012).

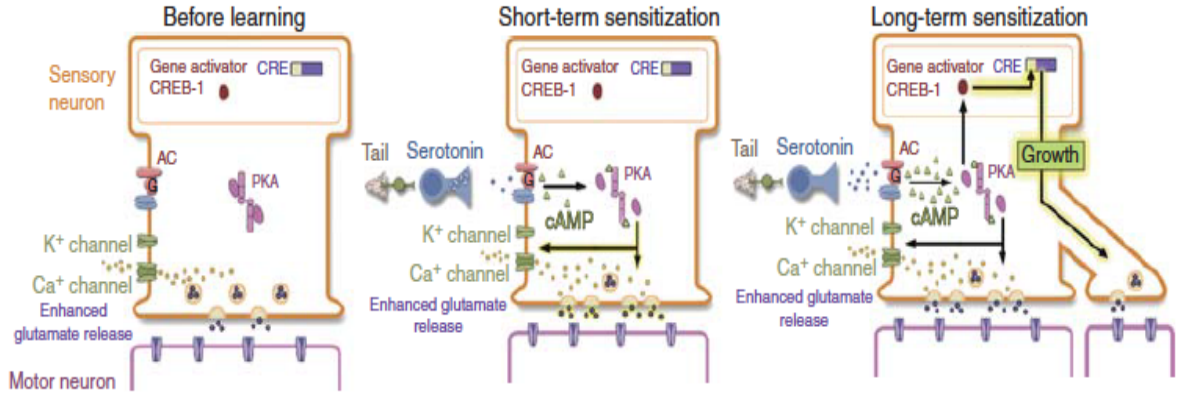

Fig.1 Short- and long-term sensitisation of the gill-withdrawal reflex in *Aplysia* (from Mayford et al. 2012). Stimulation of 5-HT neurons in response to a shock to the tail causes neurotransmitter release onto the pictured sensory neuron. G-protein mediated activation of adenylyl cyclase results in cAMP rises leading to activation of protein kinase A (PKA). Short-term changes include posttranslational modification of substrates eg. Phosphorylation of calcium and potassium channels leading to increased glutamate release onto the adjacent motor neuron. Long-term changes result from persistent cAMP rises, which leads to alterations of protein synthesis and gene transcription leading to growth of new synapses.

With regards to mammals, particularly humans, it was found from patients with lesions that declarative and procedural memory required distinct brain areas. The medial temporal lobe, particularly the hippocampus, is required for declarative memory

(Scoville and Milner 1957; Squire 1992; Schacter and Tulving 1994) whereas procedural memory is dependent on various brain areas depending on the type of skills involved (Mayford et al., 2012). The hippocampus has served as the centre for its important role on memory formation not just in humans but also in rodents and other mammals (Mayford et al., 2012). In 1973 in Lund, Sweden, Bliss and Lømo found that an applied train of high frequency stimulation (HFS) to axons in the hippocampus, in particular the Schaffer collaterals from the CA3 synapsing with CA1 pyramidal neurons (Fig.2), resulted in increased synaptic strength demonstrated by increased responses to stimuli following application of the HFS protocol (Bliss and Lømo 1973). These increases in synaptic strength were collectively termed long-term potentiation (LTP) (Lüscher and Malenka, 2012; Mayford et al., 2012). LTP furthermore usually follows the theory postulated by Hebb, however with exceptions such as the one seen in the mossy fiber pathway in the hippocampus, which is N-methyl-D-aspartate receptor (NMDAR) independent (Fig.2) and postsynaptic activity is not dependent on presynaptic stimulation in order to induce LTP (Bliss and Collinridge, 1993). Additionally, long-term depression (LTD), which is a process, by which synaptic strength is decreased following persisting low frequency stimulation of synaptic connections, was discovered (Lüscher and Malenka, 2012). LTP and LTD are thus both thought to represent molecular events of memory formation within a bidirectional model of synaptic plasticity.

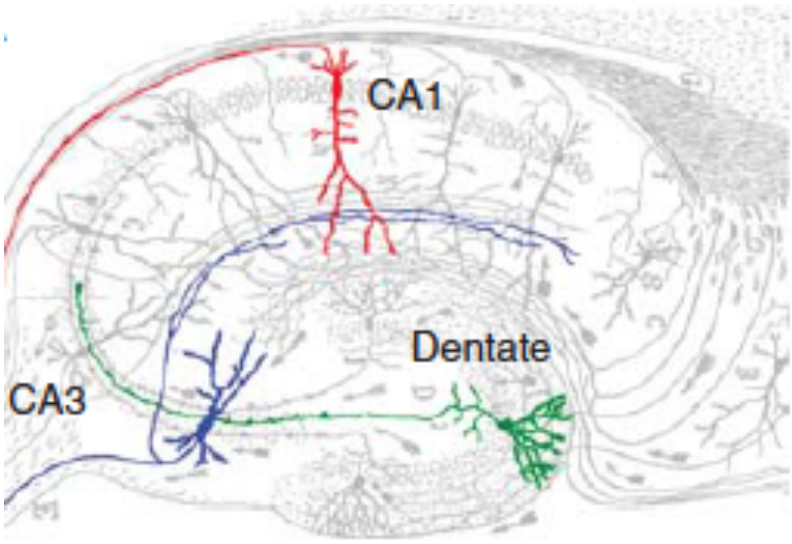

Fig.2 Historical drawing by Ramon y Cajal (1909) of the trisynaptic pathway in the hippocampus (from Lüscher and Malenka, 2012) LTP and LTD are induced by activation of NMDARs at synapses between CA3 and CA1 pyramidal neurons (blue and red). In contrast, LTP at mossy fiber synapses onto CA3 neurons (green on blue) is NMDAR-independent

Research has largely focused on Hebbian forms of LTP, such as the one found to take place postsynaptically in the CA3-CA1 pyramidal neuron synapses in the hippocampus. These are characterized by specificity, cooperativity and associativity (Mayford et al. 2012; Lüscher and Malenka, 2012). LTP is specific as it only occurs at synapses that are subject to tetanic stimulation, leaving neighbouring ones unaffected. Cooperativity means that multiple inputs are required to act together to induce LTP. Associativity comprises the finding that weak input can associate with stronger input and thus be potentiated to levels sufficient enough to induce LTP (Mayford et al., 2012). On a molecular level, it was found that LTP is induced at excitatory synapses, which mediate their actions mainly via the neurotransmitter glutamate, which is the main excitatory neurotransmitter in the brain. However, synaptic plasticity was also found to take place at inhibitory synapses termed I-LTP and I-LTD (Castillo et al., 2012).

Basal neurotransmission following presynaptic neurotransmitter release is mainly mediated by ionotropic α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs), whereas the events preceding LTP formation were found to involve NMDARs, which are another type of ionotropic glutamate receptor. NMDARs serve as molecular coincidence detectors as they are not simply activated by their agonist glutamate but instead require an additional depolarization of the resting membrane potential in order to expel a magnesium ion that occludes the channel pore due to the attractive force exerted by the normally negatively charged cellular inside as opposed to the extracellular fluid. NMDARs, as opposed to most AMPARs, provided that they contain the RNA-edited GluA2 subunit, are calcium permeable and thus their activation induces intracellular changes through calcium sensitive pathways.

Most forms of LTP and LTD were shown to depend on NMDAR-mediated calcium influx to the postsynaptic neuron in order to be induced. Activation of these different forms of memory encoding processes probably depend on an interplay of multiple factors such as the magnitude of stimulation, occurring calcium influx and previous excitation of the postsynaptic neuron (Mayford et al., 2012). Low calcium influx due to moderate NMDAR activation was shown to induce phosphatases, in particular the calcium/calmodulin-dependent protein phosphatase calcineurin and protein phosphatase 1 (PP1) (Lisman 1989), which in turn led to LTD through the

downregulation of AMPARs via endocytosis (Fig.3) (Mulkey et al. 1993, 1994: Carroll et al. 2001). In contrast, large intracellular calcium increases induce kinases leading to increased conductance and AMPAR insertion thereby leading to LTP (Fig.3) (Benke et al. 1998; Malenka, 1994)

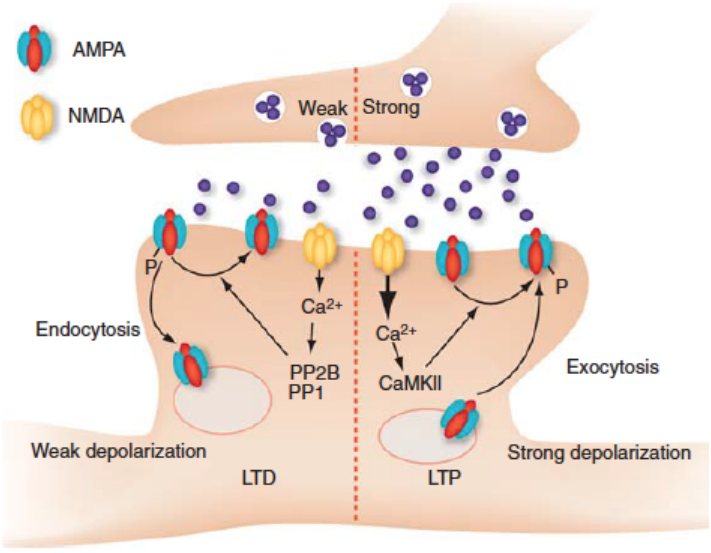

Fig.3 Postsynaptic expression mechanisms of LTP and LTD (From Lüscher and Malenka) Weak activity of the presynaptic neuron leads to modest depolarization and calcium influx through NMDA receptors. This preferentially activates phosphatases that dephosphorylate AMPA receptors, thus promoting receptor endocytosis. Strong activity paired with strong depolarization triggers LTP in part via CaMKII, receptor phosphorylation, and exocytosis

LTP and LTD were found to follow distinct temporal patterns (Silva et al., 1992a,b; Bourtchouladze et al., 1994; Huang et al., 1995; Abel et al., 1997). The early phase (minutes) is characterised by posttranslational modifications of membrane receptors through kinases and phosphatases mentioned earlier. The intermediate stage is characterised by local increases in protein translation from pre-existing mRNA, whereas the late stage (hours) is involved with increased transcription of genes such as those encoding AMPA receptors (Lüscher and Malenka, 2012).

Hippocampal slice preparations allowed for identification of protein kinases, which are activated through NMDA receptor-mediated calcium-influx. These are protein kinase C (PKC) (Routtenberg, 1986; Malinow et al., 1988), calcium calmodulin-dependent kinase II (CaMKII) (Malenka et al., 1989; Malinow et al., 1989) or the tyrosine kinase Fyn (O'Dell et al. 1991; Grant et al. 1992). Furthermore, cAMP-dependent protein kinase (PKA), mitogen-activated protein kinases, tyrosine kinases, protein kinase M-ζ

(was shown to be calcium independent – Douglas et al., 2002), and extracellular signal–regulated kinase (ERK) have also been suggested to contribute to LTP (Bliss and Collingridge 1993; Malenka and Nicoll, 1999; Salter and Kalia 2004; Sweatt 2004). Mice, in which Fyn or CaMKII were inactive due to partial deletion, showed impaired hippocampal LTP and spatial memory. Additionally, immediate early genes (IEGs) such as cfos and zif268, which are transcription factors and whose activation also depends on intracellular calcium rises and induction of constitutive transcription factors such as cAMP response element-binding protein (CREB), were shown to be necessary for long term memory (Purves et al., 2008; Mayford et al., 2012). These results also link temporal stages of LTP to those of memory formation. The insertion of new AMPA receptors through these transcriptional events in the maintenance of LTP was further supported by findings that dendritic spine excitatory synapses that undergo LTP enlarge (Mulkey et al. 1993, 1994: Carroll et al. 2001). This demonstrates that, if LTP is induced during learning and memory, and if in turn, LTP is associated with synapse growth, the resulting change in synaptic weight may serve as the biological manifestation of memory. In contrast, LTD was found to induce shrinkage of dendritic spines (Nägerl et al. 2004; Zhou et al. 2004; Wang et al. 2007; Kasai et al. 2010).

In 1986, more behavioural support for the synaptic model of memory was provided by Morris and colleagues, who found that blockage of NMDA receptors in the rat resulted in their inability to remember the location of the platform in the Morris water maze along with blockage of LTP induction. Interestingly, when the platform was visible, thus spatial memory was not required, mice were not impaired in learning how to swim to the platform. Conclusively, the hippocampus does not seem to be required for procedural memory (Morris et al., 1986). Additionally, Tang and colleagues (1999) showed that enhanced activation of NMDARs by overexpression of the NMDAR 2B subunit in transgenic mice resulted in superior performance in behavioural learning and memory tasks. In 1998, Moser and colleagues found that artificial saturation of LTP in rats with only one intact hippocampus resulted in their impaired performance in the Morris water maze spatial learning task. Interestingly, one year later, Moser and colleagues (Otnaess et al.,1999) found that pre-trained rats did not show such impairment in spatial learning upon saturation of LTP.

It was repeatedly shown that interference with kinases, IEGs or receptors associated with LTP induction results in impairment of memory types. However, the direct

induction of LTP through learning was not demonstrated until Whitlock and colleagues (2006) showed that one-trial inhibitory avoidance learning in rats induced LTP through NMDAR-dependent processes resulting in synaptic alterations of AMPAR abundance thereby mimicking the effects of high frequency stimulation (HFS) on LTP. Additionally, the learning-induced LTP was shown to partially occlude LTP induced through HFS thereafter (Whitlock et al., 2006). An attempt to demonstrate the induction of memories through LTP was published by Nabavi et al. (2014). This study used optogenetics in order to deactivate fear memory in the amygdala by inducing LTD. Subsequent induction of LTP was able to reinstate the fear memory thus showing that existing memories can be manipulated through LTD and LTP.

Distinct phases of memory consolidation

Memories can be subdivided depending on when they are retrieved after acquisition, ranging from short- (seconds), intermediate (hours) to long-term (days) memories (Kandel et al., 2014). Short-term memories are acquired constantly, but not everything that is experienced in a day will be available for recall on the following day. Thus, for a short-term memory to be represented by a long-term memory days or even years later, certain processes, some of which were discussed in the previous section, were found to be required. These processes were termed memory consolidation already very early on, with some of the first ideas originating from findings that recent memories are more vulnerable to disease or injury than those acquired long ago (Müller and Pilzecker, 1900; Ribot, 1881) While short-term memories are not reliant on *de novo* transcription and translation of new proteins, long-term memories were shown to depend on these processes (Caroni et al., 2014, Karunakaran et al., 2016; Bekinschtein, 2007). As previously discussed, the late phase of LTP is characterized by the production of new synaptic proteins and receptors leading to lasting synaptic changes. Most studies of LTP have focused on a relatively restricted time window of around 1h comprising of the processes discussed in the previous paragraphs. Importantly, IEGs such as cfos and Arc were found to be relevant for long-term memory although peaking at around 90 minutes after induction (Karunakaran et al., 2016; Katche, 2010 and 2013; Nakayama, 2015; Caroni, 2014; Purves et al., 2008; Mayford et al., 2012). Two time-windows during which cascades of transient molecular and cellular responses take place were identified. The first window is the acquisition-induced cascade, comprising of previously discussed post-translational modifications, translation of pre-existing

mRNA such as for cfos, or transcription of brain-derived neurotrophic factor (BDNF) and Arc, allowing growth of immature synapses. The second window comprises a second wave of IEGs and other transcription factors and occurs at 12-15h after acquisition of the learning and relies on BDNF and local dopamine (DA) D1/5 receptor signaling (Caroni et al., 2014; Redondo et al., 2011; Bekinshtein et al., 2007; Katche 2010; Trifilieff 2006;; Ressler et al., 2002). Strengthening of new synapses was found to take place between 11-18 hours after learning induction. At this time point, long-term memory consolidation is thought to have completed essentially. However, it was found that even thereafter, elimination of previous and some new synapses takes place and cell assemblies thought to comprise of the memory are reactivated during non-REM sleep and quiet wakefulness in order to further consolidate these memories (Girardeau et al., 2014; Ego-Stengel et al., 2010; Nakashiba et al., 2009; Jadhav et al., 2012; Singer et al., 2013; Caroni et al., 2014; Buzsaki, 2015).

Memory processes on a systems level

In addition to cellular mechanisms of consolidation, systems consolidation is thought of as the phenomenon that describes how the contributions of brain regions to a particular memory change over time. This phenomenon was first observed by Brenda Milner whose patient H.M. had undergone resection of the medial temporal lobe (MTL) and in particular the hippocampal formation, which caused both anterograde and retrograde amnesia (Scoville and Milner, 1957). However, the retrograde amnesia was restricted to a few years and memories that had been acquired in the distant past were unaffected. Early studies analysing physiological changes over time measured 2-deoxyglucose (2-DG) uptake in different brain regions as a result of memory induction (Squire et al., 2015; Bontempi et al., 1999). Increased 2-DG uptake was prominent in the hippocampus shortly after testing, but decreased with time when testing was delayed for several weeks. Concomitantly, there was a time-dependent increase in 2-DG uptake found in the neocortex (Bontempi et a., 1999). Some years later, the same pattern was discovered for cfos relating to remote fear-memory in the anterior cigulate cortex (Frankland et al., 2004).

Considering that the human nervous system contains between 300 and 500 billion neurons and that each neuron forms thousands of synaptic connections, the vast capacity for memory storage becomes evident. Across brain structures, specific connectivity allows for functional specification and yet the possibility to represent

countless different memories within confined circuits. Moving from the synapse level to a circuit level, a specific memory trace is thought to be encoded by a distinct subset of neurons, which, if interfered with, results in a loss of the corresponding memory (Han et al., 2009; Josselyn, 2010). Fear memory responses were not only shown to be reduced by inactivation of a fear-memory corresponding engram, it was also shown that the induction of a fear memory response can be elicited by reactivation of the corresponding engram in the absence of any context associated with fear (Liu et al., 2012). This was done by labelling hippocampal cells in the dentate gyrus of mice with channelrhodopsin-2 during a fear conditioning paradigm (Boyden et al., 2005). Subsequent reactivation by light stimulation of these cells caused freezing behaviour in a neutral context thus showing that a defined sparse population of neurons within a likely much larger systems-wide engram is sufficient for eliciting a specific memory (Liu et al., 2012).

Investigation of memory processes in animals

The application of definitions of types of memory, which are defined according to humans, poses difficulties when being tested for in animals as ie. semantic memory does not allow for identical application to animals as a distinct language is absent in the latter. However, even complex types of memory such as episodic memory have been characterised in animals in slightly varied ways.

Tulving's original definition of episodic memory as "receiving and storing information about temporally dated episodes or events and temporal-spatial relations between them" (Eacott and Norman, 2004) requires the individual to be aware of oneself in the episode that is being remembered.
However, this proves difficult to assess in animals whereupon Clayton and Dickinson coined the term episodic-like memory that overcomes the need for a conscious recollection and instead relies on an animal's capacity to remember 'what' happened 'when' and 'where' (Clayton, Salwiczek and Dickinson, 2007). Dickinson and colleagues studied the food storing behavior of scrub jays (*Aphelocoma californica*) and found that the birds exhibited memory about where particular types of food were stored and for how long it was stored until retrieved for consumption. Therefore, it was claimed that because they exhibited all three requirements for episodic-like memory

('what', 'where' and 'when'), scrub jays were the first animal to be found to exhibit episodic-like memory (Clayton et al., 2001).

Most studies investigating different types of memory over the past years were conducted in rodents and employed selective lesions or temporal inactivation of brain areas and their influence on animals' performance in different memory tasks. The 'when' element of the definition of episodic-like memory has been proved to be difficult to assess in rodents (Babb and Crystal, 2005, Babb and Crystal, 2006a; Babb and Crystal, 2006b) which led Eacott and Norman (2004) to develop a novel task, which includes a substitution of the 'when' element by introducing a 'context' element (Eacott and Norman, 2004). This novel idea was based on Gaffan's (1994) claim that by introducing a background which serves as a context to the 'what' and 'where' elements, episodic memory could be modeled and assessed via employment of this new model which he called 'scene memory' (Gaffan, 1994; Eacott and Norman, 2004).

Familiar object recognition task

In 1988, Ennaceur and Delacour introduced a method to assess memory in rodents that is based on the inherent curiosity that these animals exhibit about their environment (Ennaceur and Delacour, 1988). When animals encounter a novel object in the presence of a familiar object, thus implying that they remember the latter, they will explore the novel object more thoroughly. This task and its variants are frequently referred to as novel object recognition (NOR) task, however, the term familiar object recognition (FOR) task, accounting for the fact that it is the familiar object, which is recognized rather than the novel one, is used throughout this thesis. The version of the task which is used predominantly in this thesis tests whether mice recognize familiar objects in the absence of any changes to the context in which mice are exposed to them.

Contextual fear conditioning

A frequently used method to assess memory in a Pavlovian conditioning paradigm is the contextual fear conditioning (cFC). In this paradigm, mice are exposed to a neutral context (the conditioned stimulus, CS) in which they receive an aversive footshock (the unconditioned stimulus, US) after a delay period (Maren, 2001; LeDoux, 2000). Upon subsequent reintroduction to the context, mice exhibit a fear memory response

detected as freezing in the absence of a footshock because they have made an association between CS and US (LeDoux, 2000). The fear memory can further be modified by prolonged (30mins) context exposure without the appearance of any shocks. This process is termed extinction learning and leads to the absence of a freezing response when tested for on the next day (Myers and Davis 2002). However, after a period of 10 or more days, the behavioural response to context exposure, again, results in a freezing response, which is termed recovery of fear. The cFC task is valuable in dissecting the influence of brain regions on specific parts of the fear memory or its modification, i.e. that some regions are not required for the acquisition or the recall of fear memory but for its subsequent modification.

The Morris water maze

The Morris water maze (MWM) task is used frequently in order to assess spatial memory and navigation in rodents. The animals are required to swim within a circular pool of opaque water and to make use of environmental cues in order to navigate to an escape platform hidden just beneath the water surface (Morris, 1984). The task can be modified in many ways in order to test reference memory, short term memory or strategic navigation behaviour. Most commonly, escape latency measured as the time between entering the water and finding the hidden platform is used as a measure for successful learning. However, analyzing strategic behaviour throughout and across trials can further increase our understanding of the type of memories assessed in this task.

Brain areas involved with memory

The hippocampus represents the most widely studied brain region in relation to memory and a myriad of studies was published within the last 50 years. It was shown that the hippocampus and surrounding areas such as the lateral and medial entorhinal cortices (LEC and MEC respectively) and the peri- and postrhinal cortices are critical for various aspects of memory (Eacott and Norman, 2004; Langston and Wood, 2010; Wilson et al., 2013a and 2013b; Bussey et al., 2000)

In 1948, Tolman proposed that the hippocampus and surrounding areas formed a cognitive map of the environment but it was not until the discovery of place cells (O'Keefe and Dostrovsky, 1971) and grid cells (Hafting et al., 2005) in the rodent

medial temporal lobe that the importance of the MEC and hippocampus for spatial memory was universally accepted. Not only spatial memory, but also episodic memory has been shown to depend on hippocampal function (Tulving et al., 1972). Aspects of fear memory, the latter of which is inevitably associated with amygdala function, were also shown to depend on hippocampal function (Orsini and Maren, 2012).

Beyond the very popular brain systems, the posterior parietal cortex represents an area which is not traditionally studied regarding memory processes. Rather, the PPC is proposed to fulfil an array of functions which can be broadly grouped into four domains: sensorimotor transformations, selective attention, working memory and learning (Rawley and Constantinidis 2009). On the following pages, I will attempt to provide a summary on PPC location, connectivity and proposed functions which are relevant to the research contained within this thesis.

Posterior parietal cortex

The posterior parietal cortex (PPC) is positioned between the somatosensory cortex and the primary and secondary visual cortices across the rostro-caudal axis of the brain (Hyvarinen, 1982, Reep et al., 1994, Whitlock, 2014). Various functions of PPC have been reported throughout the literature, including cognitive, multisensory, associative and sensorimotor functions (Holmes, 1918, Bender and Teuber, 1947, Denny-Brown et al., 1952, Hyvarinen, 1982). In particular, spatial navigation, perceptual decision-making, attention, route planning, and multisensory integration were proposed to rely heavily on PPC function (Platt and Glimcher, 1999, Cui and Andersen, 2007, Andersen and Cui, 2009, Calton and Taube, 2009, Harvey et al., 2012, Hauschild et al., 2012, Carandini and Churchland, 2013, Olcese et al., 2013).

The earliest insights on PPC function resulted from clinical presentations of patients who had witnessed strokes or traumatic injuries to the brain (Whitlock, 2017). 'Bàlint's syndrome' was the first condition characterised as resulting from PPC damage in human patients described the the Austro-Hungarian physician Rezsö Bàlint in 1909. The syndrome is characterised by three key symptoms, simultagnosia, which is the inability to perceive more than one item in the visual field, oculomotor apraxia, describing difficulty in making targeted eye movements, and optic ataxia, which is the inability to make visually-guided arm and hand movements (Bàlint, 1909). These results led to the first proposal that PPC might exert functions which allow for coordinated actions within an individual's peripersonal space and the construction of a stable representation of the latter.

Further work by Macdonald Critchley described an even vaster amount of impairments regarding own body perception and motor apraxias, but also sensory disturbances, deficiencies in symbolic thought, mathematical abilities and visuospatial attention and autotopagnosia, which is the inability to correctly locate one's own body parts (Critchley, 1953).

Henry Head and Gordon Holmes proposed that PPC function is critical for plastic 'body schema', by which they mean the constant awareness of one's own bodily position and orientation of limbs in a three dimensional space across time (Head and Holmes, 1911) In simple terms, PPC function might play a key part allowing animals, including humans, to experience that they are in their own body and that the latter finds itself in different states relative to the environment across time requiring specific behaviours for each of these states in order to affect future states positively.

PPC anatomy and connectivity

Across species, the topography of PPC and surrounding areas remains largely comparable as shown in Fig.4.

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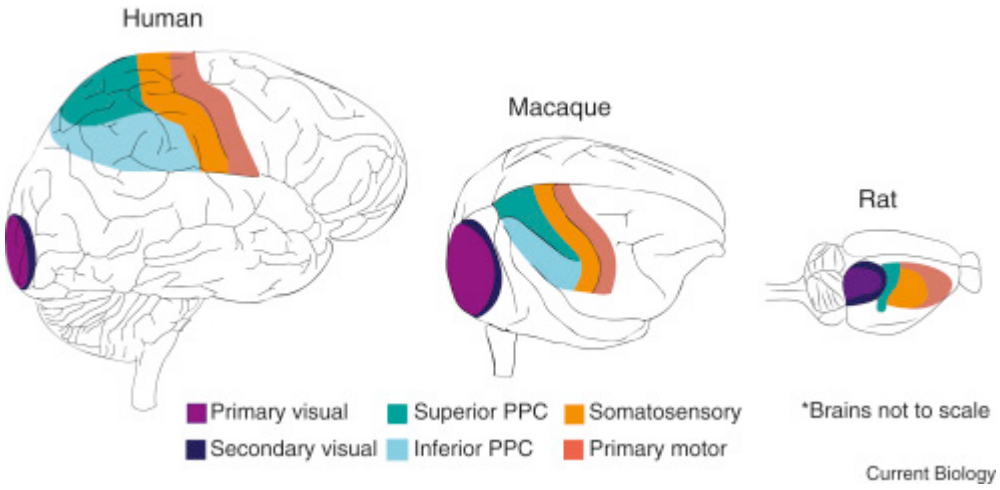


Fig. 4 Topography of posterior parietal cortex relative to other cortical areas. (from Whitlock, 2017) Lateral view of human, macaque and rat brains, showing the organization of visual, posterior parietal, somatosensory and primary motor areas of cortex. Cortical areas are arranged in the same order for all mammals, with the visual areas furthest posterior, posterior parietal cortex lying between visual and somatosensory areas, and primary motor areas in front of somatosensory cortex.

However, when it comes to defining the location of PPC in mice, variability across the literature exists regarding the exact location and terminology of the area. The Paxinos mouse brain atlas (Paxinos and Franklin, 2012), which is the atlas used as reference throughout this thesis, identifies four subdivisions of PPC according to Nissl and Acetylcholinesterase staining between -1.5mm and -2.1mm relative to bregma and 0.8mm–2.75mm lateral to bregma: MPtA (medial parietal association area), LPtA (lateral parietal association area), PtPD (posterior parietal dorsal area), and PtPR (posterior parietal caudal area). In contrast, the Allen mouse brain atlas (Dong, 2008), which uses Nissl staining only, refers to PPC as posterior parietal association area (PTLp) ranging from -2.0mm to -2.5mm relative to bregma and 1.0mm to 2.75mm lateral to bregma. The areas described in Paxinos and Allen atlases partially overlap, however, PTLp covers an area much more caudal, covering areas termed V1, V2M and V2L in Paxinos. Throughout this thesis, Paxinos mouse brain atlas is used as a reference for PPC location and all other brain areas of manipulation. When referring to the literature, the term PPC is used unless explicitly stated, that the area of reference

includes the more posterior part of PTLp corresponding to the Allen brain atlas. Importantly, the area targeted for PPC manipulation across all discussed experiments, is termed PPC according to either of the two atlases mentioned.

A more recent study by Karoline Hovde and colleagues attempted to use different approaches including laminar, and cytoarchitectonic criteria in order to define PPC (Hovde et al., 2018). Nissl, parvalbumin (PV), and type-2 muscarinic acetylcholine receptor (M2AChR) staining of coronal sections provided complementary insights on where to delineate PPC from neighboring areas (Fig.5). This figure shows very nicely the bregma levels where Paxinos and Allen atlases overlap, namely between bregma -1.91mm and -2.15mm. The value -1.91 to -2mm relative to bregma is where PPC injections discussed within this thesis were targeted along the rostro-caudal axis, thereby covering an area that is termed PPC regardless of the reference atlas. Furthermore, for discussing PPC within this thesis, no distinctions were made regarding the subdivisions within PPC itself.

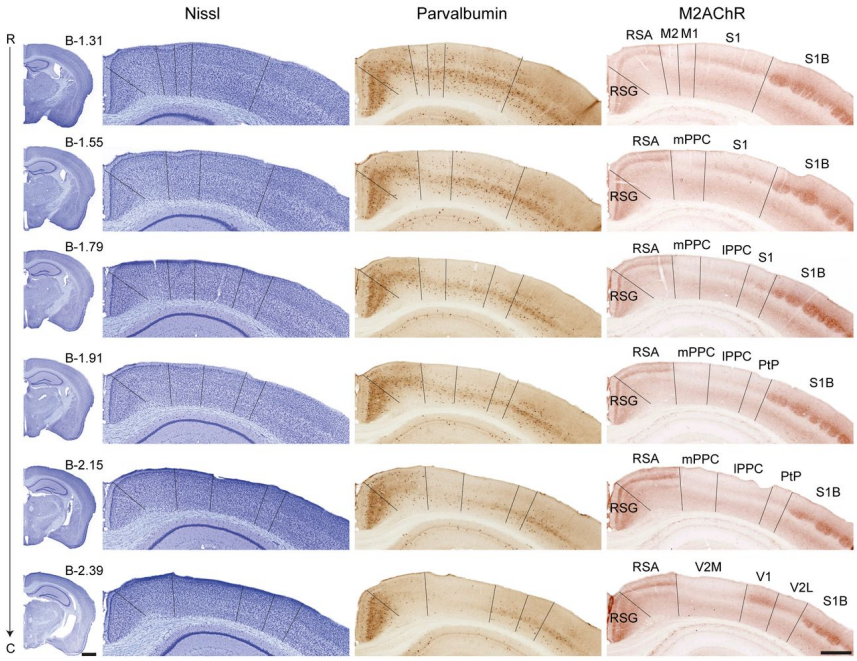


Fig.5 Coronal sections of 40microm ranging from -1.31 to -2.39 relative to bregma. (from Hovde et al., 2018) Nissl, PV and M2AChR stainings are shown on zoomed in sections referring to the bregma level shown on the left of each row.

PPC connectivity is characterised by a multitude of areas involving primary sensory and higher associative areas, but also some thalamic nuclei, the striatum and claustrum (Lyamzin and Benucci, 2019). PPC is thought to reciprocally connect visual, auditory and somatosensory areas to higher associative areas such as the orbitofrontal

(OFC), the prefrontal (PFC), retrosplenial (RSC) and anterior cingulate (ACC) cortices (Zingg et al., 2014; Wang et al., 2011; Zhang et al., 2016). PPC is also characterised by reciprocal connections with primary and secondary motor cortices. Subcortical projections include the dorsal striatum, the thalamus and the claustrum (Hintiryan et al., 2016; Harvey et al., 2012; Zingg et a., 2014).

Sensory-motor functions in PPC

As mentioned previously, the visuospatial and visuomotor deficits decribed by Bálint, such as the inability of patients with bilateral PPC lesions to grasp objects which were placed right in front of them, points towards a role of PPC in connecting visual sensory with executive motor functions (Bálint, 1909; Whitlock, 2014). The integration of perceived multisensory information into a common reference system which serves as the basis for generating goal-oriented motor commands was suggested to represent a main role of PPC function, sometimes referred to as "vision for action" (Goodale and Milner, 2002). When animals move within the environment, they must keep track of the constantly changing orientation of their body parts in relation to themselves but also to the environment which is equally changing as the animal is moving (Rawley and Constantinidis 2009). Broadly, two categories of reference systems can be distinguished, the egocentric point of view, that is the position of oneself within the perceived environment, and the allocentric point of view, within which spatial maps are constructed and which does not require one's own position within it. Both points of views require sensory-motor integration to different degrees and PPC was suggested to play a role in either reference frame for navigation, whereas the hippocampal formation, for instance, was shown to be required only for the mapping of allocentric space (Morris, Garrud, Rawlins, & O'Keefe, 1982; O'Keefe and Nadel, 1978).

A role for PPC in navigation

Studies investigating the role of PPC in navigation came largely from human functional magnetic resonance imaging (fMRI) studies. Eleanor Maguire and her colleagues published several studies proposing a role for PPC in computing sequential movements such as left or right turns in order to reach a goal, with medial PPC being more involved with immediate movement planning and lateral PPC being more involved with distant route planning (Maguire et al., 2002; Maguire et al., 2006). A study

by Ciaramelli and colleagues showed that patients suffering from PPC damage, were unimpaired in tasks relying on allocentric representations such as judging the distance between two locations within a well-known town. However, when these patients were asked to navigate through the same town, a task requiring them to use egocentric representations, they failed consistently and reported impoverished and disembodied experiences during the navigation task (Ciaramelli et al., 2010). In macaques, navigating through a virtual house in order to reach a particular location, requires PPC function (Sato et al., 2006). Rodent studies have complemented an important role for PPC in navigation, mostly through PPC lesions resulting in deficits of forming spatial representations from multisensory inputs (Whitlock et al., 2008).

However, it was also shown that PPC engages not only with navigational processes but also, as mentioned earlier, with sensory processing and dependent decision-making (Harvey et al., 2012). Because animals naturally execute their behaviours within space, one could imagine PPC to serve the purpose of generating behavioural plans which are fit to environmental stimuli ranging from simple goal-directed grasping of objects to complex navigation within an environment. In this view, the function of PPC would not be a navigational one *per se* but rather a behavioural role which manifests itself in navigational deficits if interrupted in frequently employed tests.

Thus, the question could be asked whether deficits observed by PPC lesions or inactivations in navigational or reaching tasks, could be due to a failure of deciding on the correct behaviour rather than resulting from a deficit in spatial or visual processing. This hypothesis would also fit the findings that PPC damage leads to aberrant visuo-spatial behaviour, such that although objects can be perceived and named within the peri-personal space, one is unable to grasp them precisely. Here, PPC function could represent tuning the movement of the hand to real-time visual feedback, both processes which are known to function independently, in order to ensure fine-tuned motor skills.

This scenario gives rise to a further complication when trying to understand PPC function. Assuming that we witness an event of optic ataxia, the viewer cannot distinguish whether the person is unable to 'decide' where the actual object is located with respect to its hand, or if it's the person's altered motor skills which led to a failure of execution of a correct 'decision'.

Until quite recently, proposed PPC functions were as diverse as the research questions they tried to answer. However, in order to really increase our understanding of PPC

function, it is important to attempt to reconcile the wealth of data. Medendorp and Heed suggested to view PPC function in the face of the optimal feedback control (OFC) theory (Todorov, 2004). Shadmehr and Krakauer (2008) propose a model of optimal feedback control (Fig.6), that proposes PPC function as state estimation. PPC is thought to integrate proprioceptive and visual predictions with sensory feedback to form an estimation about how previous commands affected the state of the body and the environment in which the latter exists.

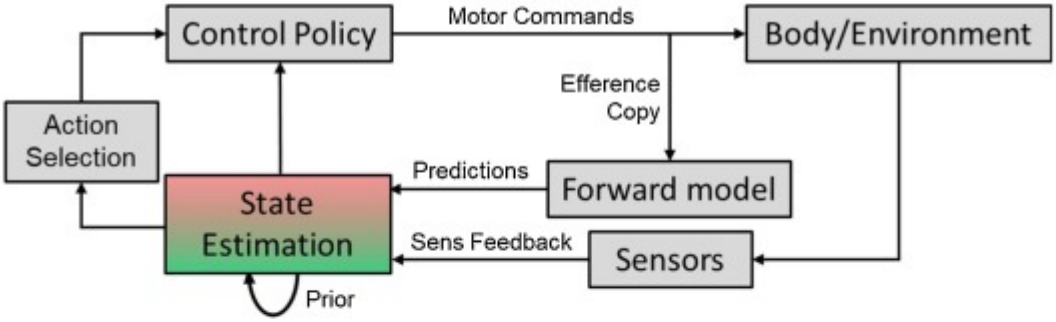


Fig.6 Illustration of optimal feedback control theory (from Shadmehr and Krakauer, 2008). The state estimator integrates current sensory feedback with predictions from previous motor commands and expected sensory state (prior) according to similar previous events. Action selection specifies the goal of the behaviour, while control policy is set in place in order to reflect general rules and feedback gains relating to current environmental and bodily states.

PPC, attention selection and working memory

Multisensory input, however, likely exceeds the capacities for simultaneous processing and attention must be channelled to relevant stimuli which are required for goal-directed behavioural decisions. It was shown that primate PPC activation is observed when animals need to direct their attention from irrelevant to relevant visual stimuli (Constantinidis, 2006). Attention selection is closely related to working memory as the two share common requirements, such as the ability to keep relevant information temporarily present during cognitive processes. The term working memory is essentially referring to short-term memory but implicating the function of actively utilizing information stored 'short-term' during cognitive processes rather than passively storing it (Baddeley, 1986; Baddeley & Hitch, 1974, 2000; Pascual-Leone, 1970). The prefrontal cortex (PFC) is thought to play a crucial role in working memory and was shown to exhibit reciprocal connections with PPC (Rawley and Constantinidis, 2009). Imaging studies investigating working memory in humans show simultaneous activation of PPC and PFC (Bunge et al., 2001; Courtney et al.,1997). However, further

studies using functional magnetic resonance imaging (fMRI) and transcranial magnetic stimulation (TMS) have shown that the roles of PPC and PFC in working memory differ and that the role of PPC must be specific to certain aspects of working memory (Sakai, Rowe, & Passingham, 2002; Koch et al., 2005). A method through which the capacity of working memory can be maximized is called 'chunking', which includes grouping of multiple pieces of information into larger larger meaningful units thereby increasing the total amount of information being utilized in working memory (Miller, 1956). PPC was shown to be involved with this and other types of organizing information into meaningful comprised structures (Bor & Owen, 2007; Bor et al., 2003; Bor et al., 2004; Wendelken, Bunge, & Carter, 2008).

Working memory and selective attention are affecting learning and long-term memory because the attention towards sensory stimuli is likely selective, while working memory affects the way stimuli are integrated in order to affect subsequent behaviour and resulting environmental feedback. Additionally, long-term memory, particular episodic memory, can be affected by altered attention and working memory as the latter influences the way those memories are retrieved and integrated with new information. Naturally, episodic memory signifies a bridge between the past and the future as the former dictates the baseline against which all new information is compared and based on which future actions are decided on (Tulving, 1985; Uncapher and Wagner, 2008). In this view, PPC function would impact even long-term memory indirectly through potentially altering environmental experiences as a result of PPC's role on attention and working memory.

Towards a model integrating sensory-motor functions with a role in attention, working memory and action selection

The involvement of PPC with decision-making processes, together with the interconnectivity of PPC with sensory and motor areas points towards a role of PPC in transforming incoming sensory information into reasonable motor commands that can be measured behaviourally. Motor commands are based on decision-making that is in turn dependent, not only on the current state of the environment, but also on previous experiences, recent as well as more distant ones.

A study from the Komiyama lab investigated the role of PPC in using recent sensory-history to shape perception and working memory that in turn is measured behaviourally

in decision-making (Hwang et al., 2017). The authors used a visually-instructed action selection task in which mice were required to move a joystick in the correct direction based on a visual stimulus. They developed a model that could most accurately predict the mice' behaviour based on 3 factors: the current stimulus at any trial, the choice outcome history of previous trials and a constant choice preference. The latter two represent the internal bias, of which first, the outcome, and second, the choice of previous trials exhibited the largest predictive power to influence future behaviour based on internal bias. Most importantly, when PPC was silenced optogenetically, they could abolish the recent history-dependent bias on decision-making. Furthermore, they could show that the subjective bias based on recent history was computed prior to stimulus presentation and thus shaping perception of the latter (Hwang et al., 2017).

Another study used an auditory working memory task in rats, where the authors demonstrated that sensory-stimulus history exerted quantifiable effects on behaviour (Akrami et al., 2018). The rats used in this study had to determine which of two tones, delivered several seconds apart, was louder. This task required rats to hold the loudness of the first tone in working memory during the delay period between the two tones, to enable comparison with the second. They found that a phenomenon termed contraction bias took place. Contraction bias was first described by Harry Hollingworth (1910) referring to the phenomenon that the representation of a stimulus in working memory is systematically biased towards the average of recent past observations. That means in this particular experiment that, in additional to having learnt the rule on how to perform this task, the decisions that the rats would make were also influenced by how the recent sensory history preceeding the current trials shaped their perceptional judgement. When the authors silenced PPC during the delay period between the two tones that rats were supposed to compare, the result was that rats performed better than with PPC being active. This outcome shows, that although PPC function clearly is involved with experiences on a short-term memory scale, PPC function is not just holding or recalling short term memory. Furthermore, they showed that silencing PPC dramatically reduced the influence of recent sensory experience on performance. Thereby reducing contraction bias and increasing the weight of the learnt rule to impact decisions.

These examples suggest that PPC function reflects internal biases related to the value of past stimuli (Akrami, 2017) or actions (Hwang, 2017). This is a central component of decision-making, that is likely to exert greater influence over decisions, the less clear the presented evidence for a certain decision is. In simple terms, behaviour that is based on a learnt rule is shaped by how successfully this rule was employed in recent cases of similar or identical sensory stimuli, in order to most accurately predict whether decisions will be advantageous. Thus, without PPC function, decision-making would be rigid, not taking into account recent dynamics or outcomes of past decisions.

Specific roles for different PPC projections

Newer research has provided not only further insight about the role of PPC in general, but has provided knowledge about distinct roles of specific projection targets of PPC. Because the last part of this thesis will focus on projection-specific roles of PPC, I would like to provide reference to some of the newest discoveries related to this question.

Striatal projections for history-dependent decision bias

A recent study investigated the roles of specific projection targets of PPC, in particular the dorsal striatum (DS) and the posterior secondary motor cortex (pM2) (Hwang et al., 2019). The authors found that the neurons projecting to either of the projection targets formed largely separate populations. While the neurons projecting to the DS received strong input from associative regions, the neurons projecting to pM2 were characterized strongly by input from sensorimotor areas. The influence these projections exerted on action-selection bias based on previous stimulus history was very different, with dorsal projections dominating. Manipulation of pM2 projections in contrast altered movement kinematics.

In the last part of this thesis, I will also discuss the role of PPC projections to DS, thus I would like to briefly introduce the dorsal striatum and its internal organization.

The dorsal striatum forms part of the basal ganglia, comprising of interconnected nuclei including the striatum, globus pallidus (internal (GPi) and external (GPe) segment), sub-thalamic nucleus (STN), and substantia nigra pars compacta (SNc) and pars reticulata (SNr). Traditionally, the basal ganglia have been studied in the face of Parkinson's or Huntington's disease with regards to their symptomatic nature

characterized by motor dysfunctions. However, more recently, a broader function emerged comprising decision-making and associative learning (Peak et al., 2018).
The dorsal striatum comprises mainly (95%) of GABAergic spiny projection neurons (SPNs) (Matamales et al., 2009). Of those, excitatory dopamine D1 receptor-expressing neurons project to the SNr and the GPi, which is termed the direct pathway. In contrast, the inhibitory dopamine D2 receptor expressing neurons project to indirectly to SNr and GPi via GPe and STN and are thus termed the indirect pathway (Matamales et al., 2009; Gerfen et al., 1990). The remaining 5% of neurons are made up by tonically active cholinergic interneurons, which are thought to modulate SPN activity, fast-spiking GABAergic interneurons, which target both types of SPNS but preferentially those of the direct pathway, and lastly low-threashold spinking GABAergic interneurons, which are thought to play a role in LTP (Peak et al., 2018; Calabresi et al., 1999; Silberberg and Bolam, 2015; Tepper and Bolam, 2004; Gittis et al., 2010). Despite the low numbers of striatal interneurons, approapriate behavioural output related to basal ganglia function is thought to be greatly affected by interneuron modification of SPN activity (Peak et al., 2018). In the appendix of this thesis, I will discuss preliminary experiments regarding manipulation of cholinergic interneurons within DS with regards to behavioural phenotypes discussed in the face of PPC manipulations. Overall, the striatum, within its immediate network of the basal ganglia is thought to represent more than a feed-forward system conveying cortical, thalamic as well as limbic input to the two main output pathways. Rather, the striatum takes on a role in integrating multiple signals into a more defined output (Peak at al., 2018)

The tail of striatum and threat-related behaviours

The tail of the striatum (TS) represents a sub region of the striatum that I will also discuss with regards to PPC function in the last part of this thesis. The TS is a relatively small area but it might have a distinct role because of its unique connectivity. TS, unlike the dorsomedial striatum, receives projections from visual, somatosensory, auditory and gustatory cortex as well as from the thalamus (Jiang and Kim, 2018). Further dopamine and serotonin neurons from the lateral part of the SNc and dorsal raphe nucleus project to TS, which contrasts the dopamine projections originating from a separate group of neurons from the SNc that target DMS. Interestingly, the basolateral amygdala (BLA) selectively innervates the TS (Jiang and Kim, 2018).

It is a well-established notion that many dopamine neurons are excited by unpredicted rewards or reward-predicting stimuli, while they are inhibited by adversity or omission of reward-prediction (Schultz et al., 1997; Cohen et al., 2012; Roitman et al., 2008; Matsumoto et al., 2016). Artificially activating these neurons was furthermore found to mimic reward whereas transient inactivation can mimic negative outcomes (Tsai et al., 2009; Chang et al. 2016). Importantly, dopamine projections to the TS were found to be activated by aversive and neutral stimuli (Menegas et al., 2017). Furthermore, it was found that dopamine responses to novel stimuli were found in TS dopamine axons and that these responses were not tuned to value-related dopamine signals in the ventral striatum (VS) (Menegas et al., 2017). When examining dopamine axon activity, it was found that TS neurons responded to tones and air puffs, but not to water delivery, whereas the activity in VS was corresponding to reward size (Menegas et al., 2018). Negative stimuli such as bitter taste or omission of reward were not reflected in TS neuron activity, whereas high intensity somatosensory, auditory, visual and olfactory stimuli strongly activated these neurons. Furthermore, dopamine axon responses scaled with novelty of the stimuli and decayed with time. Thus, specifically TS dopamine neurons were found to respond specifically to novel or high intensity stimuli (Menegas et al., 2018). When the same group used optogenetic activation of dopamine axons in TS, they could show that mice, undergoing a choice-task, showed bias to avoid the choice associated with optogenetic activation. This was the opposite when VS-axons were activated. There, activation led to a reinforcement of the choice associated with the activation. Ablation of TS-projecting dopamine neurons whose cell bodies were located in lateral part of the SNc caused mice to reduce their avoidance behaviour towards high intensity stimuli (air puffs) without affecting initial responses (retreat). However, mice showed no difference in responding to bitter taste or water reduction after ablation. In order to understand the role of novelty for TS dopamine neurons, approach behaviour towards novel objects was examined. Normal object approach takes place in bouts of approach and retreat that gradually become longer as mice show intrinsic curiosity despite the awareness of potential threat, a universal concept of internal conflict described by William James as "weal or woe" (James, 1890). After ablation of TS dopamine neurons, mice initially show normal approach-retreat behaviour to the object, but quickly display prolonged bouts of exploration and overall spend more time in the proximity of the object. Conversely, when TS dopamine

neurons were optogenetically activated, bouts of exploration of novel objects were reduced (Menegas et al., 2018).

Overall, TS dopamine neurons seem to encode physical salience of external stimuli. This function is important for risk-taking behaviour as these neurons would convey information for potential threat associated with novel or high intensity external stimuli and I will discuss their involvement with PPC function in the later parts if this thesis.

Aim and rationale of the thesis

It becomes clear that the identification of the role of PPC is complicated by the wealth of data that was published to date. The more recent results point towards a role for PPC in creating recent history-dependent bias, which could be interpreted in the way that PPC shapes attention, perception and decision-making. Thus, working memory, viewed as the process using novel information actively to adapt behaviour would be inevitably dependent on PPC function. The connections PPC has with auditory, visual, sensory, associative and motor regions make it an ideal candidate for integrating multiple sources of external information, which can be used to shape behaviour. Thus the function of PPC would range from shaping attention, which in turn influences perception to impact on decision-making based on recent history, in addition to behaviour that is based on more distant memories or baseline states of the individual guided by other brain regions. Lastly, the output pathways for behaviour influenced by PPC could depend on striatal projections, particularly the dorsal as well as the tail of the striatum, potentially fulfilling different aspects of PPC function.

The aim of this thesis was to characterise the function of PPC in mice in order to better understand and to reconcile the diversity of the discussed roles proposed in the literature. I wanted to find out whether PPC function impacts memory formation, consolidation and retrieval or modification differently. The proposed roles for inferring history-dependent bias to current decisions could be viewed as the ability to use short-term memory effectively to behave adaptively in the face of new information or such that contradicts previous memories. I have used transient silencing of the whole PPC to gain insight on the role of PPC on a temporal scale from short-term to long-term memory and its role in modification of memory-dependent and -independent behaviour. Additionally, I have used transient silencing of specific projections of PPC to provide suggestions for downstream implementation of proposed PPC function thereby putting the latter into a systems-wide context.

Results

Introduction

In order to get a first insight on the role of PPC in the behavioural paradigms that we are using in the lab, I used transient activation of parvalbumin (PV)-expressing inhibitory neurons in PPC for silencing of the latter region. This was done by bilaterally injecting PSAM, an adeno-associated virus (AAV) carrying a cre-dependent excitatory ion channel (excitation: rAAV9-CAG-flox- PSAM (Leu41Phe, Tyr116Phe) 5HT3-WPRE), into PPC (Fig.7) of PV-cre mice at least 8 days prior to behavioural procedures (Magnus et al., 2011). By injecting the synthetic ligand PSEM, PV-neurons now expressing these channels could be transiently activated, which in turn led to silencing of the PPC for the duration of the behavioural expriment. In contrast, mice in the control groups were injected with 0.9% saline solution whenever PSEM was administered in the treated groups.

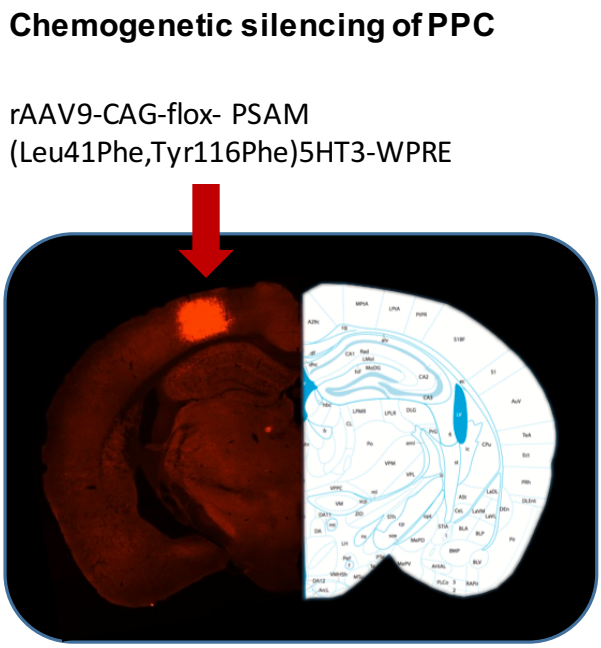


Fig.7 Chemogenetic silencing of PPC Representative image of expression of Cre-dependent AAV9 chemogenetic activator virus in PV-positive PPC neurons visualized with bungarotoxin staining. Image taken at 5x magnification. (left) Schematic representation of PPC location according to Paxinos mouse brain atlas (right)

Contextual fear conditioning

PPC is required during extinction but not for long-term retention of extinction

By examining the effects of PPC silencing at different time points within the cFC protocol, it was possible to test the influence of PPC on different components of learning and memory. From the literature, it was suggested that the role of PPC could be to influence behaviour based on recent history. Therefore, I started my experiments by silencing PPC during extinction of a previously acquired fear memory.
During the acquisition of fear memory, mice received 5 consecutive foot shocks within a conditioning chamber. After 24h, mice were injected with either PSEM or saline respectively 15mins prior to reintroduction to the conditioning chamber for fear memory recall and subsequent extinction. The first 5 minutes represent the fear memory recall, where there was no difference in the freezing behaviour between saline control mice and PPC silenced mice (Fig.8a left). However, during the course of the 30min of extinction, PPC silenced mice did not gradually decrease their freezing levels as saline controls and instead, displayed significantly higher freezing until the end of extinction (Fig.8a left). When these mice were tested for extinction memory on the following day, they showed normal retention of extinction (Fig.8a right). Thus, although mice continued to freeze during extinction itself when PPC was silenced, they still acquired and recalled this memory normally as shown by their behaviour on the day following extinction. In summary, only short-term memory recall of extinction memory was impaired as a result of PPC silencing.

I have also tested whether silencing PPC at acquisition of the fear memory affected next day memory recall. To test this, I injected mice with PSEM or saline respectively 15 minutes before cFC acquisition. In this case, there was no difference in freezing behaviour on recall after 24h (Fig.8b).

Additionally, I also silenced PPC only during retention of extinction, 24h after mice had undergone a normal extinction protocol as described before. In injected PSEM or saline respectively 15 minutes before testing retention of extinction. PPC silencing 24h after extinction did not affect the retention of extinction memory (Fig.8c).

Therefore, these first experiments showed that PPC silencing only affects behaviour during extinction online, during immediate recall of newly acquired short-term memory, but does not affect long-term extinction memory recall. Similarly, acquisition of fear memory and subsequent 24h recall is unaffected by PPC silencing.

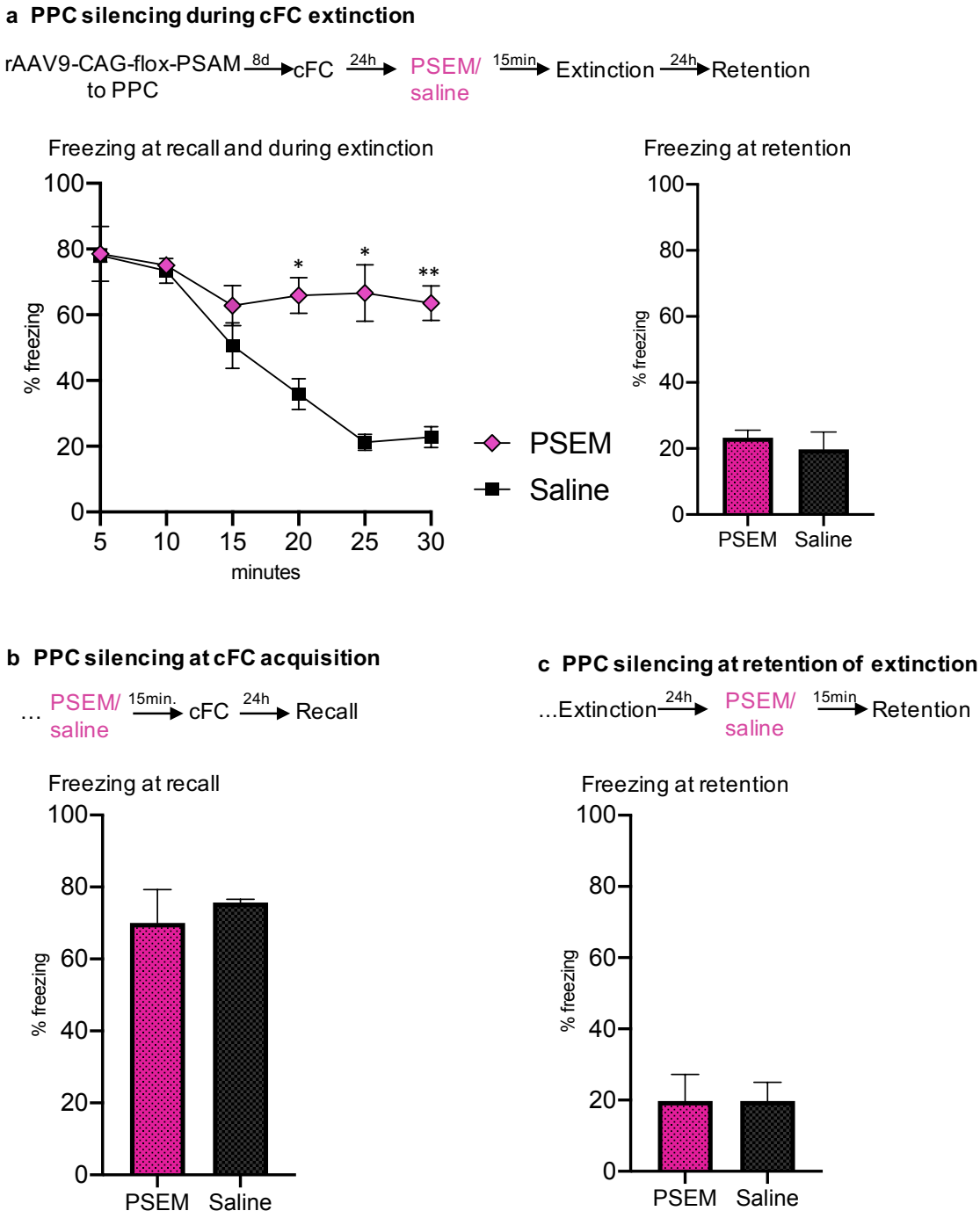


Fig.8 PPC is required for a reduction in freezing behaviour during extinction, but not for learning or recall of fear memory or extinction memory tested 24h later (a) PPC silencing prevented a normal reduction in freezing behaviour during extinction training (Two way ANOVA with Sidak's multiple comparison, interaction F (5, 45) = 8,264 P<0,0001; time F (2.483, 22.34) =19.48 P<0.0001; group F (1,9) =31.07 P=0.0003; multiple comparison PSEM vs. saline:

20mins p=0.0165; 25mins p=0.0285; 30mins p=0.0021 n=5/6) but did not affect learning of extinction tested 24h later (unpaired t-test P=0.492 n=5 **(b)** PPC silencing during acquisition did not affect 24h recall behaviour (unpaired t test P=0.5749 n=3) **(c)** PPC silencing during retention did not affect the recall of extinction memory (unpaired t test P>0.999 n=3).

In untreated animals, during extinction, previous fear memory caused freezing behaviour in anticipation of threat associated with the context. The absence of shocks was registered and newly acquired extinction memory was incorporated into behaviour across the 30min extinction period so that control mice showed low freezing behaviour towards the end of this period. PPC silencing prevented mice from changing their behaviour towards reflecting the new evidence. In other words, they failed to recall extinction memory during or immediatly after acquisition, although they were able to recall it normally during 24h retention of extinction.

PPC is required for responding normally to aversive foot shocks

I noticed that, although PPC silencing at acquisition did not affect next day recall, mice seemed more fearful on the acquisition day. Thus I decided to look more closely at the behaviour during cFC acquisition. I injected mice with either PSEM or saline 15mins prior to acquisition. In this case, control mice, which had received saline, changed gradually from an exploratory to a freezing state in response to the 5 incremental shocks appering over the course of the 5 minutes of cFC acquisition. PPC silencing during cFC acquisition caused mice to show a full freezing response already as a result of the first shock (Fig.9a left). Freezing remained high throughout the duration of acquisition after the first shock, while control mice only showed comparably high freezing levels after the fourth shock. Freezing behaviour after the fifth shock was not recorded because the acquisition paradigm ended immediately after the 5th shock. After 24h, when the fear memory was recalled, both groups of mice showed normal recall freezing (Fig.9a right). Subsequent extinction of fear memory (data not shown) took place normally and led to low freezing in both groups when retention of extinction was tested 24h later (Fig.9a right).

Recall freezing levels after 5 shocks during acquisition were generally very high (around 70-80%), therefore it was difficult to conclude whether mice in which PPC was silenced during acquisition only showed an altered response online, or whether they also valued the negative evidence more strongly in terms of the memory they formed

about it. In order to answer this, I subjected mice to a 1-shock acquisition protocol, in which mice received a single foot shock in the middle of a period of 5 min exposure to the conditioning chamber, 15 mins after injection of PSEM or saline respectively. Saline control mice did not respond with significant freezing to the appearance of the shock (Fig.9b saline). In contrast, mice in which PPC was silenced during this 1-shock acquisition, showed increased freezing immediately after the shock (Fig.9b PSEM). However, both groups did not display significant freezing upon 24h recall (Fig.9b right).

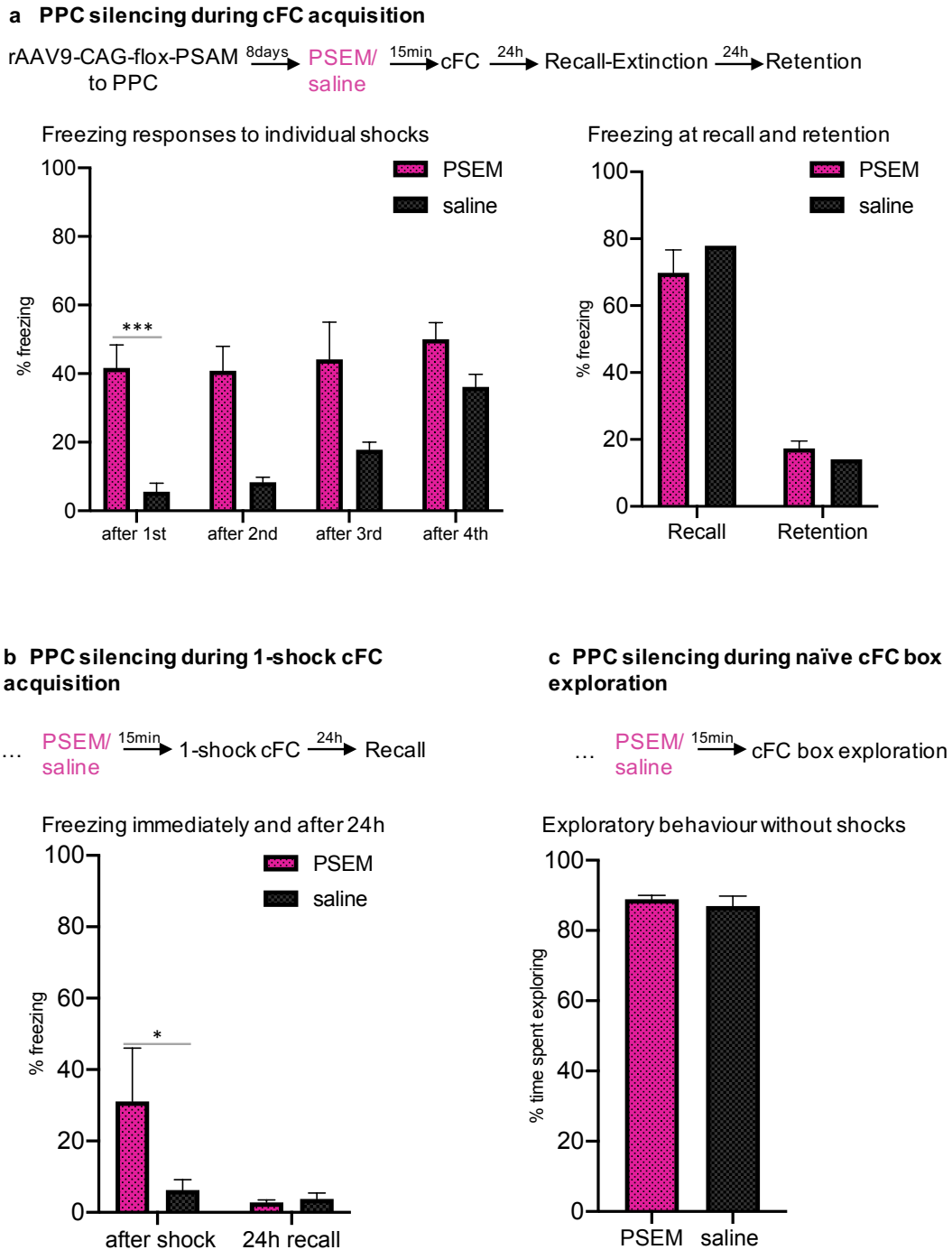


Fig.9 PPC silencing during cFC acquisition causes increased freezing response to shock(s) but leaves long term memory and its modification unaffected (a) PPC silencing increased freezing responses to consecutive shocks (Two-way ANOVA with Sidak's multiple comparison, interaction F(3,24)=4,537 P=0.0118; time F(2,215,17,72)=15,29 P<0.0001; group F(1,8)=23,19 P=0.0013 multiple comparison PSEM vs. saline: after 1st shock P=0.0320; after 2nd shock P=0.0686; after 3rd shock P=0.3153; after 4th shock P=0.2298 n=5/6) without affecting 24h recall freezing or retention after extinction (Two-way ANOVA with Sidak's multiple comparison Interaction F(1,3)=0.6622 P=0.4754; time F(1,3)=69,53 P=0.0036; group F(1,3)=0.07277 P=0.8048 multiple comparison PSEM vs. saline: recall P=0.7544; retention P=0.9547 n=4/1). **(b)** PPC silencing during 1-shock cFC acquisition increased immediate freezing response but did not affect the absence of freezing behaviour on the next day recall (Two-way ANOVA with Sidak's multiple comparison Interaction F(1,5)=4,692) P=0.0826; time F(1,5)=6,328 P=0.0535; group F(1,5)=4,252 P=0.0942 multiple comparison PSEM vs. saline: after shock P=0.0270; 24h recall P=0.9952 n=4/3) **(c)** PPC silencing during naïve cFC box exploration did not affect spontaneous exploration of cFC conditioning box (unpaired t-test P=0.2607 n=3)

In order to show that without the appearance of shocks, PPC silencing does not cause any behavioural effects, mice were exposed to the conditioning chamber and left to explore freely for 5 min after having received PSEM or saline 15min earlier. PPC silencing did not affect exploratory behaviour compared to saline controls (Fig.9c).

Immediately after a foot shock, PPC silenced mice seemed unable to respond appropriately to previously acquired memory for safety within the context, as of the 2 or 2.5mins preceeding the shock in a normal and a 1-shock acquisition respectively. Instead, mice reacted to the aversive foot shock exclusively and hence showed strong freezing. After 24h, both, the memory about safety, as well as about the single or multiple foot shocks were not PPC-dependent for their recall and thus PPC silencing had no effect.

The results so far indicate that PPC is not required for short-term memory recall *per se*, as the foot shock(s) are recalled strongly. However, it seems rather that the combined memories about the shock(s) and the time in the context during which mice experienced no aversive events were not integrated normally and short-term memory-dependent behaviour was focused on the aversive event only.

PPC is required for retention of extinction after 15mins but not after 24h

In order to test whether PPC silencing also affects short term retention of extinction after the latter has been completed, PPC was silenced right after a normal extinction protocol and mice were tested for retention. To test this, I divided mice into two groups after completed extinction and injected them with PSEM or saline respectively. 15 mins later, I tested retention of extinction. Although both groups of mice showed low freezing levels at the end of extinction (Fig.10a left), silencing PPC 15 minutes after the end of extinction reinstated freezing behaviour to levels close to normal cFC recall levels (Fig.10a middle). In saline controls, freezing behaviour remained at low levels during retention test at 15mins. Interestingly, when PPC was silenced again at long term retention of extinction 24h later, PSEM treated mice were comparable to saline controls and showed normal retention of extinction memory displayed by low freezing levels (Fig.10a right).

Fig.10 PPC silencing post extinction after 15mins and 24h post-extinction, reinstated freezing, despite normal retention after 24h (2-way ANOVA with Sidak's multiple comparison, Interaction F(2,8)=42,02 P<0,0001; time F(1,288, 5,151)=35,70 P=0,0014; group F(1,4)=26,64 P=0,0067; multiple comparison PSEM vs. saline, last 5 mins of extinction P=0.9905, PSEM recall 15 mins P=0.0470, PSEM recall 24h P=0.8374; n=3)

PPC is required for retention of extinction until after 11h but not after 18h or longer

With the aim of better understanding the time scale after extinction learning, during which retention of extinction memory is still PPC dependent, a range of time points for silencing PPC after extinction was selected. This was done the same way as described for silencing 15mins post-extinction. For every time point of post-extinction retention test, there was a separate group of mice, which was recorded for freezing at the end of extinction, at retention at the particular time point, and also at retention at 24h. For every group, after extinction, mice were divided into two groups, receiving either PSEM or saline 15min before both retention tests.

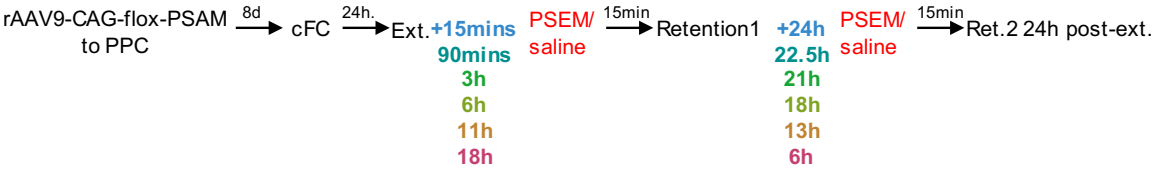
At the end of extinction, all groups of mice had extinguished well (Fig.11a). At this point, there had been no treatment carried out yet.

When the first retention was tested at 15mins, 90mins, 3h, 6h and 11h, PSEM treated mice showed a significant reinstatement of freezing behaviour, compared to those mice that had received saline prior to retention testing (Fig.11b). Freezing was lower in the group tested at 11h post-extinction, although still significantly higher than saline controls. PPC silencing at 18h post-extinction did not cause a freezing reinstatement (Fig.11b right).

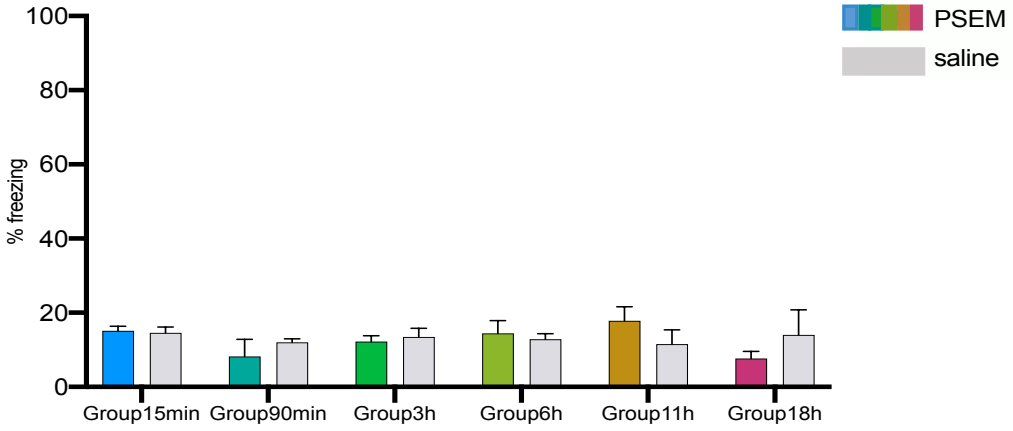
When retention 2 was tested 24h post-extinction for every group, there was no difference between the groups that had received PSEM or saline before either of the retentiont tests (Fig.11c).

These experiments show that, in addition to PPC being required during cFC extinction for immediate recall of the new extinction memory, it is also required for retention of extinction memory right after acquisition until at least 11h afterwards. However, at 18h post-acquisition, retention of extinction has become PPC independent.

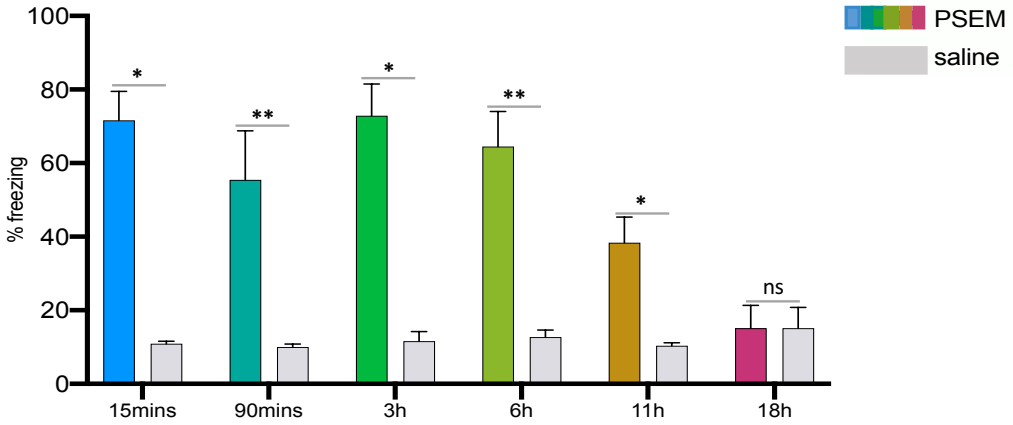
PPC silencing at different time points after completed extinction



a Freezing during the last 5 min of extinction (before any treatment)



b Freezing during retention1 at 15min, 90min, 3h, 6h, 11h and 18h post-extinction (PSEM/saline)



c Freezing during retention2 at 24h post-extinction (PSEM/saline)

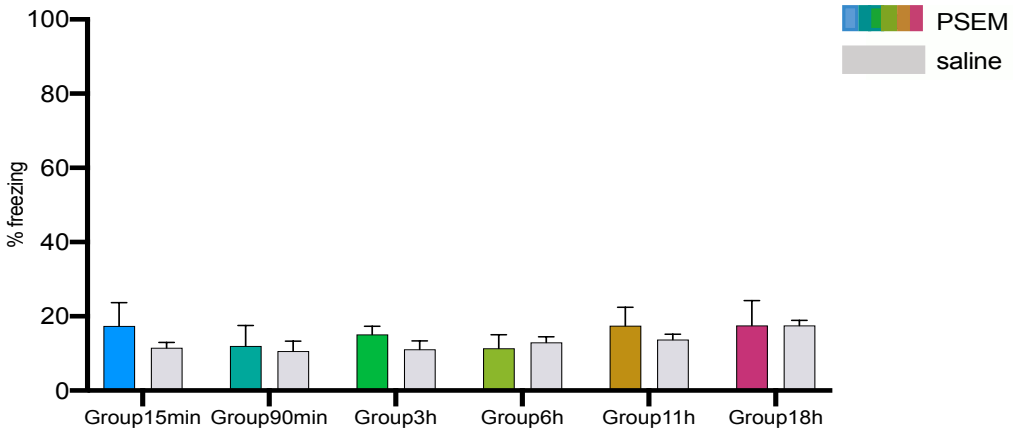


Fig.11 (a) Individual groups of mice showed normal freezing levels at the end of extinction before any treatment (mixed effects analysis with Sidaks's multiple comparison: interaction F(2,037, 8,147)=0,7591 P=0,5008; time F(1,8)=0,09919 P=0,7609; group F(5,20)=0,7327 P=0,6074) **(b) PPC silencing at different time points post-extinction** has different effects depending on the time point of comparison. Silencing reinstated freezing up until 11h after the end of extinction. After 18h, PPC silencing had no effects (mixed effects analysis with Sidak's multiple comparison: Interaction F(1,872, 11,23)=3,386 P=0,0730; time F(1,30)=72,86 P<0,0001; group F(5,30)=3,3031 P=0,0074. Multiple comparison PSEM vs. saline: @15mins P=0.0470 n=3/3; @90mins P=0.0095 n=3/2; @3h P=0.1211 n=3/2; @6h P=0.0073 n=6/4; @11h P=0.0292 n=6/4; @18h P>0.9999 n=3/3) **(c) PPC silencing 24h post-extinction** did not reinstate freezing in any of the groups (mixed effects analysis with Sidak's multiple comparison: Interaction F(5,22)=0,5598 P=0,7295; time F(1,8)=0,6569 P=0,4411; group F(5,22)=0,2358 P=0,9425)

To conclude from the cFC experiments, during acquisition of fear memory, PPC silencing led to a full freezing response already after 1 foot shock. This suggests that observed behaviour is only a response to the foot shock, rather than a combination of the latter together with memory recall of previous evidence for safety acquired within the minutes before the shock. After 24h, PPC silencing did not cause a difference between groups, because at this time point, memory-based behaviour has become PPC independent. Thus PPC silencing does not abolish short-term memory recall *per se*, rather it seems that while the shock is remembered well and acted upon, the evidence acquired for safety while there was no shock, is not taken into account for the behavioural response.

During extinction, when PPC was silenced, mice could normally acquire extinction memory but could not recall this new memory until 18h after acquisition. This was independent of whether PPC was silenced during the acquisition of the extinction memory, or afterwards, as long as it appeared within 11h of acquisition.

When PPC was silenced during 24h cFC recall, during 24h extinction retention, or during naive box exploration, there was no <18h memory recall required for normal performance, and hence there was no difference between PSEM treated and saline control mice.

Overall, the cFC experiments suggest that PPC does not influence memory acquisition but memory recall until at least 11h after acquisition. Before completed consolidation, PPC could thus play a role in assigning value to individual or multiple newly acquired memories to influence behaviour adaptively. At 18h after memory acquisition, memory recall and corresponding memory-based behaviour is no longer dependent on PPC.

Familiar object recognition (FOR) task

In order to investigate whether the effects of PPC silencing only appear in relation to aversive events, PPC silencing was induced in the familiar object recognition (FOR) task.

The FOR task utilizes the innate curiosity that mice exhibit towards novel objects in their environment. In the task employed, mice were exposed to two identical objects within a rectangular box for a period of 10 minutes on day 1. On the following day, mice were exposed to one of the objects from day 1 and to a novel object. Normally, mice spent more time exploring the novel object because they remembered the familiar one from day 1. Day 2 can be utilized not only to test recall of day 1 object memory, but also to test the acquisition of memory about the novel object if the latter is tested together with an object which is novel on day 3. This modified version of the FOR task was used initially to test the effects of PPC silencing on object discrimination on day 2, hence testing day 1 recall and day 2 memory acquisition for day 3. I did this because I wanted to test whether mice are able to recall previous memory and to acquire new memory during PPC silencing.

PPC is required for normal exploration of objects

PPC silencing on day 2 of a 3-day FOR task

PPC silencing was first carried out on day 2 of the 3-day version of the FOR task (Fig.12). I analyzed behavioural parameters such as object exploration, context exploration and repetitive behaviour, which represents mostly immobile behaviour and grooming. Additionally, I quantified a discrimination index that describes the preference for either the novel (positive values) or the familar (negaitve values) object as shown in Fig.12d left).

On the first day of the task, where there was no treatment induced, both groups of mice showed comparable behaviour regarding all parameters measured (Fig.11 left of each diagram). On day 2, 15mins prior to testing, PSEM or saline was injected to the treated and the control group respectively. Silencing PPC on day 2 of this task reduced object and context exploration and increased repetitive behaviour (Fig.12a-c middle of each

diagram). Furthermore, mice showed no discrimination for any of the objects as shown by the discrimination index being close to zero (Fig.12d middle of diagram). On day 3, when PPC was no longer silenced, mice behaved comparable to controls regarding all parameters (Fig.11 right of each diagram). Strikingly, mice also showed normal discrimination for the novel object on day 3 (Fig.11d right of diagram), which demonstrates that mice, despite their lack of physical exploration, formed a good memory about the objects they were presented with on day 2.

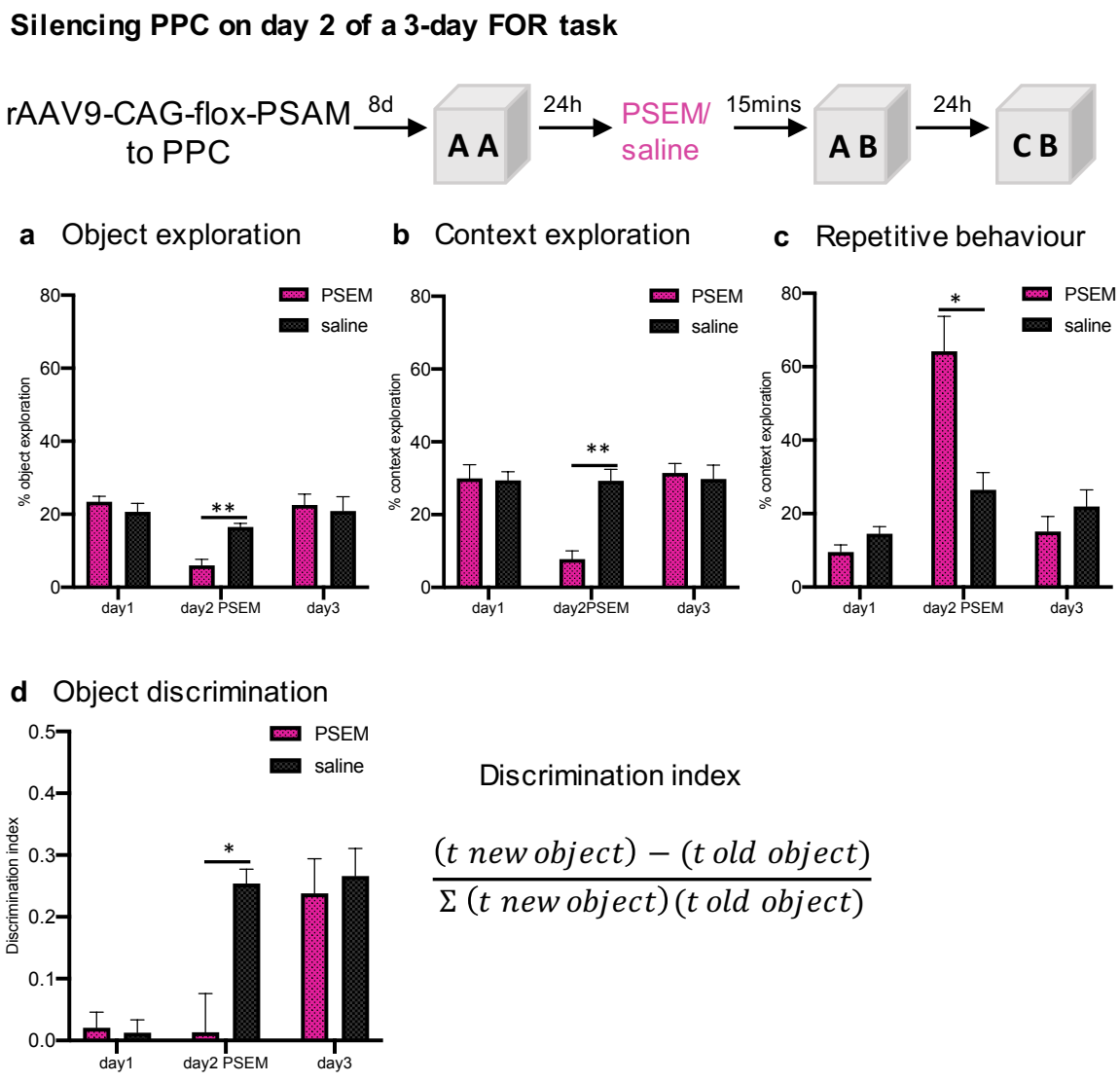


Fig.12 Silencing PPC on day 2 of a 3-day FOR task PPC silencing **(a)** reduced object exploration (2-way ANOVA with Sidak's multiple comparisons test: Interaction F(2,20)=8,197 P=0,0025; time F(1,418, 14,18)=22,86 P=0,0001; group F(1,10)=0,5438 P=0,4778; multiple comparison PSEM vs. saline, day1 P=0.7145, day2PSEM P=0.0018, day3 P=0.9839 n=6) **(b)** reduced context exploration (2-way ANOVA with Sidak's multiple comparison test: Interaction F(2,20)=12,23 P=0,0003; time F(1,742, 17,42)=12,95 P=0,0005; group F(1,10)=4,300

P=0,0649; multiple comparison PSEM vs. saline, day 1 P=0.9991, day2PSEM P=0.0011, day3 P=0.8902 n=6). **(c)** but increased repetitive behaviour (2-way ANOVA with Sidak's multiple comparison test: Interaction F(2,20)=16,63 P<0,0001; time F(1,348, 13,48)=32,74 P<0,0001; group F(1,10)=2,754 P=0,1280; multiple comparison PSEM vs. saline, day 1 P=0.2824, day2PSEM P=0.0259, day3 P=0.6431 n=6). **(d)** abolished object discrimination on the day of silencing but not thereafter (2-way ANOVA with Sidak's multiple comparison test: Interaction F(2,20)=6,596 P=0,0063; time F(1,949, 19,49)=20,27 P<0,0001; group F(1,10)=4,345 P=0,0637; multiple comparison PSEM vs. saline day1 P=0.9934, day2PSEM P=0.0305, day3 P=0.9743 n=6).

This experiment showed that PPC silencing affected familiar and novel object as well as context exploration, and instead caused mice to remain largely immobile. Importantly to keep in mind, PPC did not affect context exploration in the absence of an object as was described in the previous section on fear conditioning experiments. Thus, the role of the context during PPC silencing will also be addressed later in this chapter.

Normally, when mice approached an object, they were cautious initially, and explored the object in short bouts of exploration. These became longer, as mice acquired memory about the object being safe. When PPC was silenced, a few exploratory bouts, did not lead to increased object interaction, although there was no danger found in relation to the object.

In other words, mice were unable to recall the recently acquired memory about the object and hence failed to adjust their behaviour appropriately. However, after 24h, mice were able to recall memory about object identity from the previous day normally.

PPC silencing on day 1 of a 2-day FOR task

Next, I wanted to test whether PPC silencing caused the same phenotype when it was done on day 1 instead of day 2 in order to make sure that the effect was not dependent on pre-exposure on day 1. To test this, I used a shortened FOR version only consisting of day 1 and 2. I injected either PSEM or saline 15 mins prior to testing on day 1 to treated and control groups respectively. Silencing PPC caused a similar phenotype of reduced object and context exploration, concomitant with an increase in repetitive behaviour (Fig.13a-c). Similarly, object discrimination on the day following silencing remained unaffected (Fig.13d).

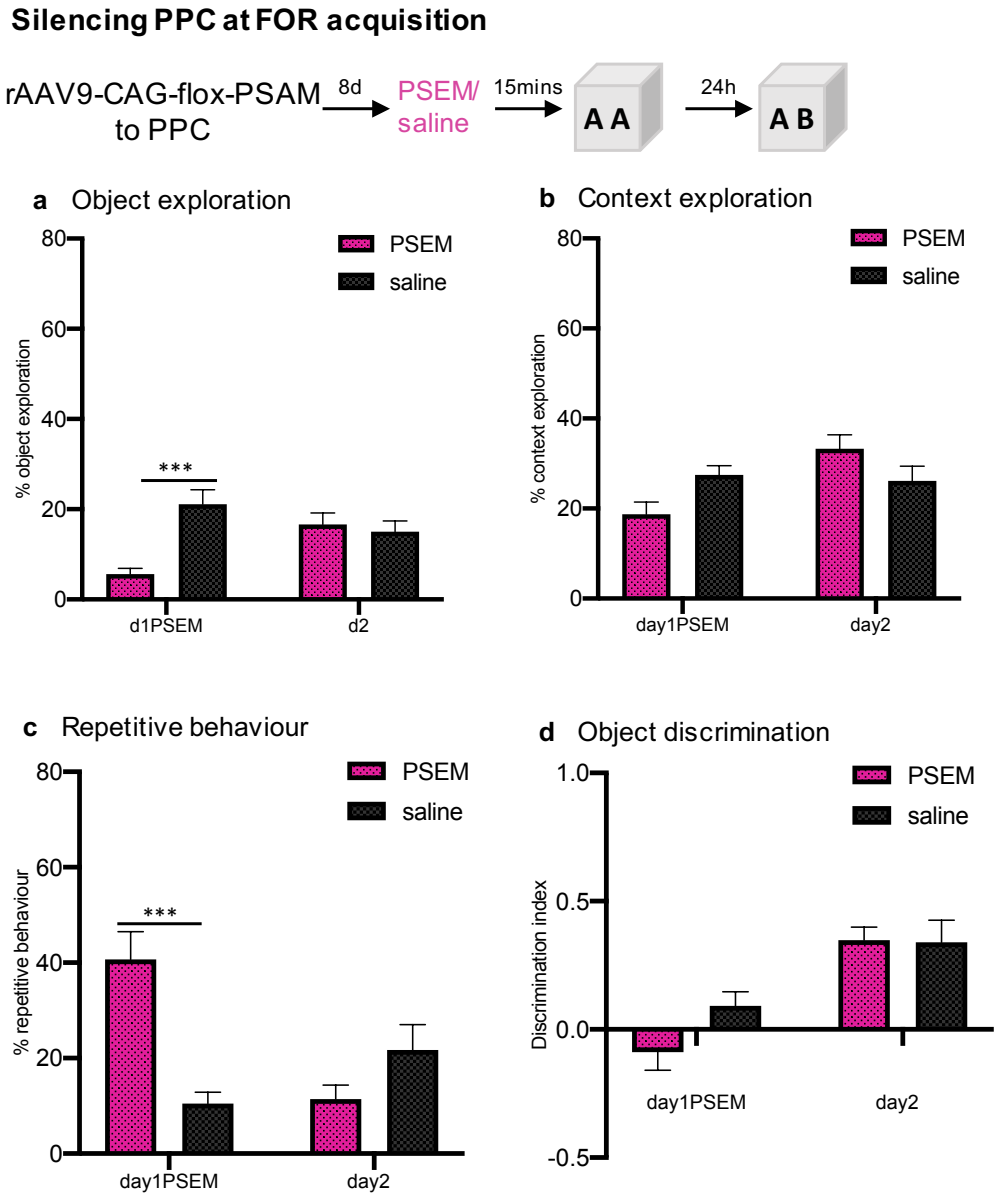


Fig. 13 Silencing PPC at FOR acquisition PPC silencing **(a)** reduced object exploration (2-way ANOVA with Sidak's multiple comparisons test: Interaction F(1,8)=53,09 P<0,0001; time F(1,8)=4,284 P=0,0723; group F(1,8)=4,516 P=0,0663; multiple comparison PSEM vs. saline, day1PSEM P=0.0008, day2 P=0.8790 n=5) **(b)** reduced context exploration (2-way ANOVA

with Sidak's multiple comparison test: : Interaction F(1,8)=7,804 P=0,0234; time F(1,8)=5,435 P=0,0481; group F(1,8)=0,07906 P=0,7857; multiple comparison PSEM vs. saline, day 1PSEM P=0.0865, day2 P=0.1768 n=5). **(c)** but increased repetitive behaviour (2-way ANOVA with Sidak's multiple comparison test: Interaction F(1,8)=38,41 P=0,0003; time F(1,8)=7,574 P=0,0250; group F(1,8)=3,559 P=0,0959; multiple comparison PSEM vs. saline l, day1PSEM P=0.0003, day2 P=0.2167 n=5). **(d)** did not affect day2 object discrimination (2-way ANOVA with Sidak's multiple comparison test: Interaction F(1,8)=2,476 P=0,1542; time F(1,8)=32,49 P=0,0005; group F(1,8)=1,347 P=0,2792; multiple comparison PSEM vs. saline day1PSEM P=0.1471, day2 P=0.9946 n=5).

Because mice acquired normal memory about the objects despite lack of exploration on the day of silencing, I decided to do a control experiment, in which the two objects were only distinguishable by odour but looked identical. Assumingly, silenced mice would be unable to form a memory about the objects unless they approached them. As expected, mice which had received PSEM prior to day 1 displayed lower exploratory behaviour, concomitantly preventing exposure to the two different odours that the objects were carrying (Fig.14a). In contrast, saline controls showed a significantly higher object exploration (Fig.14a). On the following day, one odour remained with one of the objects, while the other object was now carrying a novel odour. Control mice spent more time exploring the object carrying the novel odour (Fig.14b), whereas mice in which PPC had been silenced previously, showed no discrimination for the object carrying the novel odour (Fig.14b), despite normal exploratory behaviour (Fig.14a).

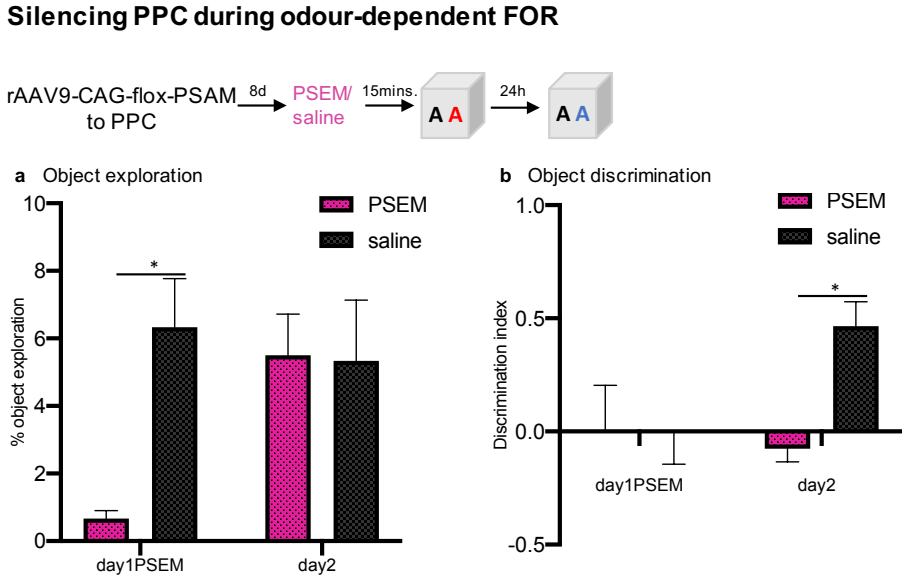


Fig. 14 Silencing PPC during odour-dependent FOR (a) decreased object exploration on the day of silencing (2-way ANOVA with Sidak's multiple comparison: Interaction F(1,6)=4,115 P=0,0888; time F(1,6)=1,777 P=0,2309; group F(1,6)=5,585 P=0,0560; multiple comparison PSEM vs. saline: day1PSEM P=0.0196; day2 P=0.9951 n=4) **(b)** abolished discrimination for the novel odour-carrying object on day 2 (2-way ANOVA with Sidak's multiple comparison;

Interaction F(1,6)=4,232 P=0,0854; time F(1,6)=2,198 P=0,1887; group F(1,6)=3,458 P=0,1123; multiple comparison PSEM vs. saline day1PSEM P>0.9999; day2 P=0.0342 n=4).

This experiment showed that, although PPC silencing does not change long term memory formation as shown by the other experiments before, PPC silencing affects memory formation if it changes a behaviour, which is required for acquiring the same information as control animals. Hence, PPC silencing affected memory acquisition indirectly through its impact on short-term memory recall which was required for exploratory behaviour in the presence of objects.

The role of PPC on behaviour is context-independent

PPC silencing affected exploratory behaviour in the presence of objects and mice displayed excessive caution that prevented them from exploring the objects and also the context normally. I was curious to test whether doing the 2-day FOR in the home cage would change the effects of PPC silencing under the assumption that mice would feel safer in this context. I injected mice with PSEM or saline respectively, 15 mins prior to day 1 exposure. On day 2, there was no treatment done in either group. Silencing PPC on day 1 of the 2-day FOR caused a significant decrease in object and context exploration and an increase in repetitive behaviour compared to saline controls (Fig.15a-c). Object discrimination on the day following silencing was unaffected (Fig.15d).

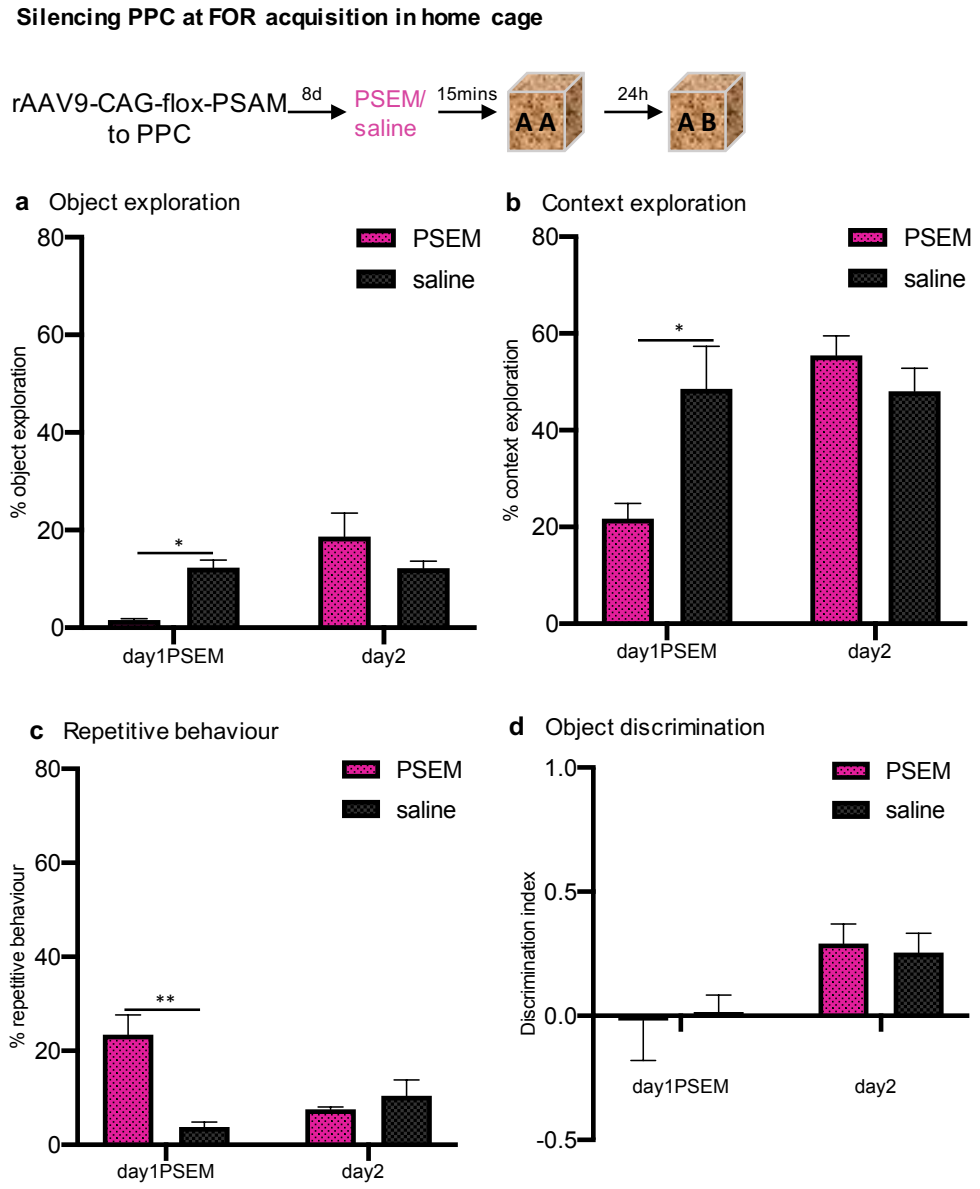


Fig.15 Silencing PPC at FOR acquisition in the home cage PPC silencing **(a)** reduced object exploration (2-way ANOVA with Sidak's multiple comparisons test: Interaction F(1,4)=9,156 P=0,0389; time F(1,4)=8,921 P=0,0405; group F(1,4)=0,8195 P=0,4165; multiple comparison PSEM vs. saline, day1PSEM P=0.0395, day2 P=0.2279 n=3) **(b)** reduced context exploration (2-way ANOVA with Sidak's multiple comparison test: Interaction F(1,4)=25,33 P=0,0073; time F(1,4)=23,29 P=0,0081; group F(1,4)=1,827 P=0,2478; multiple comparison PSEM vs. saline, day 1PSEM P=0.0194, day2 P=0.6171 n=3). **(c)** but increased repetitive behaviour (2-way ANOVA with Sidak's multiple comparison test: Interaction F(1,4)=25,38 P=0,0073; time F(1,4)=4,242 P=0,1085; group F(1,4)=6,952 P=0,0578; multiple comparison PSEM vs. saline, day1PSEM P=0.0020, day2 P=0.7285 n=3). **(d)** did not affect day2 object discrimination (2-way ANOVA with Sidak's multiple comparison test: Interaction F(1,4)=0,1638 P=0,7064; time F(1,4)=10,04 P=0,0339; group F(1,4)=8,056e-005 P= 9933; multiple comparison PSEM vs. saline day1PSEM P=0.9685, day2 P=0.9645 n=3).

Thus, changing the context from a neutral to a familiar one did not affect the effects of PPC silencing on behaviour.

So far we found that empty context exploration was not affected by PPC silencing (Fig.9c) and that FOR done in the home cage did not change the effects of silencing PPC (Fig.15). Thus it seems that the context itself does not play a role for PPC function. As a control, I wanted to test whether PPC silencing had any effects in contexts that are not uniform, which means that mice can chose between parts of the context that are different.

First, I tested mice in the elevated plus maze, a test frequently employed in order to test anxiety and anxiolytic compounds measured in relation to exploratory behaviour of the mice. The test consists of an elevated cross of which two opposing arms have walls, whereas the other two arms have no walls. Typically, mice spend more time in the closed arms but not exclusively. I wanted to test whether silencing PPC in this task led to an increased amount of time that mice spend in the closed arm. To test this, I injected mice with PSEM or saline 15mins before being placed into the maze for 5 minutes. PPC silencing did not affect the behaviour of the mice compared to saline controls as measured by the times mice spent in each type of arm (Fig.16a). Hence, PPC silencing does not induce anxious behaviour.

Another test investigating the role of context included a large box, which was divided by a transparent wall with a small opening through which mice could freely move from one side to the other. One of the sides was filled with home cage bedding, whereas the other side was completely empty. I injected mice with PSEM or saline 15mins before being placed into the empty side of the context. Mice were left to freely explore

the context for 5 mins. PPC silencing did not affect the time mice spent in each of the sides of the context compared to saline control mice (Fig.16b).

Hence, PPC function influences object and context-related exploratory behaviour differently and the latter is only affected in the presence of an object or as a response to external threat.

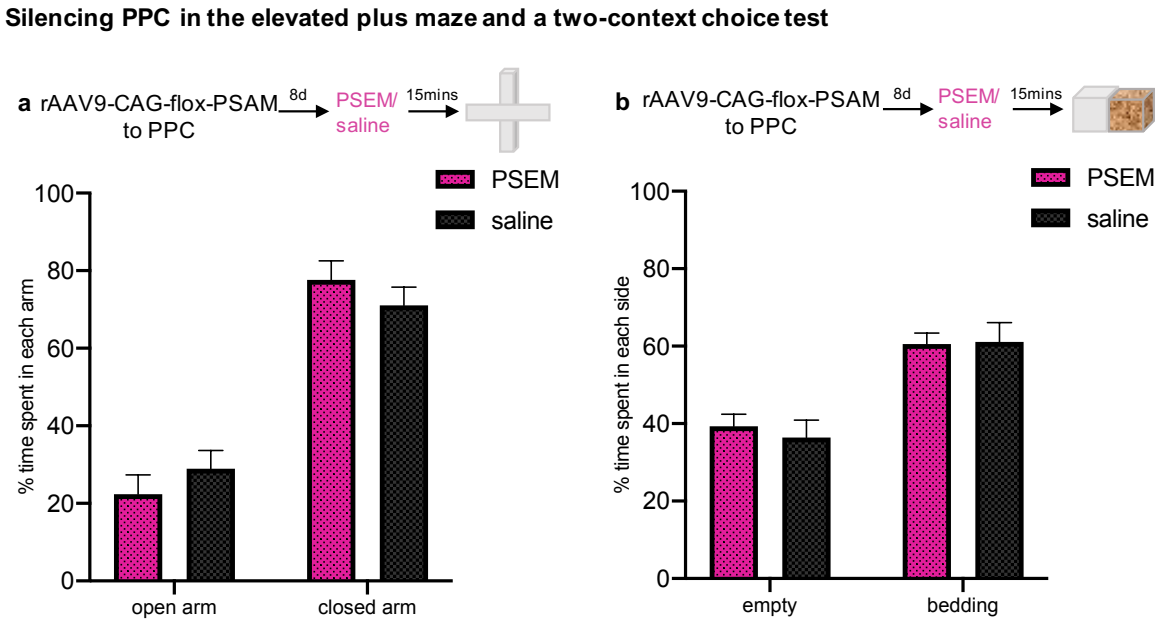


Fig.16 (a) Silencing PPC in the elevated plus maze did not affect behaviour compared to control mice (2-way ANOVA with Sidak's multiple comparison: Interaction F(1,9)=0,8965; P=0,3684); time F(1,9)=49,60 P<0,0001; group F(1,9)=0,000 P<0,9999; multiple comparison PSEM vs. saline: open arm P=0.5856; closed arm P=0.5856, n=6/4) **(b) Silencing PPC in a two context choice test** did not affect behaviour compared to controls (2-way ANOVA with Sidak's multiple comparison : Interaction F(1,5)=0,07972; P=0,7890; time F(1,5)=14,15 P=0,0131; group F(1,5)=0,1743 P=1743; multiple comparison PSEM vs. saline: empty context P=0.8742, bedding context P=0,9955, n=3/4).

I decided to do another control experiment considering the finding that context seemed to play a role only in relation to object presence. I wanted to test whether mice would avoid one of two contexts if one contained an object whereas the other one would be empty.

To test this, I used the same box as for the two-context choice task, however, without any bedding material present. One side was empty, whereas the other side contained an object, and mice were free to spend time on either of the sides for a duration of 5 minutes. I injected mice with PSEM or saline 15mins prior to being placed into the side

containing the object. Silencing PPC caused mice to spend more time in the context, which did not contain the object, whereas saline control mice spent more time in the context with the object (Fig.17a). Concomitantly, silenced mice also explored the object much less compared to saline controls (Fig.18c).

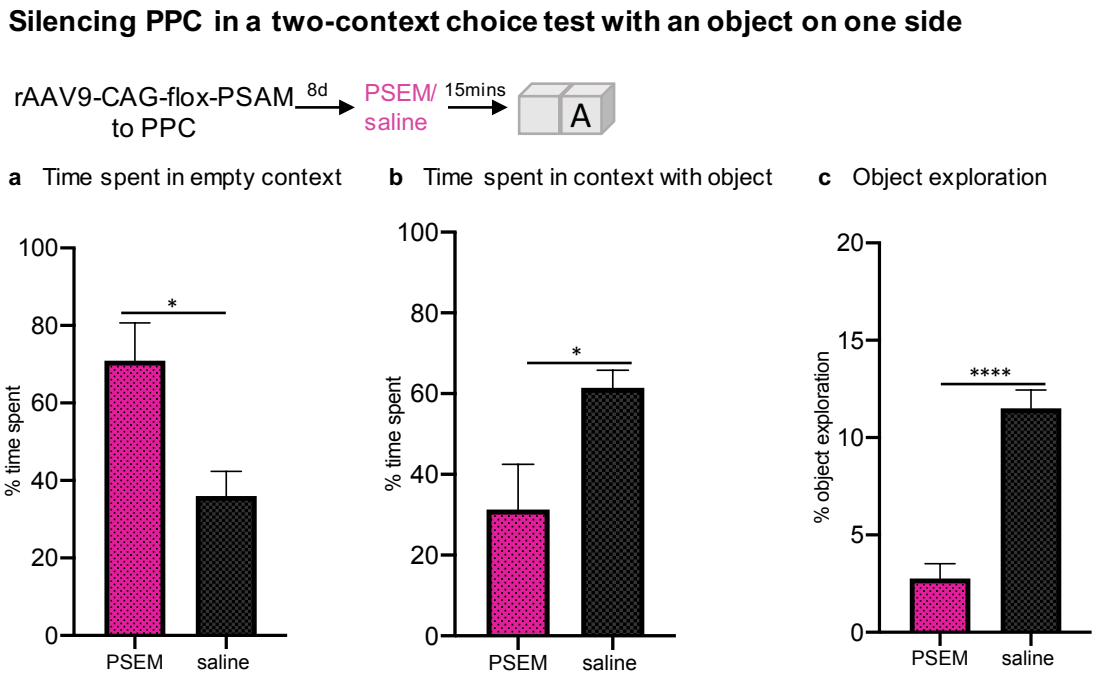


Fig.17 Silencing PPC in a two-context choice test with an object on one side (a) increased the time spent in the empty context (unpaired t-test P=0,0145 n=6) **(b)** decreased the time spent in the context containing the object (unpaired t-test P=0,0388 n=6) **(c)** reduced object exploration (unpaired t-test P<0,0001 n=6)

To conclude from the experiments discussed so far, PPC function is required for normal behaviour in the presence of objects, independent of contextual features of the environment. The behavioural effect of PPC silencing on object exploration seems to result from the inability to recall newly acquired information about object safety thus preventing a progressive object interaction with exposure time.

Social behaviour is independent of PPC function

With the purpose of understanding whether PPC silencing affects social behaviour, I replaced 1 of the 2 objects on FOR day 1 with a social intruder mouse, which was placed under a small cage at the position where the second object would have been. I divided mice into two groups receiving PSEM and saline respectively 15 min prior to exposure to the context with an object and the intruder mouse. PPC silencing did not affect social interaction compared to saline controls (Fig.18a), while replicating the phenotype regarding object and context exploration as well as regarding repetitive behaviour (Fig.18b-d).

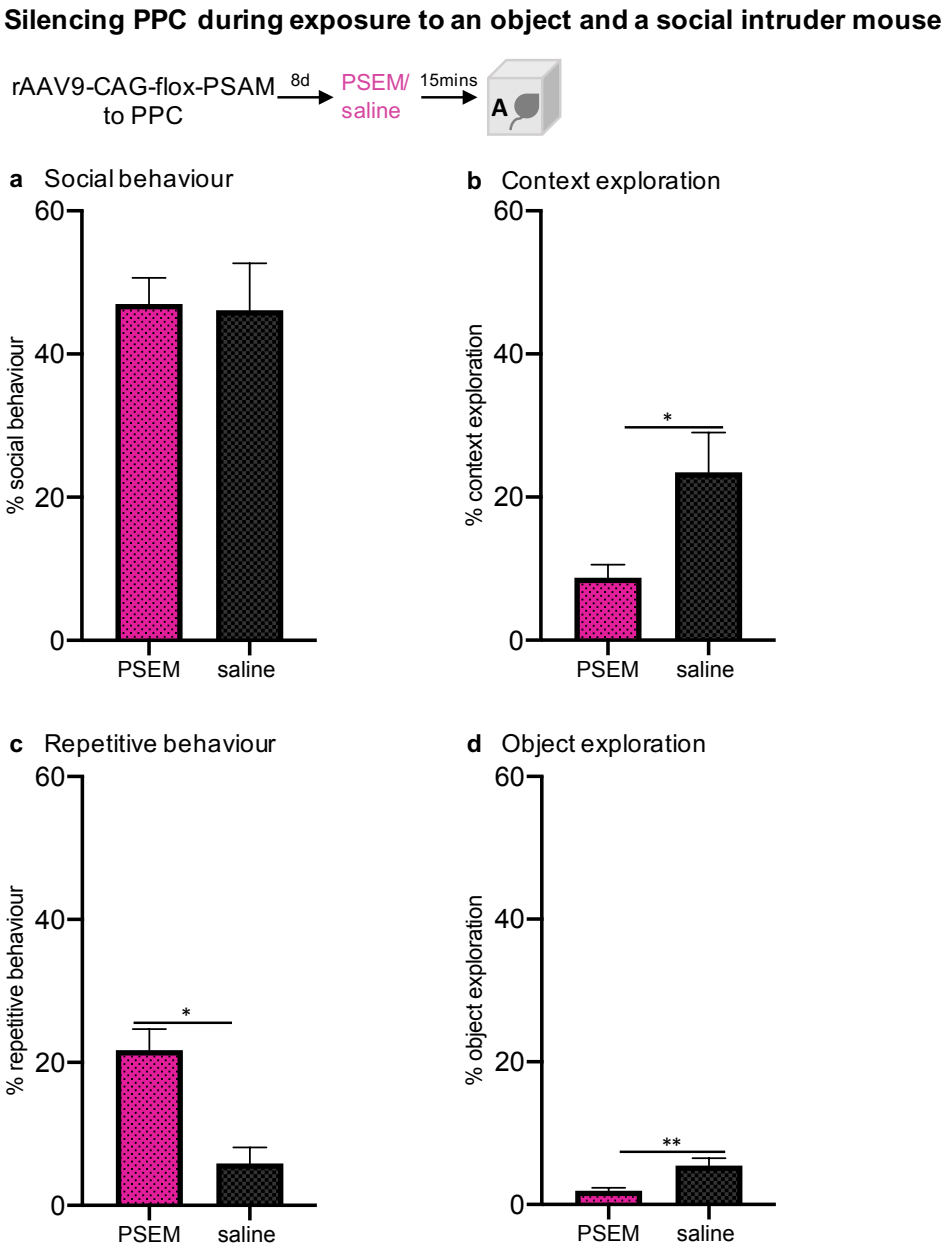


Fig.18 Silencing PPC during exposure to object and social intruder mouse in a neutral context (a) did not affect social interaction time with the intruder mouse (unpaired t-test P=0.9003 n=6/3) **(b)** decreased context exploration (unpaired t-test P=0.0144 n=6/3) **(c)** increased repetitive behaviour (unpaired t-test P=0.0102 n=6/3) **(d)** decreased object exploration (unpaired t-test P=0.0068, n=6/3).

To conclude from this experiment, PPC silencing only affects context and object exploratory behaviour in the presence of an object, but does not affect social interaction. If short-term memory recall in the presence of a potentially threatening object is required for normal exploratory behaviour, it could mean that for social interaction to take place no potential threat needs to be overcome and thus short term memory recall is not crucial.

PPC does not affect behaviour in the presence of a familiarized object

Mice avoid unknown objects during PPC silencing, even if they have seen them before for a duration of 10 minutes (Fig.12, 3-day FOR). However, I was curious to test whether a longer familiarization to the object in the home cage could affect the effects of PPC silencing in a neutral context. After 3 days, mice know the object well and might not consider it potentially threatening anymore. To test this, I placed an object into the home cage for 3 days, just before exposing mice to the same object in a neutral context after injection of PSEM or saline respectively. Indeed, when PPC was silenced during exposure to the familiarized object in a neutral context, mice showed behaviour that was no different from saline controls (Fig.19). I measured object exploration (Fig.19a), context exploration (Fig.19b) and repetitive behaviour (Fig. 19c).

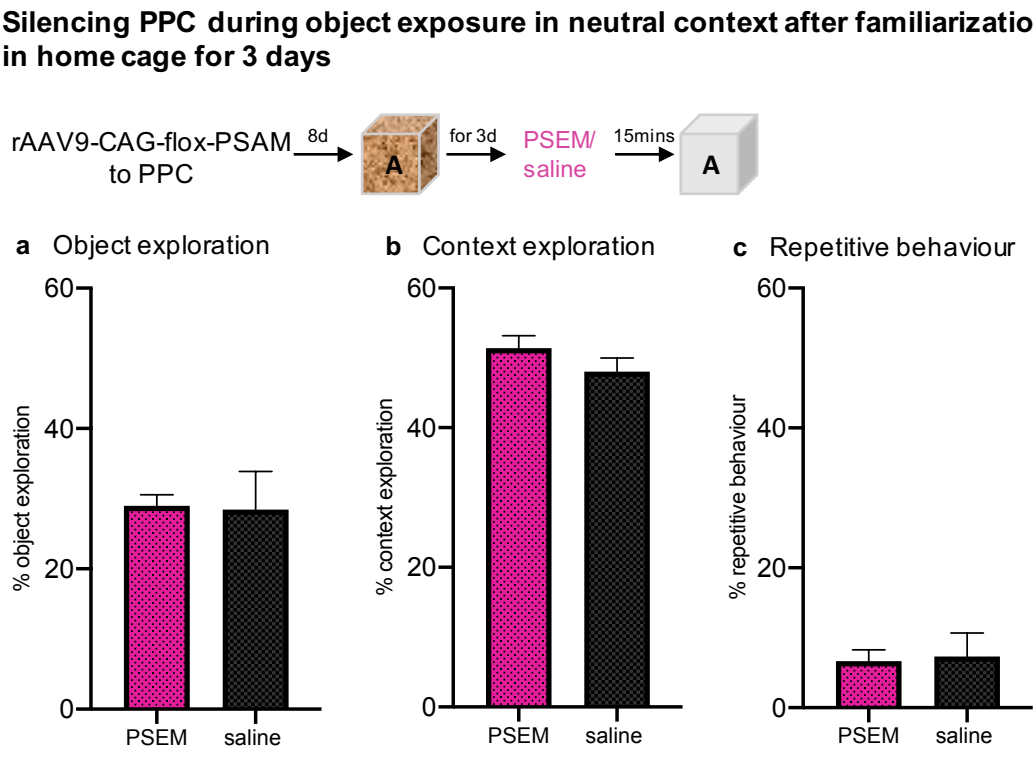


Fig.19 Silencing PPC at object exposure in neutral context after 3-day home cage familiarization to the object PPC silencing **(a)** did not affect object exploration (unpaired t-test P=0.8993 n=6/3) **(b)** did not affect context exploration (unpaired t-test P=0.2863 n=6/3) **(c)** did not affect repetitive behaviour (unpaired t-test P=0.8420 n=6/3)

Next I wanted to find out for how long an object needed to be present in order to abolish the effects of PPC silencing. I tested the effects of familiarization for 24h, 6h and 3h right before testing object exposure in a neutral context.

I followed the same procedures as described for the 3-day familiarization, varying only the familiarization time in the home cage. 15 mins before testing, I removed the mice from the home cage and injected PSEM or saline respectively before testing exposure to the home cage object in a neutral context. PPC silencing after 24h object exposure did not result in a behaviour that was distinguishable from saline controls regarding context and object exploration, as well as repetitive behaviour (Fig.20a-c). The same held true for silencing PPC after 6h of object exposure where silencing did not affect object and context exploration, nor repetitive behaviour significantly (Fig.20d-f).

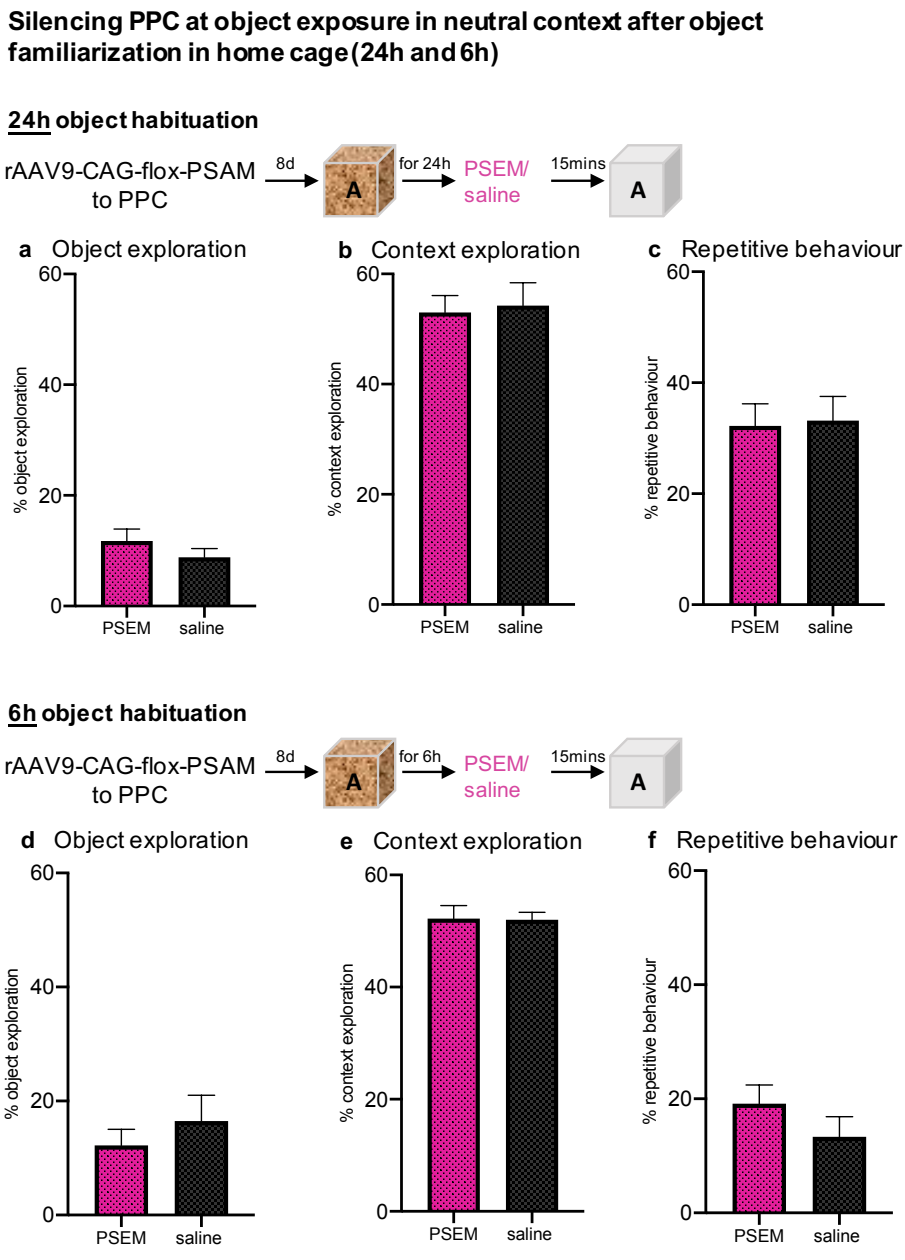


Fig.20 (a-c) Silencing PPC at object exposure in neutral context after 24h home cage familiarization to the object PPC silencing **(a)** did not affect object exploration (unpaired t-test P=0.3064 n=3/5) **(b)** did not affect context exploration (unpaired t-test P=0.8309 n=3/5) **(c)** did not affect repetitive behaviour (unpaired t-test P=0.8839 n=3/5) **(d-f) Silencing PPC at object exposure in neutral context after 6h home cage familiarization to the object** PPC silencing **(d)** did not affect object exploration (unpaired t-test P=0.4399 n=7/3) **(e)** did not affect context exploration (unpaired t-test P=0.9503 n=7/3) **(f)** did not affect repetitive behaviour (unpaired t-test P=0.3282 n=7/3)

However, PPC silencing after 3h of object exposure, led to decreased object and context exploration, as well as increased repetitive behaviour (Fig.21a-c). This phenotype resembled closely the one that was observed when PPC was silenced during naive exposure to an object in a neutral context (Fig.21d-f).

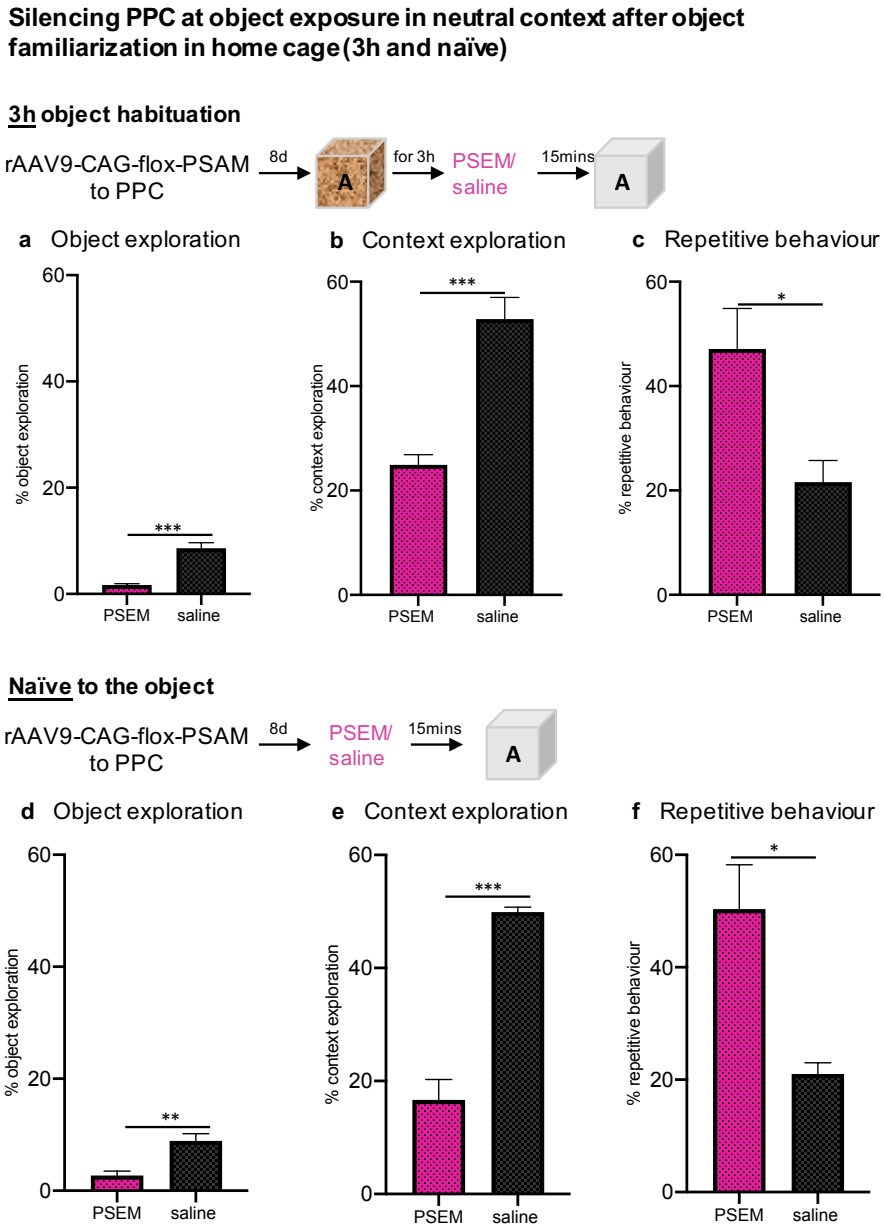


Fig.21 (a-c) Silencing PPC at object exposure in neutral context after 3h home cage familiarization to the object PPC silencing **(a)** reduced object exploration (unpaired t-test P=0.0007 n=4/6) **(b)** reduced context exploration (unpaired t-test P=0.0009 n=4/6) **(c)** increases repetitive behaviour (unpaired t-test P=0.0137 n=4/6) **(d-f) Silencing PPC at naïve object exposure** PPC silencing **(d)** reduced object exploration (unpaired t-test P=0.0025 n=7/3) **(e)** reduced context exploration (unpaired t-test P=0.0004 n=7/3) **(f)** increased repetitive behaviour (unpaired t-test P=0.0475 n=7/3)

To conclude from this paragraph, home cage object exposure from a duration of 6h onwards rescues the effects of PPC silencing during exposure to the object in a neutral context. This result suggests that the effects of PPC silencing during exposure to novel objects is indeed due to the inability of mice to acquire memory about these objects, i.e. their safety.

Object familiarization affects not only behaviour towards the same object but also permits general object exposure during PPC silencing

Having found that object familiarization rescued behaviour towards the same object under PPC silencing conditions, I was curious to test whether object familiarization leads to a general ability to explore objects without PPC function, regardless of whether the familiarized object is present or not. To test this, I injected mice with PSEM or saline respectively 15min prior to exposure to a novel object after 24h familiarization to another one (Fig.22a-c). Familiarization was carried out over 24h across the following experiments for practical reasons. PPC silencing did not affect object or context exploration, nor repetitive behaviour compared to saline controls. Hence, the previous object familiarization in the home cage was sufficient to abolish the effects of silencing PPC during novel object exposure.

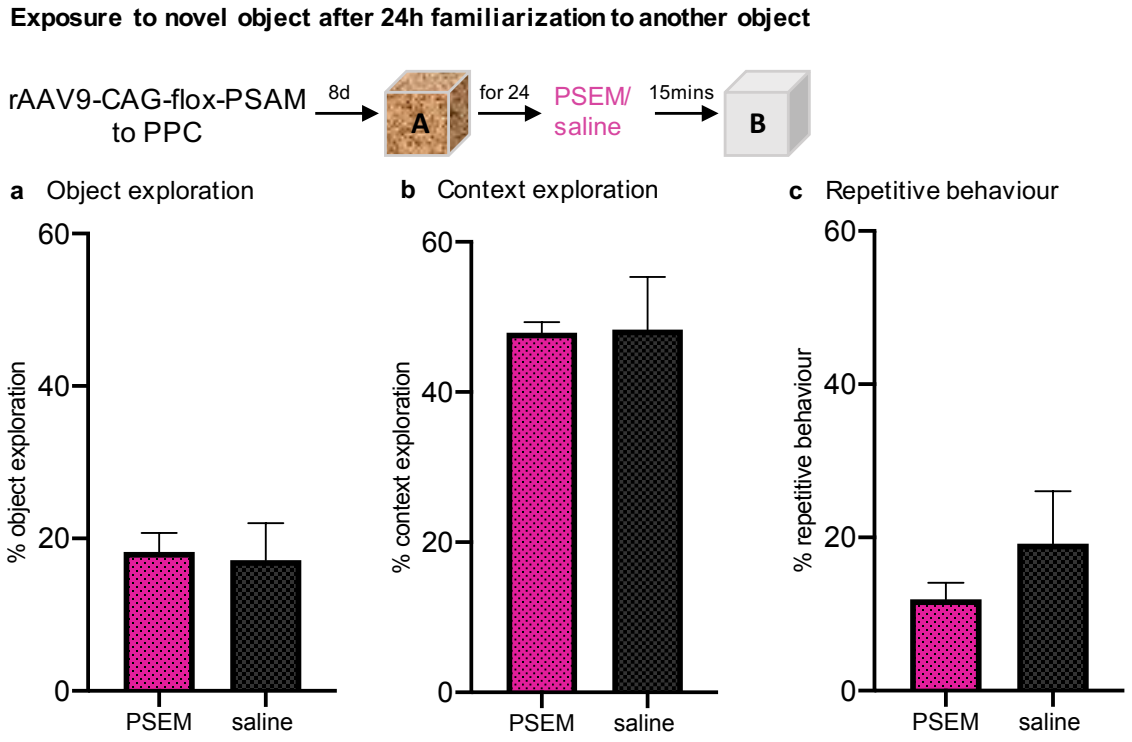


Fig.22 Silencing PPC during exposure to a novel object after 24h familiarization to another object PPC silencing **(a)** did not affect object exploration (unpaired T-test P=0.8306 n=4/2) **(b)** did not affect context exploration (unpaired t-test P=0.9347 n=4/2) **(c)** did not affect repetitive behaviour (unpaired t-test P=0.2430 n=4/2)

I was curious to test how mice would behave if they were exposed to two objects, one identical in appearance to the familiarized one, together with a new one during the test. After 24h familiarization, mice were injected with PSEM or saline respectively and exposed to the two objects in a neutral context (Fig.23a-c). PPC silencing did not have any effect on behavioural parameters measured. Mice discriminated well for the novel object and showed comparable object and context exploration times, as well as no difference in repetitive behaviour. This result is important as it shows that mice are able to use short-term memory about object features acquired in order to reach a good discrimination index during silencing. Thus, the phenotype resulting from PPC silencing without familiarization possibly arises through a failure to ascribe value to short-term memories rather than from an inability to recall short-term memory *per se.*

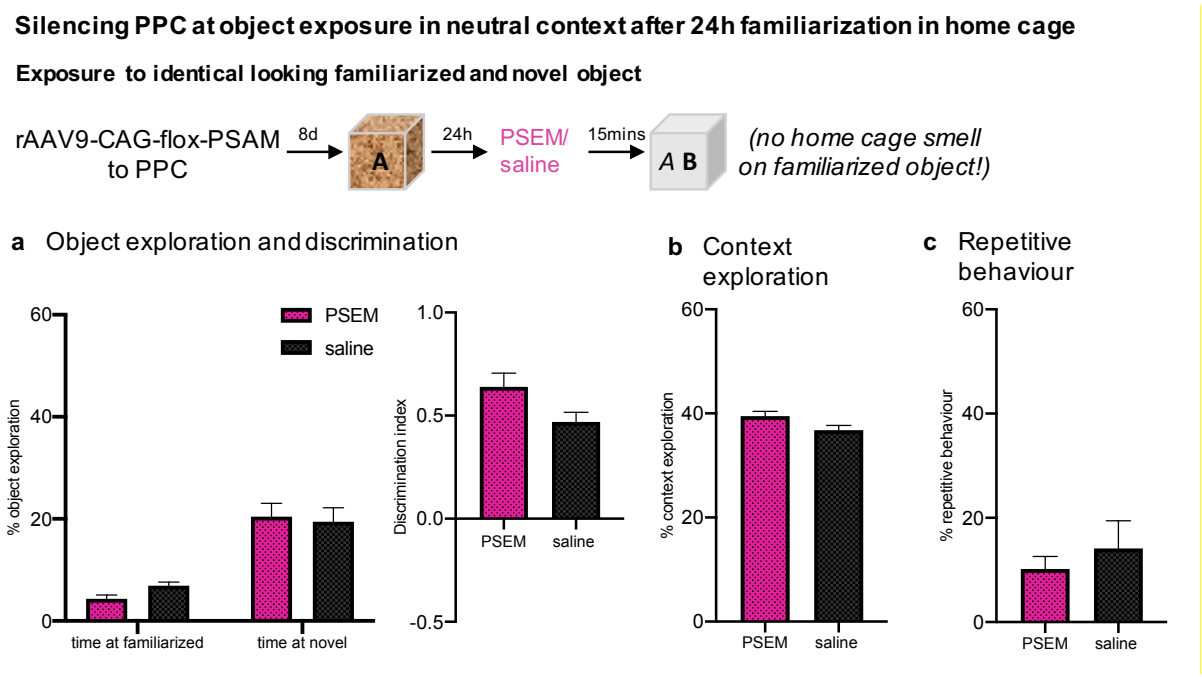


Fig.23 Silencing during exposure to identical looking familiarized and novel object after 24h exposure PPC silencing **(a)** did not affect object exploration (2-way ANOVA with Sidak's multiple comparison PSEM vs. saline: time at other identical P=0.6230; time at new P=0.9310 n=4/3) Object discrimination was not affected (unpaired T-test P=0.1099 n=4/3 **(b)** did not affect context exploration (unpaired t-test P=0.0935 n=4/3) **(c)** did not affect repetitive behaviour (unpaired t-test P=0.4912 n=4/3)

Additionally, I wanted to test wheter the familiarization effect on object exposure would be long-lasting after object removal from the home cage following the 24h familiarization and testing in a neutral context at a later time. To test this, mice were subjected to 24h object exposure in their home cage, followed by a gap of 5 days while there was no object present (Fig.24a-c). PSEM or saline respectively was injected 15min prior to neutral context exposure containing a novel object. PPC silencing during novel object exposure after this delay, caused mice to show the phenotype which was characterized by decreased object and context exposure, along with increased repetitive behaviour. Thus, the effect of home cage familiarization to an object only transiently affected the ability of mice to explore other objects and the surrounding contexts normally despite PPC silencing.

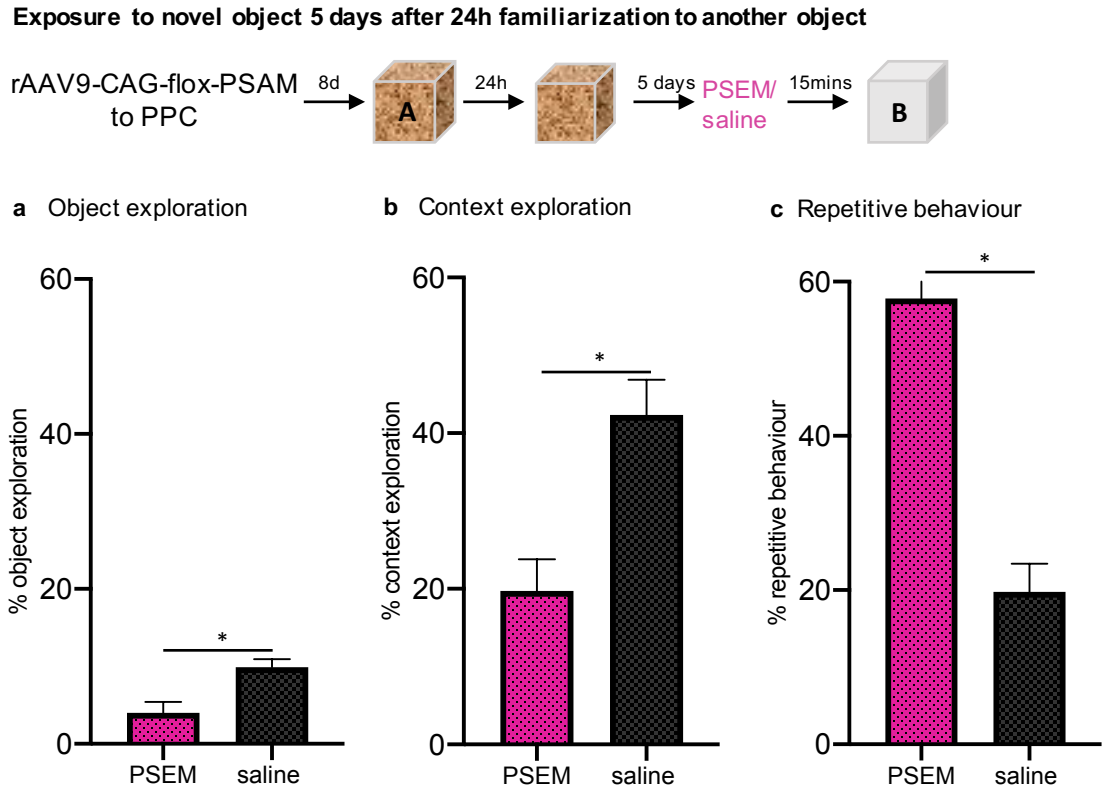


Fig.24 Silencing during exposure to new object 5 days after 24h exposure to another object PPC silencing **(a)** decreased object exploration (Unpaired T-test P=0.0256 n=4/3) **(b)** decreased context exploration (unpaired t-test P=0.0141 n=4/3) **(c)** increased repetitive behaviour (unpaired t-test P=0.0199 n=4/3)

In summary, the experiments in this last paragraph showed that PPC function affects behaviour in the presence of objects, but not in relation to contextual features of the environment or social behaviour. Object familiarization of 6h or longer permits normal

exploratory behaviour towards objects in general. This effect however, is only temorary and mice return to require PPC function for object exploration 5 days after familiarization has finished.

Excessive freezing in 1-shock cFC can be shifted towards object interaction through familiarization

Considering that mice did not express excess caution towards novel objects after immediate familiarization of 6h or longer, I wanted to investigate the behaviour of mice towards known objects under threatening conditions. To find out, whether a sudden threat would make them resort back to excess caution during PPC silencing. Further, to test whether excessive freezing is a general response to an aversive foot shock under PPC silencing conditions, or whether this could be shifted to any other behaviour.

In order to test this, I pre-exposed all mice to an object in their home cage for 24h. Mice were then divided into those that received PSEM or saline respectively 15 min prior to 1-shock cFC acquisition in the presence of the object from the home cage (Fig.25). I measured object and context exploration as well as repetitive behaviour in the period before and after the shock for the PSEM and saline groups. The shock appeared at 2.5min whereas the whole duration of the protocol was 5min.

PPC silencing did not affect the behaviour of the mice prior to the appearance of the shock (Fig.25a-c). However, once the shock appeared, mice in which PPC was silenced, turned to the object more frequently and spent more time at and even on it (Fig.25a). Freezing behaviour and context exploration remained unchanged (Fig.25b,c).
We know from previous experiments, that without the presence of an object, the occurrence of a single foot shock made PPC silenced mice react with excessive freezing. In this case, because there was an object that mice regarded as familiar and safe, the excessive freezing seemed replaced by an increased object interaction. Thus, although objects naturally evoke more caution than contexts, that is likely due to their intrinsic potential threat, this balance could be altered by familiarizing mice with the object prior to subjecting mice to aversive stimuli within a previously neutral context. Thus, excessive freezing is not a general response to threat resulting from PPC silencing. Rather, if available, PPC silencing biases mice to behaviour that is dependent on previously acquired memories that can be acted upon independent of PPC silencing.

Silencing PPC during a 1-shock cFC acquisition after 24h object familiarization in home cage

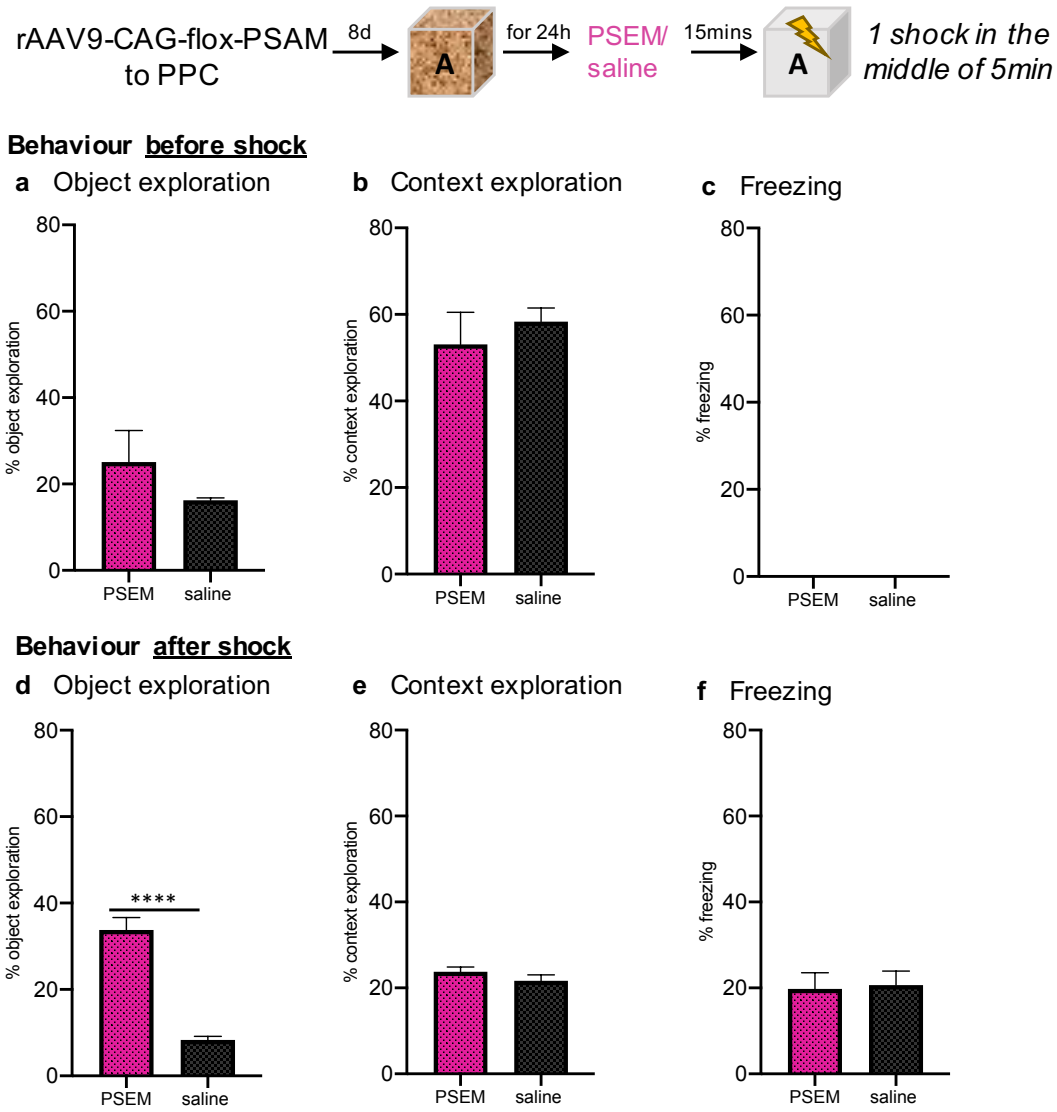


Fig.25 Silencing PPC during a 1-shock cFC acquisition after 24h object familiarization in home cage. Before appearance of the shock (a) object exploration was unaffected (unpaired t-test p=0.1089 n=3/6), **(b)** context exploration was unaffected (unpaired t-test p=0.4604 n=3/6) and **(c)** freezing behaviour (no freezing at all) was unaffected. **After appearance of the shock (d)** object exploration was increased (unpaired t-test p<0.0001 n=3/6) **(e)** context exploration was unaffected (unpaired t-test p=0.3597 n=3/6) **(f)** freezing behaviour was unaffected (unpaired t-test p=0.8736 n=3/6).

In order to show that the increased object interaction time during PPC silencing is not normally a result of the experience of a foot shock in the presence of an object, mice were subjected to a 1-shock cFC acquisition in the presence of the same, but without prior exposure as a control experiment. Mice were injected with either PSEM or saline 15 mins prior to being placed into the conditioning chamber for a 1 shock cFC acquisition in the presence of the same type of object as used in the previous experiment. Again, behaviour in the 2.5min before and after the appearance of the shock was analysed.

Before the appearance of the shock, PPC silencing caused a reduction in object and context exploration compared to saline controls but did not causes any freezing (Fig.26a-c). This was expected as prior to the shock this experiment essentially represents an FOR setting with one object present.

After the appearance of the shock, PPC silenced mice reduced their object exploration even further, whereas saline control mice only mildly reduced their object exploration as a result of the shock (Fig.26d). Context exploration was already lower compared to saline controls before the shock. After the shock, both treated and control mice reduced their context interaction further (Fig.26e). Saline control mice did not show any considerable freezing after they received the foot shock, whereas PPC silenced mice responded with increased freezing to the shock (Fig.26f).

Strikingly, in the previous experiment, freezing behaviour was elevated also in the control group after the appearance of the shock when mice had been previously familiarized with the object, an effect that could be attributed to mice expecting safety in the presence of the object and hence respond more to unexpected threat. In contrast, saline control mice in the group without prior object exposure, do not respond with freezing to the foot shock, hence the behaviour is very similar to the one observed after a 1-shock protocol without object presence.

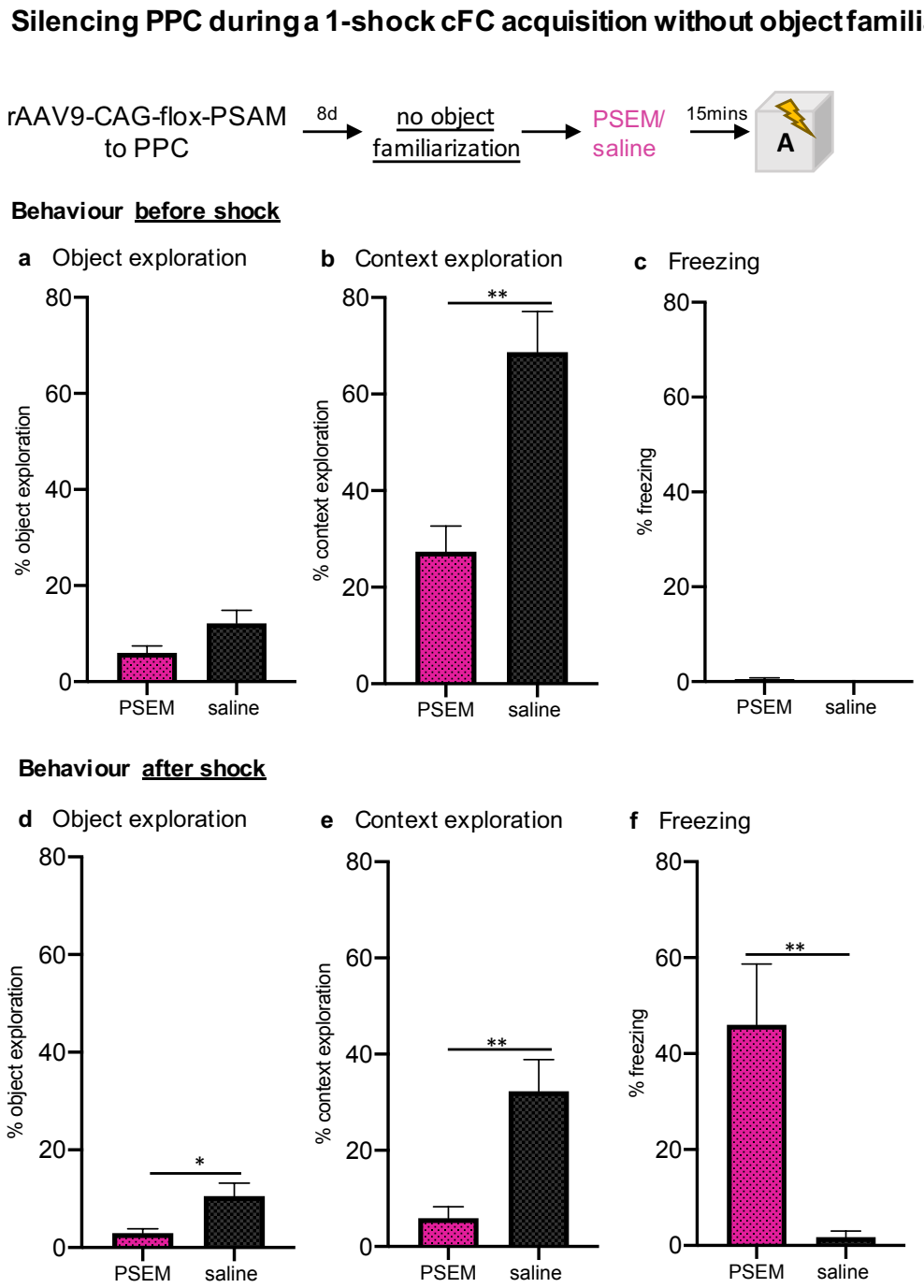


Fig.26 Silencing PPC during a 1-shock cFC acquisition without prior object exposure **Before shock (a)** object exploration was reduced (unpaired t-test P=0.0810 n=5/5), **(b)** context exploration was reduced (unpaired t-test P=0.0032 n=5) and **(c)** freezing behaviour (no freezing) was unaffected (unpaired t-test P=0,3466). **After shock (d)** object exploration was reduced (unpaired t-test P=0,0267 n=5) **(e)** context exploration was reduced (unpaired t-test p=0.0056 n=5) **(f)** freezing behaviour was increased (unpaired t-test p=0.0083 n=5).

To conclude, PPC function is required to behave normally towards novel objects, but not towards objects that mice have been familiarized with. In cFC, PPC silencing causes mice to react with excessive freezing towards external threat immediately after occurrence of the shock(s), but not after 24h.

When cFC occurs in the presence of a familiarized object, behaviour is altered due to the long-term memory about object safety. Thus reactions to an aversive foot shock under PPC silencing can be influenced by available long-term memories.

Excessive freezing to the foot shock under PPC silencing may thus be due to the lack of appropriate use of short-term memory about safety experienced despite the shock(s) and instead, behaviour is a result of instinct only as there is no relevant long-term memory available.

The Morris water maze

The Morris water maze (MWM) is an incremental learning task, which can be used to assess spatial learning and memory in rodents (Morris, 1984; D'Hooge and De Deyn 2001). The MWM requires mice to learn how to navigate to a hidden platform in opaque water within a circular pool. Mice achieve this by finally establishing a spatial map from visual cues, which are placed around the pool. There are different behavioural phases that characterise learning of this task eventually leading to spatial navigation, each of which will be discussed in detail later in this chapter.

The type of navigation used in this task is termed allocentric navigation, meaning that mice use the environment to create a spatial map, which serves as a guide for movement behaviour with the purpose of finding a particular location within this map, in this case the hidden platform. While performing the task, mice need to use their previous long-term memory together with current feedback in order to solve the task and to learn further. Given that PPC function seems crucial to recall and behaviourally act upon new short-term memories, I was curious to see whether PPC silencing affected performance in this task.

Analysis of MWM learning in WT mice

Mice received 4 trials of training per day, with changing entry points to the pool so that they were required to learn how to use the environmental cues for navigation (Fig.28a). Training was carried out daily over a period of 8 days. If mice did not find the platform within 60 seconds of a trial, they were guided to the platform location and taken out the water from there. Learning was measured by latency to platform (average seconds of 4 trials for each mouse) (Fig.28a) and swimming strategy (Fig.28b,c). By having two measures for learning, we gain insight on how successful the mouse is in escaping the pool (latencies) but also how the mouse attempts to achieve this (strategies). Escape latencies decreased gradually across training days as shown in Fig.28a.

Swimming strategy development in the MWM

Swimming strategies were previously described in detail (Garthe, Behr and Kempermann; 2009). Usually, one strategy was assigned per trial through manual rating based on watching the recorded training videos. However, there were cases, in which mice switched from one strategy to another within one trial. If those strategies

were displayed for approximately equal amounts of time, two strategies were assigned for one trial.

Initially, when being placed into the water for the first time, mice were swimming along the wall of the pool frequently, possibly in an attempt to find an escape. This strategy appeared in the first trials in many mice and is called 'thigmotaxis' (Fig.28b,c). Fig.28b shows examples for each strategy. 'Thigmotaxis' was proposed to be a first reaction to a new and stressful situation (Garthe, Behr and Kempermann, 2009).

Thigmotaxis was quickly replaced by 'random swim', a strategy characterised by mice covering the entire pool, with no recognizable pattern, often characterized by multiple bumps into the pool wall. As training continues, random swim was increasingly replaced by 'scanning', a strategy appearing similar to 'random swim' but within a more confined area distant from the pool wall. It is thought that at this point during learning the task, mice scan the environment for landmarks in order to establish a spatial map that can be used for navigation (Garthe, Behr and Kempermann, 2009). Both 'random swim' and 'scanning' are lacking spatial components.

'Chaining' is the first strategy that is considered to exhibit some spatial component, that is awareness of the platform position to be at a particular distance from the wall. Thus mice swim in circles along this particular distance from the wall. 'Chaining' appeared highest between days 3-5 of training.

Spatial strategies evolve with further training and mice show directional preference through the use of distant visual landmarks. 'Directed search' is the first of three spatial strategies that appeared considerably from day 3 and kept increasing until the end of training. Here, mice swim with a clearly recognizable directional aim for the hidden platform.

'Focal search' is more advanced as a spatial strategy and mice spend most of the search in close proximity of the hidden platform. 'Focal search' appeared gradually and was highest on the last day of training.

The most proficient spatial strategy is termed 'direct swim', characterised by mice navigating directly to the platform regardless of starting position. As the other spatial strategies, 'direct swim' appeared gradually and was highest on the last day of training.

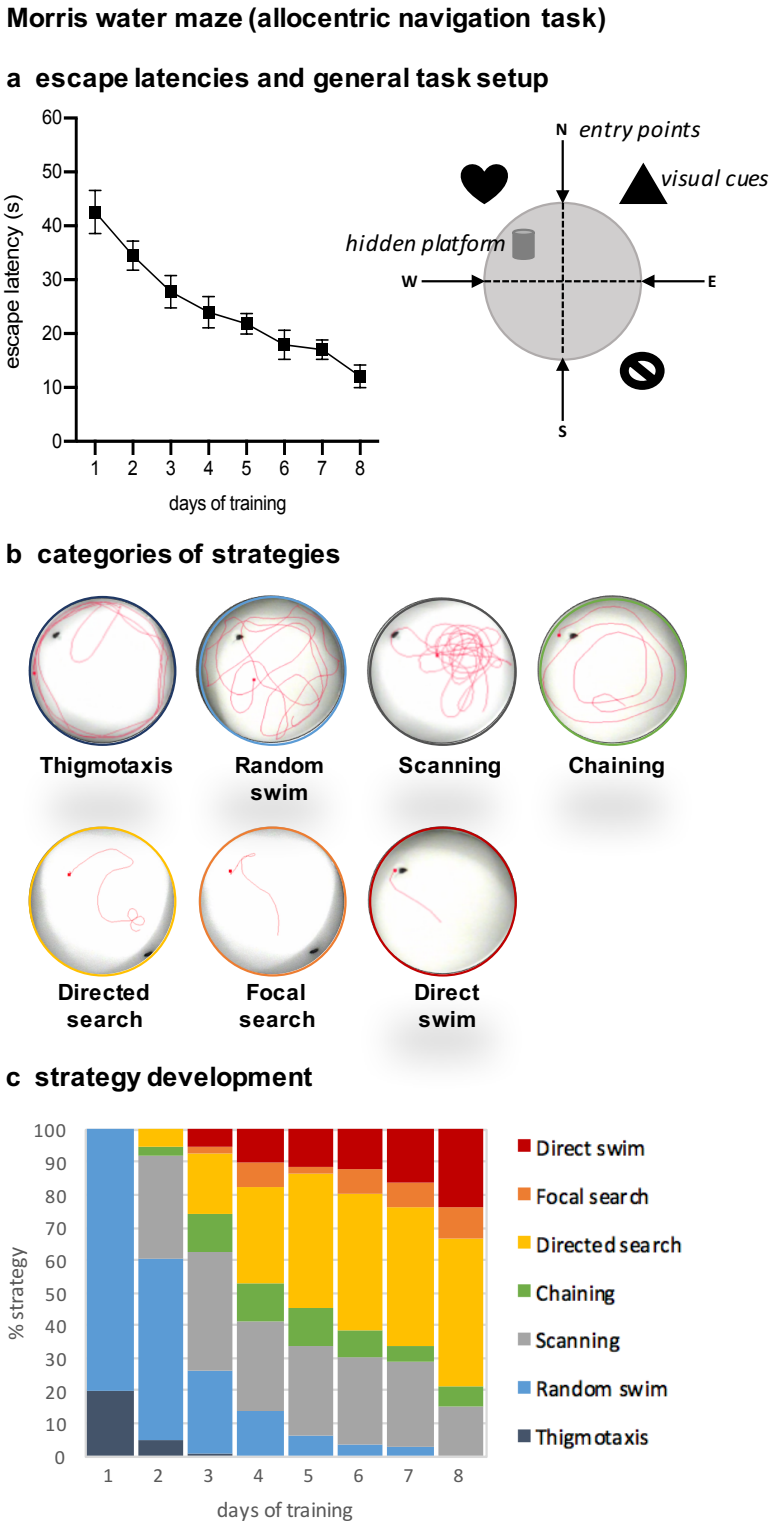


Fig.28 Morris water maze (a) Escape latencies across training days for WT mice and schematic representation of task design with a circular pool surrounded by three visual cues. Pool entry points varied across each trial and the order of entries was changed across days. **(b)** Representative images of strategy categories **(c)** Development of strategy use across training days in WT mice.

PPC silencing from day 4 on impairs recall of previous and further learning

I have found in the previous experimental paradigms, that PPC function seems important for short-term but not for long-term memory recall. The MWM, as an incremental learning task, requires mice to use long-term memory from previous training days together with short-term memory in order to improve across training days (long-term memory) and also across individual training sessions within one day (short-term and long-term memory). Additionally, when performing the MWM using long-term memory regarding the hidden platform location within the spatial map and previously used stratategies, it is important for mice to also respond appropriately to currently acquired short-term memory as they swim within the maze. Therefore, even performance based on long-term memory in general could potentially be affected by impairments regarding the use of short-term memory recall.

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I decided to silence PPC from day 4 owards because at this point, WT mice were already able to use previous long-term memories while performing the task. At the same time, WT mice were also at a point at which they improved relatively fast compared to later time points. Thus, silencing from day 4 onwards seemed a useful time point for starting PPC function interference. Mice were injected with PSEM or saline on day 4 and all following training days 15 mins prior to starting the four daily trials (Fig29).

When PPC was silenced on every day of training from day 4 onwards, escape latencies improved much less across days compared to saline injected control mice (Fig.29a).
Saline injected mice were comparable to WT mice regarding latencies as well as strategies. WT mice strategy development was discussed in the previous paragraph and is hence not discussed in detail again.
On the first day of silencing PPC (day4), mice failed to perform equal or better in terms of spatial strategies compared to the previous day (Fig.29b-c). This means that, long-term memory recall was not sufficient to perform the task to previously achieved levels of spatial search strategies. Nevertheless, mice in which PPC was silenced remained able to perform spatial strategies to a reduced extent, which was further increasing across training days towards reaching pre-silencing (day3) levels by the end of training.

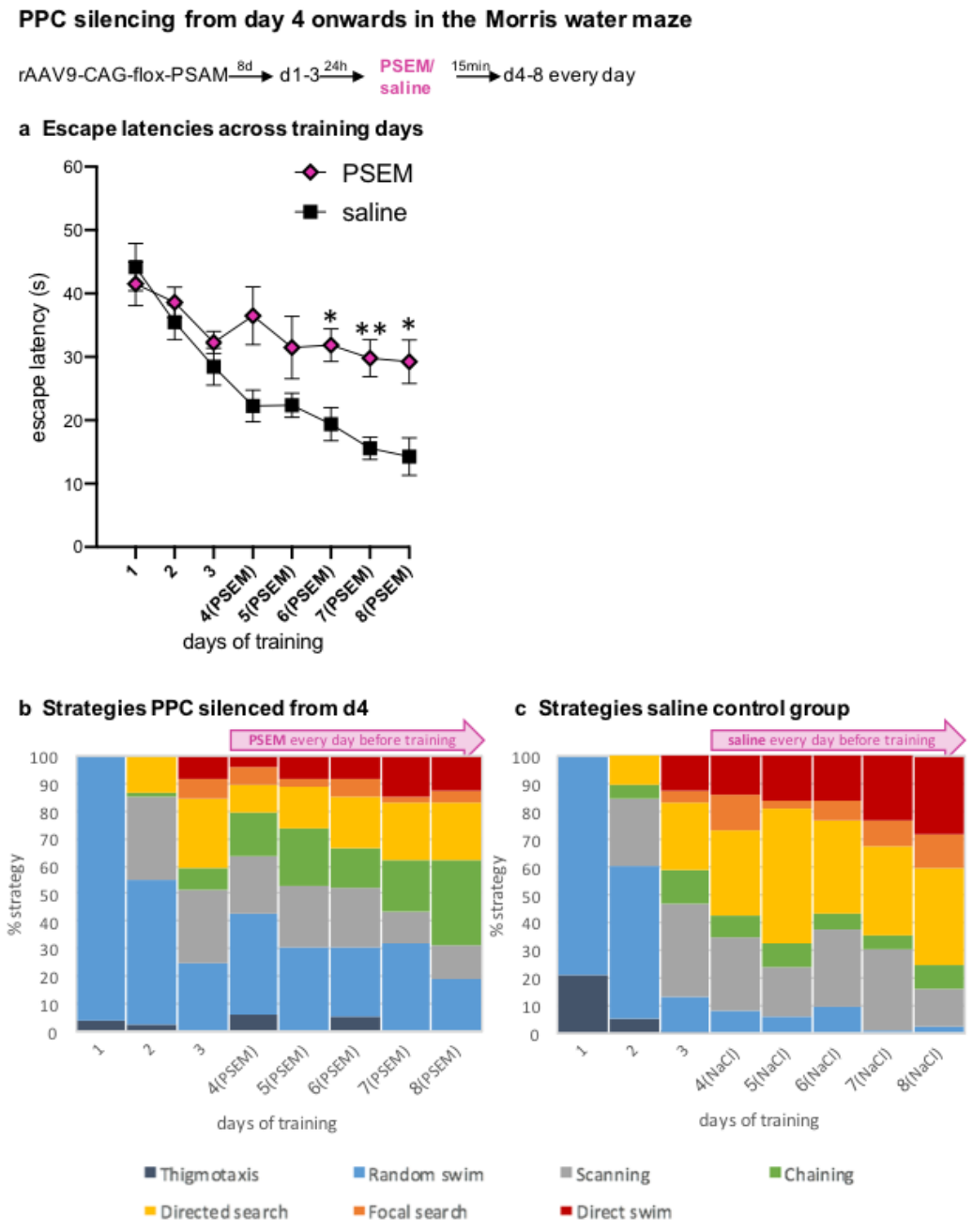


Fig.29 PPC silencing from day4 onwards in the Morris water maze (a) escape latencies across training days developed more slowly and to a lesser extent when PPC was silenced from day 4 onwards (2-way ANOVA with Sidak's multiple comparison, Interaction F (7,112) = 2,975, p=0,0067; time F (4,993, 79,89) = 8.063 p<0.0001; group F= (1,16) = 7,524 p=0,0144; Multiple comparison PSEM vs. saline day6 p=0,0366; day7 p= 0.0061; day8 p=0,0371, n=12 for PSEM/ n=6 for saline) **(b)** strategies developed very little from the onset of silencing until the end of training. Spatial searches were displayed to a lesser extent initially than before silencing, whereas the use of chaining increased across days. Random swim was displayed to a large extent across days of silencing **(c)** Saline control strategies developed normally across days characterised by an increase in the use of spatial search strategies.

Possibly, swimming behaviour is not only directed by long-term memory about the task, rather a combination of the latter, together with currently acquired short-term memory leads to further improvement, or the absence of the latter during PPC silencing.

Strikingly, mice displayed a large fraction of random swim behaviour, that could represent low confidence to stick with a strategy provided through long-term memory by means of lacking appropriate feedback on its successful application. Thus, any strategy, even if correct, would be abandoned prematurely through lack of short-term memory recall-dependent confirmation of success. Interestingly, mice increased their use of chaining across days of silencing. This may be because chaining, as a strategy is rigid in the sense that it does not require short-term memory-dependent feedback, rather it can be carried out regardless of circumstance. Importanly, this result shows that mice are able to change their behaviour across days, in other words, long-term memory formation about advantages of a particular strategy are possible, as seen with the chaining. However, the consequent use of a particular successful strategy may nevertheless fail due to impairment of short-term memory recall while performing long-term memory-guided behaviour.

PPC silencing from day4 on causes a stronger impairment, the less mice have learnt until the onset of silencing

Taking a closer look at individual mice in which PPC was silenced from day4 onwards, I noticed that, while some mice remained able to use spatial strategies quite well, others failed to use them almost completely and instead increased their use of chaining strategies with days of silencing. Nevertheless, all mice used random searches more frequently than saline controls across all days. In order to understand this better, I have decided to divide mice into those which learn to display more than 50% of spatial strategies, and those that display less than 50% of spatial strategies by the end of training on day 8 (Fig.30).

This showed that mice reaching 50% or more of spatial strategy use by the end of day8, were also the mice that had reached a level that allowed them to successfully perform spatial strategies before the onset of silencing (Fig.30a). However, despite the advanced learning stage of these mice, once mice failed to find the platform after some time within a trial, they nevertheless turned to random strategies, a behaviour which is not normally seen with mice having reached this stage of learning. Escape latencies were higher than for saline controls, however not to statistical significance.

In contrast, those mice which performed less than 50% strategy use by day 8 were also the mice that had only learnt little about spatial strategies until day 3 prior to

silencing onset (Fig.30b). These mice were unable to successfully perform spatial strategies and were also unable to learn how to improve them. Instead, they used chaining, which they had used for finding the platform before silencing. This behaviour was increasingly used across training days. However, also in those mice, the amount of the use of random search strategies remained high throughout training. Escape latencies were significantly higher as a result of PPC silencing compared to saline controls.

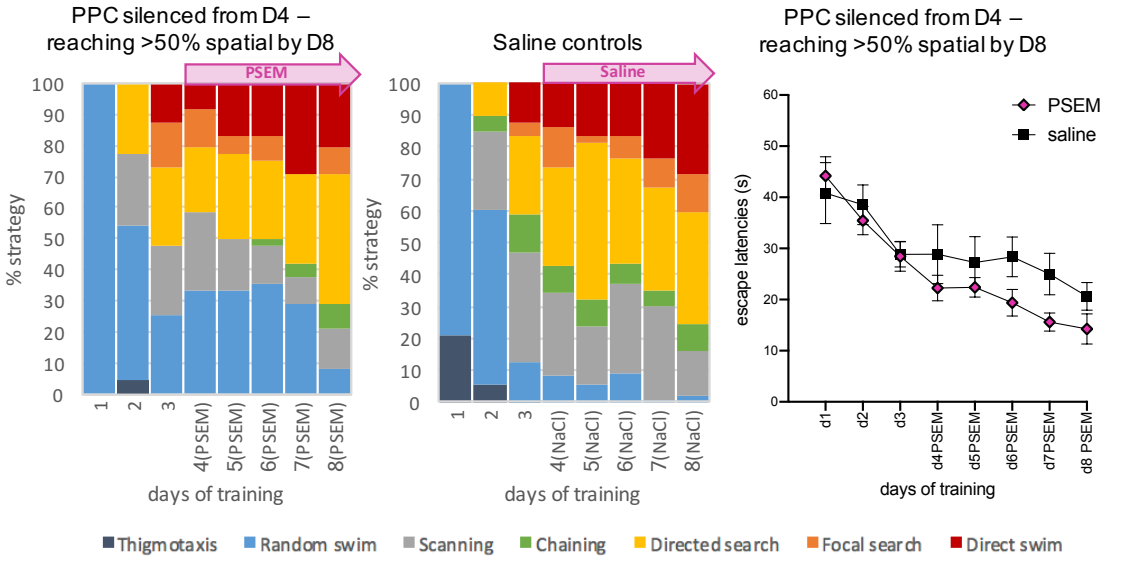
Taken together, PPC silencing in this task led to impaired performance as a function of previous progress in learning spatial strategies in the MWM task.
The more advanced the performance preceeding the onset of silencing was, the less mice were affected by the latter. Spatial strategies that were well developed before the onset of silencing, were affected little by silencing. Interestingly, mice in this group have not used chaining before the onset of silencing. Nevertheless, despite the large fraction of spatial strategy employment, random swim was also performed to large extents, unlike in the saline control group.
In contrast, those mice having displayed inferior performance relating to spatial strategies prior to silencing, were unable to recall or further improve them with PPC silencing. Instead, these mice relied on using the chaining strategy, which they had used successfully prior to the onset of silencing. Strikingly, all mice displayed a larger fraction of using random swim across all days of silencing.

In the previous experiments, PPC silencing seemed to affect short-term- but not long-term memory recall acquired more than 18h ago. This MWM task is more complex regarding the requirement for using long-term memories, not only in a pure recall manner, but also requires mice to adapt or change strategy behaviour depending on feedback during the task itself. Mice are not unabe to perform spatial or chaining strategies *per se*, nor are they unable to increase their use across days. For those mice, that are not developing spatial strategies and instead increase the use of chaining strategies, it could be because they had not previously ascribed enough confidence to this strategy and hence show conservative rather than explorative behaviour. In contrast, mice which had used spatial strategies confidently prior to silencing keep using the latter also during silencing.

Comparison of strategies depending on the percentage of spatial strategies displayed by the end of training with daily PPC silencing from day 4 onwards

rAAV9-CAG-flox-PSAM $\xrightarrow{8d}$ d1-3 $\xrightarrow{24h}$ PSEM i.p. $\xrightarrow{15min}$ d4-8 PSEM every day

a mice reaching >50% spatial at the end of training showed superior learning prior to silencing



b mice reaching <50% spatial at the end of training showed inferior learning prior to silencing

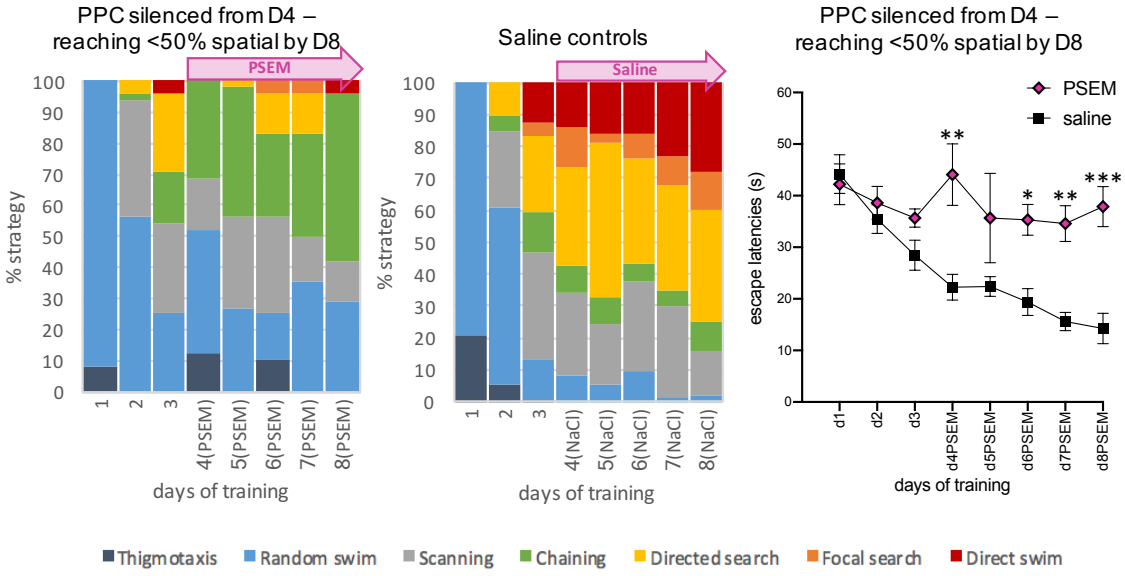


Fig.30 Comparison of strategies depending on the percentage of spatial strategies displayed by the end of training with daily PPC silencing from day 4 onwards (a) grouping mice according to their performance at the end of silencing, those which had displayed 50% or more spatial strategy use, remained able to display spatial strategies across days of silencing and even improved further until the end of training. Random swim was displayed across days. Escape latencies were not significantly different between PSEM and saline treated mice (2-way ANOVA with Sidak's multiple comparison, Interaction F (7,70) = 0,7867, p=0,6008; time F (4,059, 40,59) = 11,73 p<0.0001; group F= (1,10) = 3,633 p=0,0858 (PSEM treated n=6; saline controls n=6) **(b)** grouping mice according to their performance at the end of silencing, those which had displayed less than 50% of spatial strategy use, failed to perform

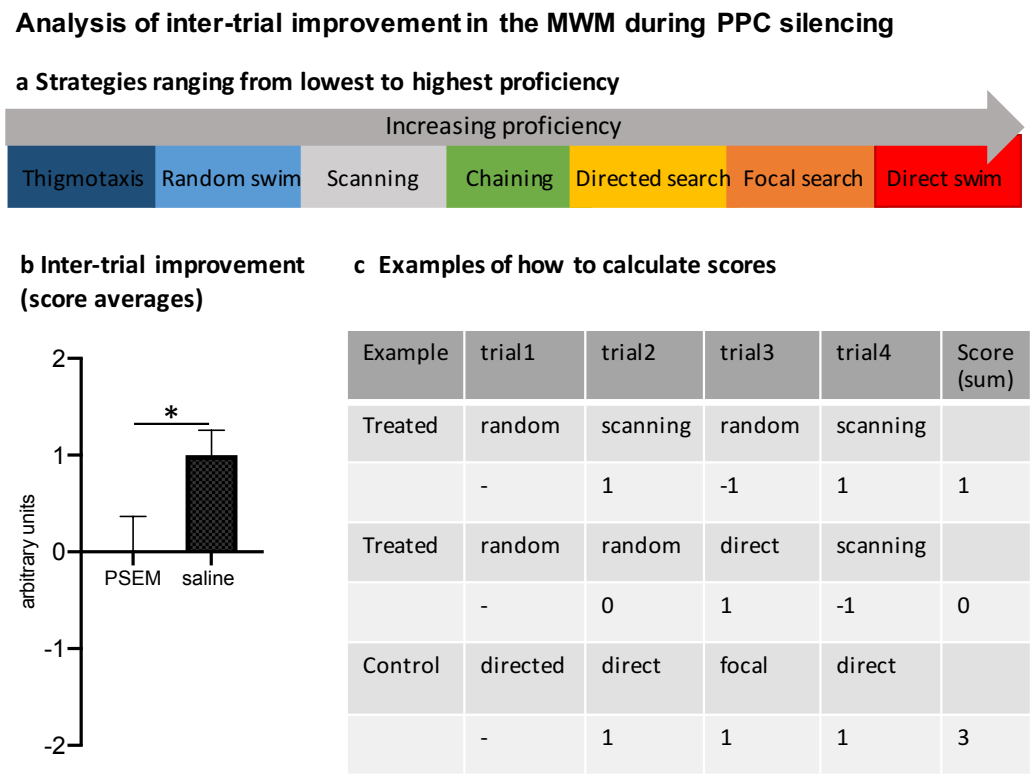
to levels prior to silencing. Instead, these mice displayed a large and increasing fraction of chaining across days of silencing, which was concomitant with a large fraction of random swim (2-way ANOVA with Sidak's multiple comparison, Interaction F (7, 70) = 3,294, p=0,0044; time F (7, 70) = 5,907 p<0.0001; group F (1, 10) = 23,38p=0,0007; Multiple comparison PSEM vs. saline: day4 p=0,0010; day6 p=0,0336; day7 p=0,0060; day8 p=0,0003; n=6)

PPC silencing impairs improvement from trial to trial within one training session

The finding that mice can improve or alter their behaviour across days, made me wonder whether and how mice behave across trials within a training session. Normally, control mice were more likely to prefer a previously used successful strategy in future trials over one, which proved unsuccessful. In other words, control mice did not only show improvement across days but also within training sessions on one day.
In order to answer the question, whether successful performance in the previous trial of one session, can predict the success of the following trial in a short-term memory-dependent fashion, I came up with a way to quantify strategy shifts. Because I wanted to investigate whether there is an inter-trial improvement, regardless of the performance level per se, I used the first of four trials on day 4 to set the baseline, against which future trials would be compared to. I picked day 4 in the middle of an 8-day training paradigm because on this day, the room for improvement is comparably large and previously acquired memory was unaffected by PPC silencing (Fig.31).

The first trial was assigned one of the strategies, which were rated in performance from lowest to highest as follows: thigmotaxis, random swim, scanning, chaining, directed search, focal search and direct swim (Fig.31a). Focal search and direct swim were considered equally in terms of proficiency. Mice were then assigned a value for each of the following trials depending on whether they improved (+1), stayed with the previous choice (0) or fell back (-1) in strategy proficiency (Fig.31c). Once mice had reached the most proficient strategies, displaying them again in the following trial also resulted in an assigned value of +1 in order to avoid rating plateaued performance negatively. The sum of the values assigned for the three trials provide an estimate of the ability of mice to improve their performance based on previous trial performance without taking into account the general proficiency.
Silencing PPC reduces the inter-trial improvement scores compared to saline controls (Fig.31b). Even when PPC silenced mice used a proficient strategy in one instance, they were not more likely to keep performing better as a result of previous success. In turn, control mice tended to improve across trials. This result suggests that mice in which PPC is silenced show an impairment in recalling short-term memory about success from a previous trial in order to use the latter to keep or even further improve in the following trial. Nevertheless, improvement or increase in using a particular

strategy across days, in a long-term memory-dependent manner, as discussed in the previous paragraph, is possible without PPC function.



Example	trial1	trial2	trial3	trial4	Score (sum)
Treated	random	scanning	random	scanning	
	-	1	-1	1	1
Treated	random	random	direct	scanning	
	-	0	1	-1	0
Control	directed	direct	focal	direct	
	-	1	1	1	3

Fig.31 Analysis of inter-trial improvement in the MWM (a) strategies ranging from lowest to highest in proficiency **(b)** Inter-trial improvement was larger in the control group than in the PPC silenced group significance (unpaired t-test P=0,0493 n=6) **(c)** Examples for how to calculate the inter-trial scores. Trial 2-4 were assigned values based on whether mice performed better, equal or worse in terms of strategies compared to the previous trial. These values were added to generate the scores for each mouse which were averaged across groups.

Overall, the water maze results so far indicate that PPC function is not required to recall and to perform a specific strategy from long-term memory, but it is crucial to first, enable mice to learn from current behaviour to the immediately following behaviour, i.e. to improve across trials, and second, to remain using a strategy consistently after failing initially. These findings point towards the necessity of PPC function for recalling short-term memory, in other words, to be able to use newly acquired learning immediately for the following trial on the same day and to appropriately react to failure when using a particular strategy. Possibly, PPC silencing does not cause a failure of short-term memory *per se*, but impairs the ability to ascribe value to newly acquired memory, thereby preventing appropriate behavioural adaptations to a changing environment.

The egocentric water maze

In contrast to allocentric navigation, which can be used for completion of the previously discussed MWM task, there is egocentric navigation, which is a type of navigation that is not dependent on visual cues, but rather relies on own-body movements within an environment that are known to lead to a certain goal, in this case the hidden platform (Milner and Goodale, 1993; Maguire et al., 1998; Burgess, 2006; Avraamides and Kelly, 2008). Considering that allocentric memory can be implemented under PPC silencing conditions, provided that there is previous long-term memory associated with it, I was curious to see whether PPC silencing would affect egocentric navigation differently. Assuming that once a certain egocentric route was learnt, it should be recalled normally during PPC silencing as this would entirely rely on long-term memory recall istead of using new information from allocentric cues that add reliance on short-term memory.

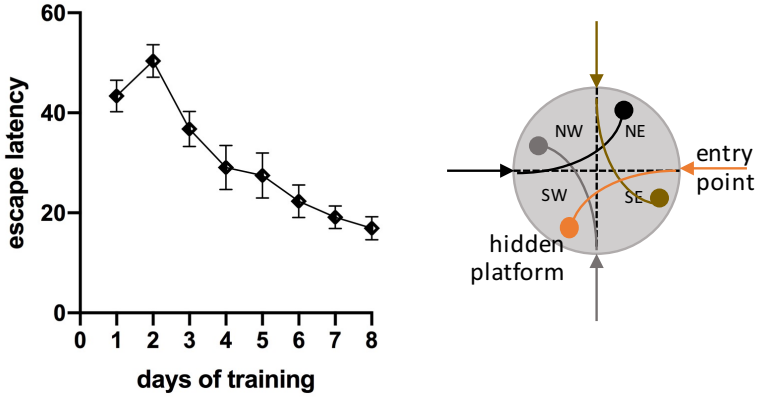
I have developed an egocentric version of the standard MWM task, in which I removed spatial cues and instead mice were required to learn a particular trajectory between the pool entry point and the hidden platform regardless of the surroundings of the pool (Fig.32a). Mice received 4 trials per day with 4 different entry points. However, the platform position was also changed in every trial in order to keep the relationship between pool entry and the former constant (Fig.32a right). This method served the purpose of making mice unable to use any remaining spatial cues such as camera cables on the ceiling to find the hidden platform.

In WT mice, the development of latencies to find the platform across days was comparable to the allocentric paradigm (Fig.32a). Regarding strategies, initially control mice behaved as mice in the allocentric MWM, characterized by previously discussed thigmotaxis, random swim and scanning behaviour (Fig.32b,d). Chaining was also present to extents that were comparable to the allocentric MWM. Therefore, these four strategies were displayed by identical colours and names. However, in addition to these, mice developed characteristically different strategies compared to the standard MWM.

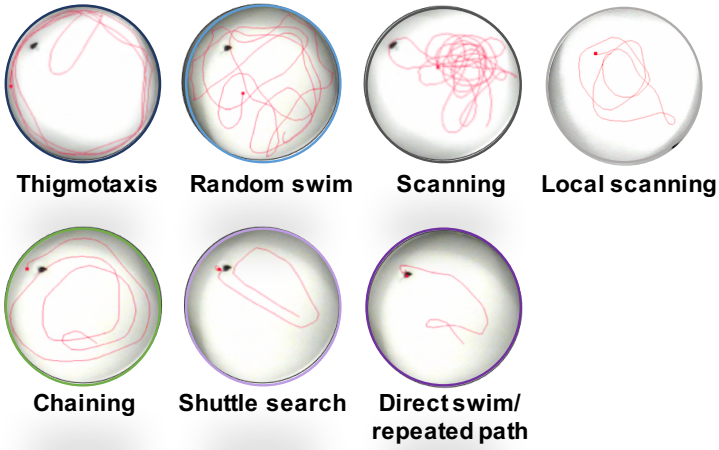
An additional strategy, which appeared rarely, was termed local scanning. It was ascribed to those behaviours, which were in the correct quadrant but not very precise around the platform.

Egocentric water maze

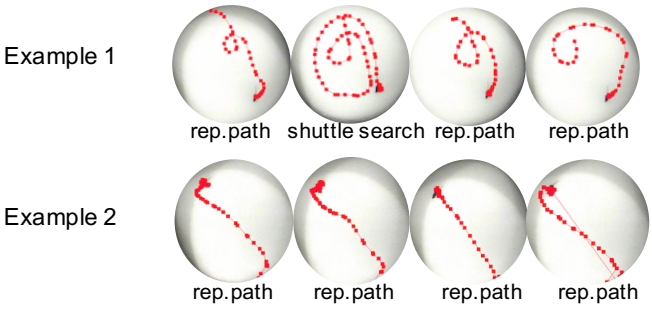
a escape latencies and general task setup



b categories of strategies



c Examples of repeated paths for individual mice



d strategy development

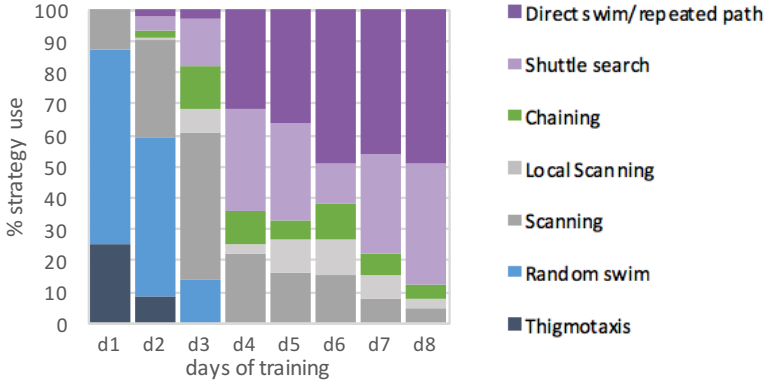


Fig.32 Egocentric water maze (a) Escape latencies across training days for control mice and schematic representation of task design with a circular pool devoid of visual cues. Pool entry points varied across each trial and the order of entries was changed across days. Platform position is adjusted to entry to keep relationship between the two constant **(b)** Representative images of strategy categories **(c)** Examples of 4 consecutive trials on day 8 of training in two different mice. Mice developed their personalised egocentric paths to the platform **(d)** Development of strategy use across training days in control mice.

Spatial search strategies varied considerably across the two paradigms. Egocentric spatial strategies included shuttle search and direct swim. The shuttle search was characterized by a correct trajectory with a narrowly missed platform and a subsequent return to the pool entry followed by another attempt of correct trajectory. This shuttle search could be repeated several times before it led to success. The putative explanation of this behaviour is that mice are under constant risk of losing their only reference point, which is the pool entry. By returning to this point, they keep track of it rather than increasing their risk of losing reference by turning around too many times in the expected platform location. Lastly, the most proficient strategy was direct swim, with the exception that in this paradigm, mice did not always use the shortest trajectory possible. Each mouse learnt a slightly different trajectory. Fig.32c shows examples of direct swim trajectories which were reproduced across days, containing small turns which keep appearing at the same locations.

Verification of egocentric behaviour in the egocentric MWM

In order to demonstrate that shuttle search reflected an egocentric behaviour, I conducted an experiment, in which the pool entry was permanently highlighted with a visual cue, while all other parameters remained the same (Fig.33a). This experiment tested whether mice still returned to the entry if they were sure to be able to find it reardless of their swimming behaviour. Thus, if mice did not show the shuttling search is this case, I would conclude that the purpose of it was to keep track of the entry point to the pool.

In this modified version, mice developed a flatter latency curve, which was very different from untreated control mice trained in the normal version of the egocentric task (Fig.33b).

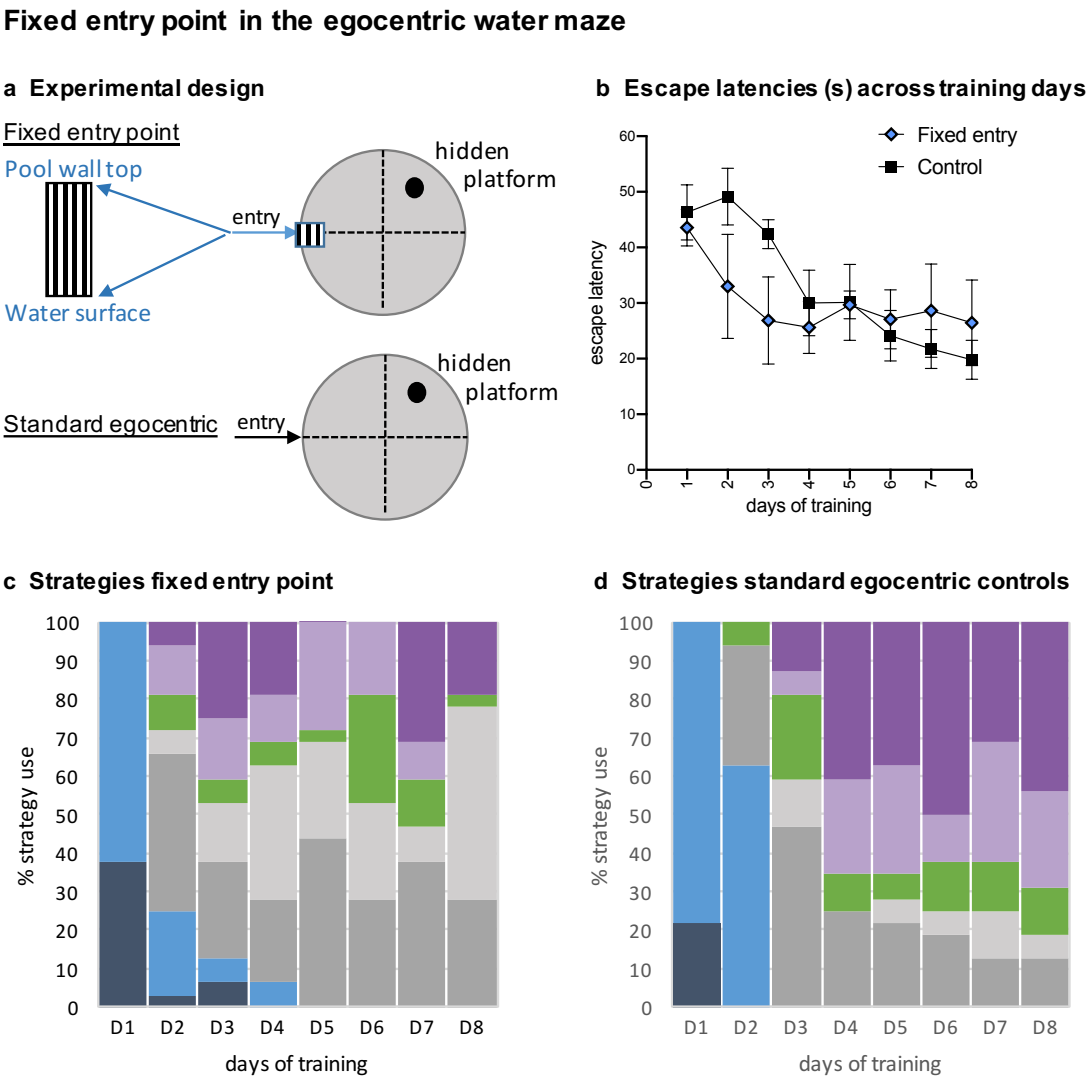


Fig.33 Fixed entry point in the egocentric water maze (a) experimental design comparing latency and strategy development during learning of the standard egocentric paradigm and a

modified version where the entry point is permanently marked with a visual cue **(b)** escape latencies were not significantly affected by permanently highlighting the entry point to the pool with a visual cue (2-way ANOVA, Interaction F (7,42) = 1,374 p=0.2415; time F (3,612, 21,67) = 4,328 p=0.0118; group F (1, 6) = 0,5349 p=0,4921 n=4). **(c,d)** swimming strategies developed differently depending on the experimental designs. In the fixed entry goup, mice used scanning and local scanning predominantly and failed to develop shuttle and direct swim strategies normally.

Escape latencies were lower initially but failed to develop further. Similarly, strategy use did not follow a clear development with mice using most strategies to different extents across days. Strikingly, mice displayed a lower fraction of shuttling behaviour, concomitant with an increased use of local scanning. This effect could result from the circumstance that in this paradigm, mice do not risk losing their reference point by initiating multiple turns around the expected platform position. Surprisingly, mice failed to increase their direct swim fraction although the task should provide ideal circumstances to improve direct swim across days.

Overall, the much lower total amount of direct swim or correct compensatory behaviour through shuttle search seems to point towards the conclusion that in contrast to the standard egocentric paradigm, mice are not using egocentric navigation in this modified version of the task. Potentially, mice are trying to use the visible entry point as a proximal cue, together with distal cues originating from the environment, such as camera cables on the ceiling. The changing of the entry and platform position accordingly across trials makes the use of distal cues for spatial navigation impossible. If the mice were to attempt to combine distal (nonsensical) with proximal (reliable and constant) cues for spatial navigation, this could explain why mice were impaired in learning this task, which at first sight seems much easier than the standard egocentric task.

PPC silencing from day4 onwards in the egocentric paradigm strongly impairs performance

I decided to silence PPC the same way as I did for the allcoentric paradigm discussed before. I trained all mice normally from day 1 to day 3, and started silencing on every day from day 4 onwards by injecting PSEM or saline respectively 15min before starting the training sessions.

Silencing PPC from day 4 in the egocentric water maze affected mice even more strongly than when silencing was done in the standard allocentric version discussed before (Fi.34). Escape latencies failed to develop beyond the point that mice had reached on day 3 prior to silencing onset (Fig.34a). Regarding strategy development, there was a strong reduction in the use of spatial strategies, especially the use of shuttle search (Fig.34b,c). Direct swim was displayed to small extents across days of silencing, demonstrating that it is neither a general failure of knowing where the platform is hidden nor to perform the trajectory. Chaining, scanning and random swim made up the largest components of behaviour displayed across silencing, while chaining was highest on the last day of training. The use of chaining increased across days of silencing PPC. This could be due to the assumption that in order to implement chaining, no short-term dependent memory is required. Instead, this strategy can be fully performed from long-term memory. Saline injected control mice, in contrast, displayed consistent and increasing use of spatial strategies across training.

There are essentially two phases that take place in an egocentric trial. The initial phase, right after being placed into the water is mainly dependent on long-term memory about the platform position in relation to the pool entry, in other words the start-goal trajectory. The compensatory phase follows the initial phase only if the platform was missed as a result of the initial attempt to find it. This phase is dependent on both long-term and short-term memory, as the knowledge about the trajectory needs to be integrated with current feedback and most importantly, previous swimming trajectories as these are essential in order to be able to navigate to the goal.

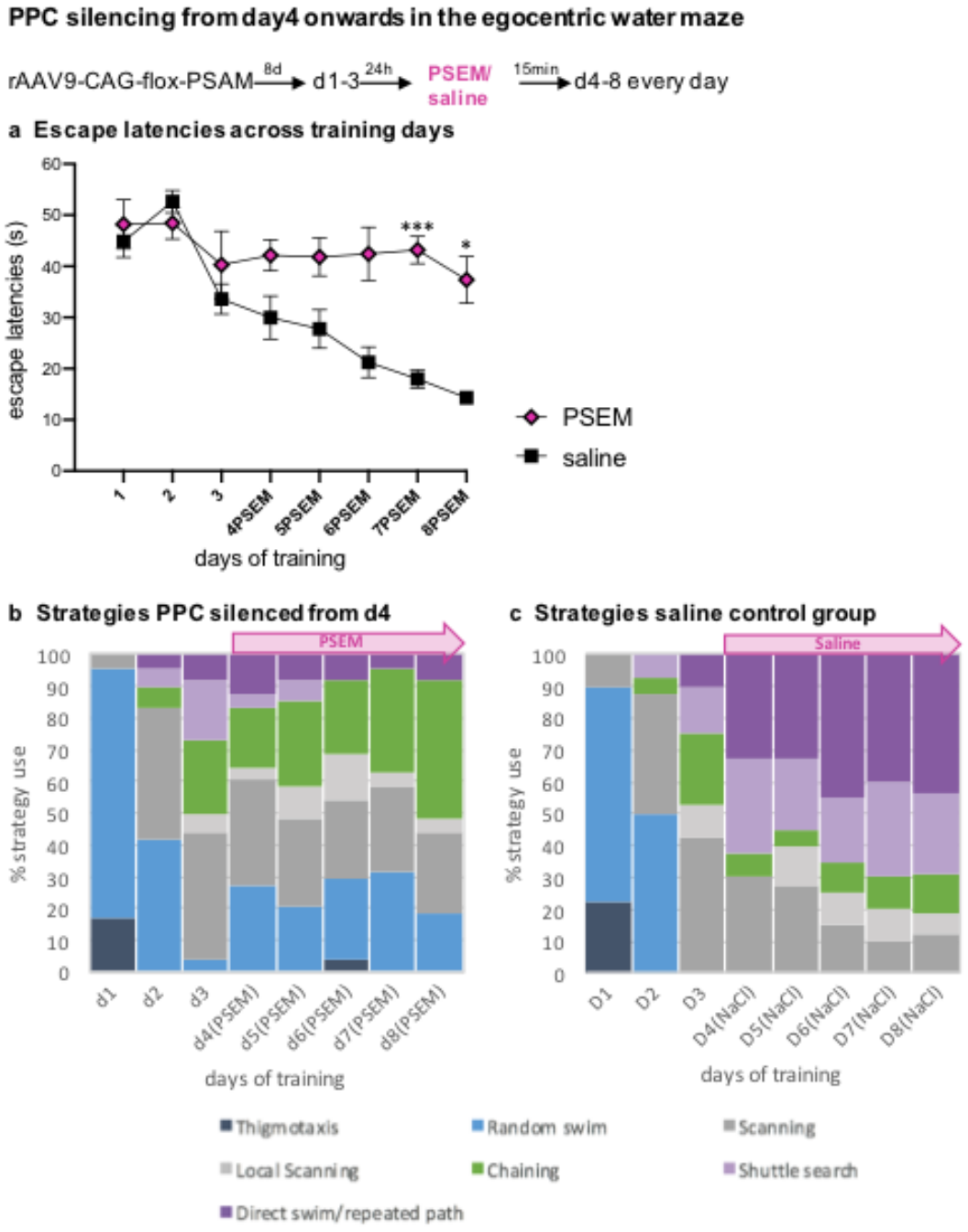


Fig.34 PPC silencing from day4 onwards in the egocentric water maze (a) escape latencies across training days failed to develop when PPC was silenced from day 4 onwards (2-way ANOVA with Sidak's multiple comparison: interaction F(7,70)=4,497 P=0,0003; time F(3,334, 33,34)=11,36 P<0,0001; group F(1,10)=22,65 P=0,0008; multiple comparison treated vs. Control: day7 PSEM P=0,0003; day8PSEM P=0,0280; n=6/6) **(b)** spatial strategies decreased from the onset of silencing until the end of training. The use of chaining was high and increased across days of silencing. Random swim and scanning was also displayed to a large extent across days of silencing **(c)** saline control strategies developed normally across days characterised by an increase in the use of spatial search strategies.

PPC silencing does not affect long-term memory recall but prevents short-term memory-dependent behavioural adaptation

I decided to look at the initial swimming behaviour, approximately the first 5 seconds of each trial, as this, if performed correctly, can be constant across trials and training days, thus representing a behavioural component of performance that is detached from short-term memory (Fig.35). In saline control mice, initial swimming demonstrated knowledge about the correct heading and angle towards the platform already on day 3, further increasing to almost all trials by the end of training on day 8 (Fig.35b). When comparing initial swimming to general strategy use it becomes clear that only less than 50% of these correct initial headings result in a successful direct swim, with most of the other trials resulting in shuttle search, followed by a smaller component of chaining or scanning behaviours.

When PPC was silenced from day 4 on, initial direct swim attempts were only reduced very little in the first days of silencing, with a gradual increase across further days (Fig.35a). One insight this result provided is, that silencing PPC did not affect the long-term memory recall of the platform position and the direct behaviour associated with reaching it. Further it showed that mice could learn across days of silencing shown by a gradual change of the initial behaviour displayed across days. This makes sense as mice, despite knowledge about the correct location, were unable to reach it successfully unless through direct swim attempts in the initial phase. In short, long-term memory recall was unaffected but short-term memory-dependent adjustments required in the compensatory phase failed. Instead, mice learned across days that chaining, a behaviour that is independent on short-term memory-dependent feedback, proved more successful under the circumstamces of PPC silencing and and increased use of the latter.

Examining compensatory phases, that is all strategies that were no successful direct swim, it becomes clear that although direct swim attempts were not strongly affected already at the onset of PPC silencing, shuttle search behaviour was dramatically reduced (Fig. 35c,d). This result demonstrates strongly that strategies that are reliant on short- as well as long-term memory guidance such as shuttle search are most strongly affected by PPC silencing.

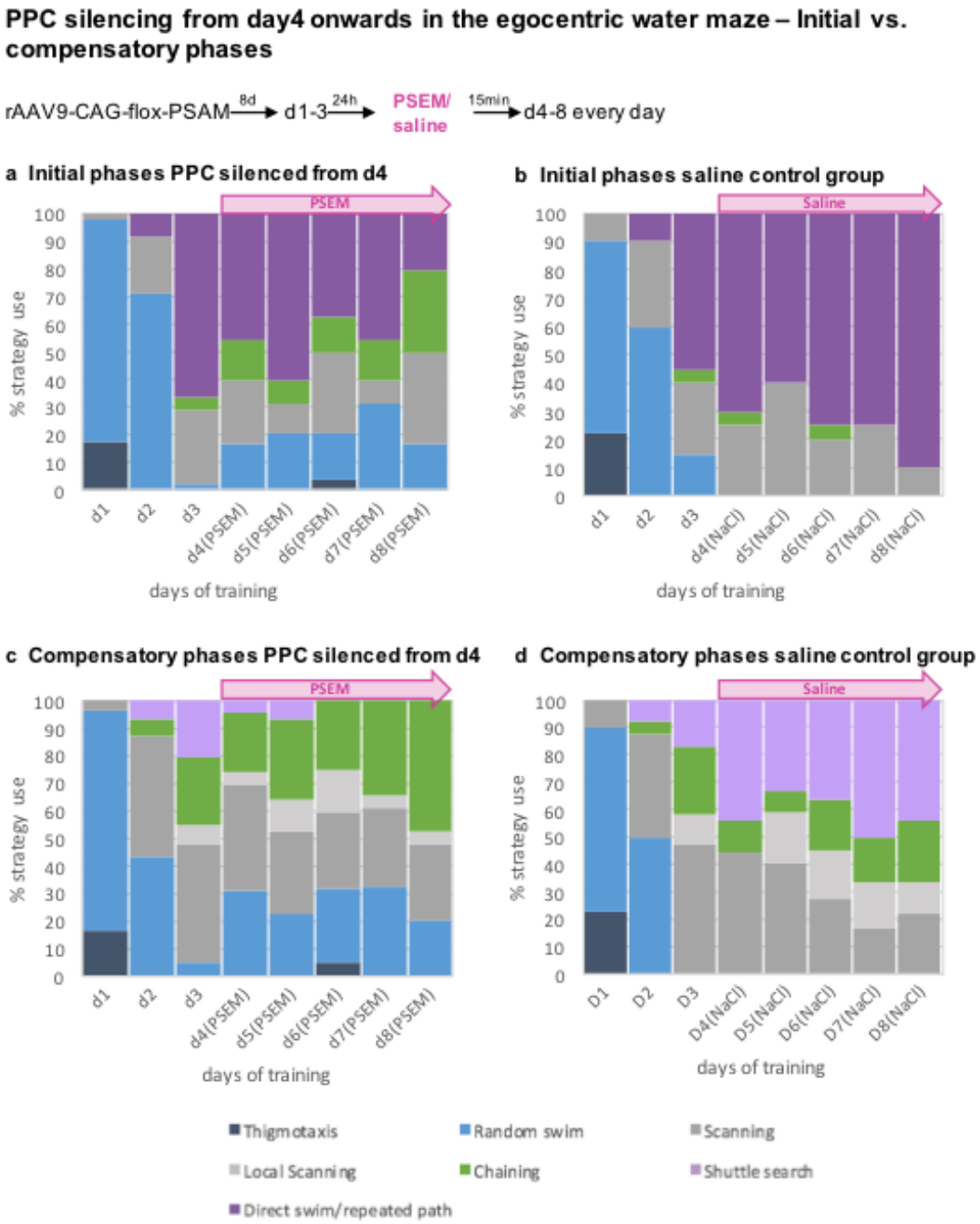


Fig.35 PPC silencing from day4 onwards in the egocentric water maze – initial attempts per trial (a) initial phases per trial during PPC silencing from day 4 onwards were characterized by large proportions of direct swim, which decreased across days of silencing. Random swim and chaining increased with silencing. **(b)** control mice improved their direct swim initial phases further with days of training **(c)** compensatory phases were characterised by a dramatic reduction in shuttle search, instead chaining as well random swim was much higher compared to saline controls. **(d)** compensatory phases in saline control mice were characterised by a dominant use of shuttle search.

PPC silencing impairs improvement from trial to trial within one training session also in the egocentric paradigm

Lastly, I decided to test whether mice in the egocentric paradigm were able to improve across trials within one training session the same way as for the allocentric paradigm. I compared the inter-trial improvement on day 4 between saline controls and PPC silenced mice without prior treatment on day 1-3.

When comparing behaviour across trials in PPC silenced mice and saline controls, the same picture emerged as for the allocentric task. Not only were mice, as shown previously, unable to learn from experience within a trial, they were also unable to carry on their experience from a previous trial to influence behaviour of the following trial (Fig.36a).

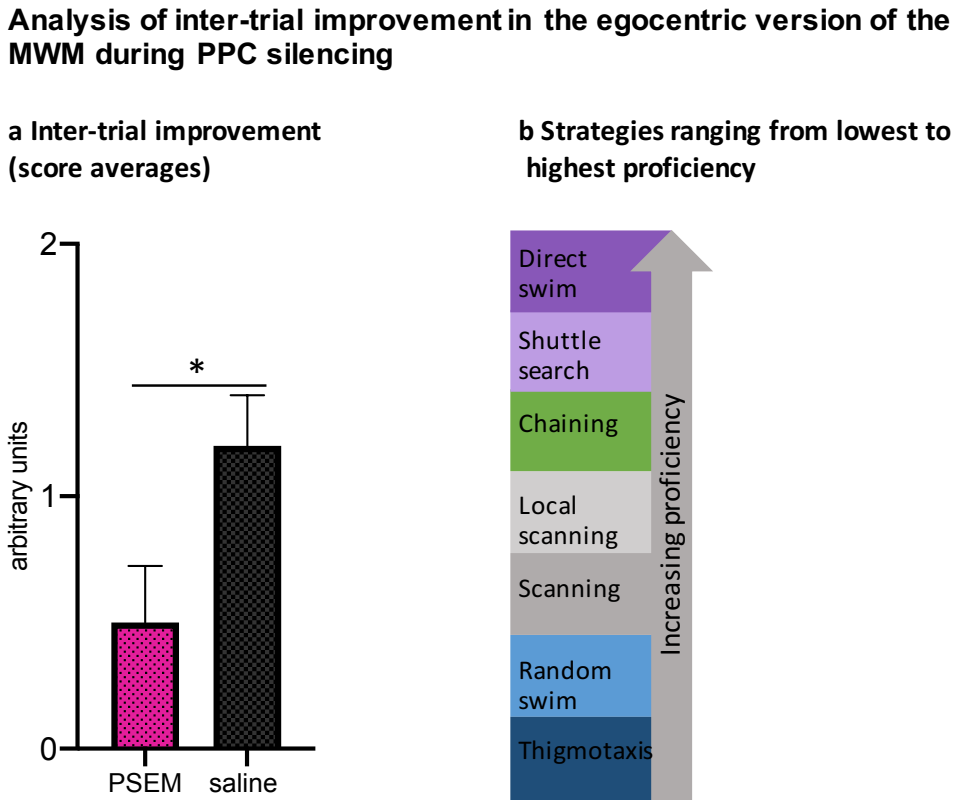


Fig.36 Analysis of inter-trial improvement in the MWM (a) Inter-trial improvement is larger in the control group than in the PPC silenced group (unpaired t-test P=0,0480 n=6) **(b)** Strategies ranging from lowest to highest in proficiency.

Taken together, the results obtained from the allocentric and egocentric water maze experiments point towards a role of PPC in recalling short-term memory that is required to behave adaptively in situations in which long-term memory recall is not sufficient for

adaptive behaviour within a changing environment. As this task requires a complex interplay of long-term memory and short-term memory both guiding behaviour, mice were strongly impaired as soon as immediate short-term memory recall was required for adapting behaviour.

1-side and alternation rule

In order to understand better, how short- and long-term memory recall is affecting behaviour with and without PPC function. I developed a set-up in which mice were required to swim to the correct one of two chambers from a starting position within a pool filled of water where they would find a platform to climb onto before being taken out of the water (Fig.37). Mice were habituated to the task on the previous day, where they learnt to swim and climb onto a green platform positioned randomly within an open pool. Once on the platform, mice were taken out of the water immediately.
On the following day, mice were placed into the starting chamber of the pool, which now contained the starting chamber, from which two additional chambers were accessible, one of which contained the escape paltform (Fig.37a). The platform was positioned in a way so that mice could only see it once having entered the correct chamber. If mice took a wrong choice of chamber they were required to return to the starting chamber and chose the other chamber where they would find the platform. Thereby the motivation to find the correct platform lied in the shorter amount of time required to swim. During training, each trial lasted for a duration of 60 seconds. If after this time, mice were unable to swim to the correct chamber with the platform present, they were taken out of the water from their current position. Mice were trained until they reached criterion, which was defined by 8 correct trials out of 10 subsequent trials on each day and in each condition. However, mice never received more than a maximum of 25 trials per day.

The 1-side rule

The first version of this task taught mice to swim to one side constantly, the 1-side rule task (Fig.37). I decided to answer three different questions in separate groups of mice.

PPC silencing impairs acquisition of the 1-side rule

The first question asked whether the acquisition of the 1-side rule was impaired by PPC silencing. Here, mice are unable to use long-term memory and instead are reliant on short-term memory to behave appropriately. I habituated all mice to the pool and platform on the day prior to the experiment. I injected PSEM or saline respectively 15min before starting the trails for each mouse. PPC silencing during the initial learning

of the 1-side rule impaired performance significantly while saline control mice learned the task quickly (Fig.37a).

PPC silencing does not affect 24h recall of the 1-side rule

The next question asked whether PPC silencing would impair recall of the 1-side rule learnt on the previous day. In this test, mice are able to perform correctly based on long-term memory only. I habituated mice to the pool and platform normally on the first day. On the second day, I trained both groups to criterion without any treatment. On the third day, I injected PSEM or saline respectively 15mins before recalling the rule learnt on the previous day. There was no effect of PPC silencing as both groups of mice recalled and performed the 1-side rule perfectly (Fig.37b).

PPC silencing impairs adaptation of a previously learnt rule

Lastly, in yet another set of mice, I asked the question as to whether PPC silencing would affect switching from one side to the other after having learnt the rule on the previous day. In this scenario, mice can recall the correct rule and side from long-term memory, but are required to use short-term memory to amend the rule for the currently correct side. Again, I habituated all mice to the pool and the platform on the first day. On the second day I trained both groups to criterion in learning the 1-side rule without any treatment. On the third day, I injected PSEM or saline respectively 15min prior to recall. However, this time during recall on the third day, I changed the platform position to the opposite chamber and mice were required to switch the rule to the other side. PPC silencing during this task impaired the switch significantly and mice were not able to reach criterion, whereas saline control mice learned the new rule effectively after a few wrong trials (Fig.37c).

Together, these experiments demonstrate that PPC silencing affects any behaviour that is dependent on short-term memory recall and its potential integration with long-term memory, but does not affect such behaviours that can be performed from long-term memory alone.

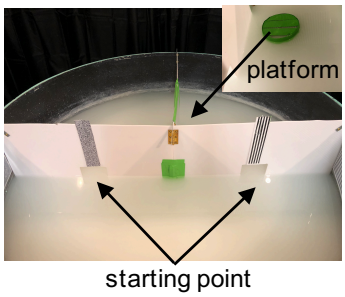
PPC silencing in the 1-side rule task

for a) rAAV9-CAG-flox-PSAM —8d→ habituation —24h→ PSEM/ saline —15min→ **acquisition**

for b) rAAV9-CAG-flox-PSAM —8d→ habituation —24h→ acquisition —24h→ PSEM/ saline —15min→ **recall**

for c) rAAV9-CAG-flox-PSAM —8d→ habituation —24h→ acquisition —24h→ PSEM/ saline —15min→ **switch**

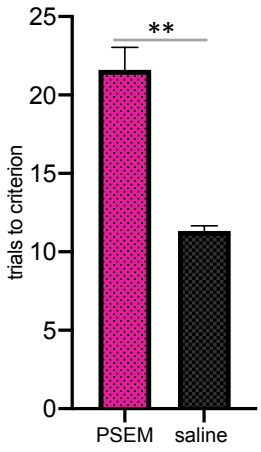
Task setup



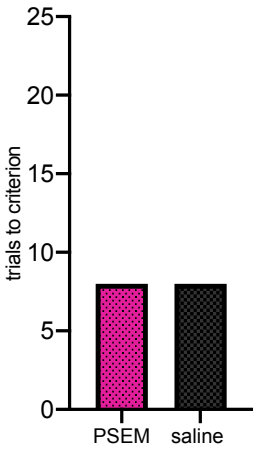
3 groups – habituation on D0

Group a) PPC silencing on **D1 acquisition**
Group b) Normal D1 acquisition – PPC silencing on **D2 recall**
Group c) Normal D1 acquisition – PPC silencing on **D2 switch**

a PPC silencing during rule acquisition



b PPC silencing during rule recall



c PPC silencing during rule switch

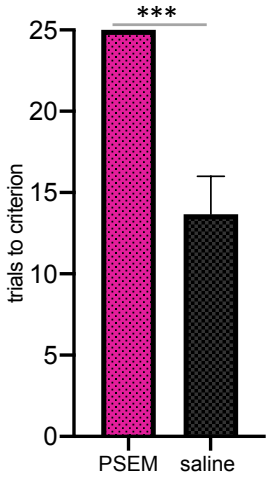


Fig.37 PPC silencing in the 1-side rule. Task setup with a pool filled with opaque water consisting of two chambers to chose from, one containing the visible platform, the other one being empty. Mice were habituated prior to training to swim to the visible platform in an open pool. **(a)** PPC silencing impaired acquisition of the 1-side rule (unpaired t-test P=0,0018, n=5/3). **(b)** PPC silencing after normal rule acquisition on the previous day, did not affect recall (n=5/3) **(c)** PPC silencing after normal rule acquisition on the previous day, impaired learning of the rule switch (unpaired t-test P=0,0006, n=5/3).

The alternation rule

A variation of the task is the alternation rule, in which mice were trained to alternate between the chambers of the pool to find the platform. The habituation and training procedures were carried out the same way as described for the 1-side rule previously, with the only difference that the platform position changed from every trial to the following (Fig.38). I decided to use this version in order to create a rule in which long-term and short-term memory need to work hand in hand in order to fulfil the task once the rule has been learnt.
During acquisition, there is no long-term memory available, thus behaviour is entirely short-term memory dependent. However, after acquisition is completed, long-term memory provides the rule itself, whereas short-term memory provides an immediate history of the choice and outcome of the preceeding trial in order to make the correct choice for the current trial.

PPC silencing impairs acquisition as well as short- and long-term recall of the alternation rule

First, I investigated whether PPC silencing would impair the learning of the alternation rule. After habitation on the previous day, I injected PSEM or saline 15mins prior to acquisition training. PPC silencing prevented learning of the rule, whereas saline control mice learned the rule quickly as shown in Fig.38a.

In another set of mice, I trained both groups to learn the alternation rule without any treatment. 90 minutes after mice had reached criterion, I injected PSEM or saline respectively and tested for recall of the alternation rule. Although mice had performed well just 90min before, PPC silencing impaired performance significantly, whereas saline control mice were unaffected (Fig.38b).

Next I was wondering whether this effect could have been due to the fact that the rule memory was only acquired 90 minutes before recall. Yet in another group of mice, I trained mice to reach criterion in the acquisition, but this time I waited for 24h until I silenced PPC during the recall of the alternation rule. In this case, mice were also

significantly impaired in recalling the previously learnt rule while saline control mice were unaffected (Fig.38c).

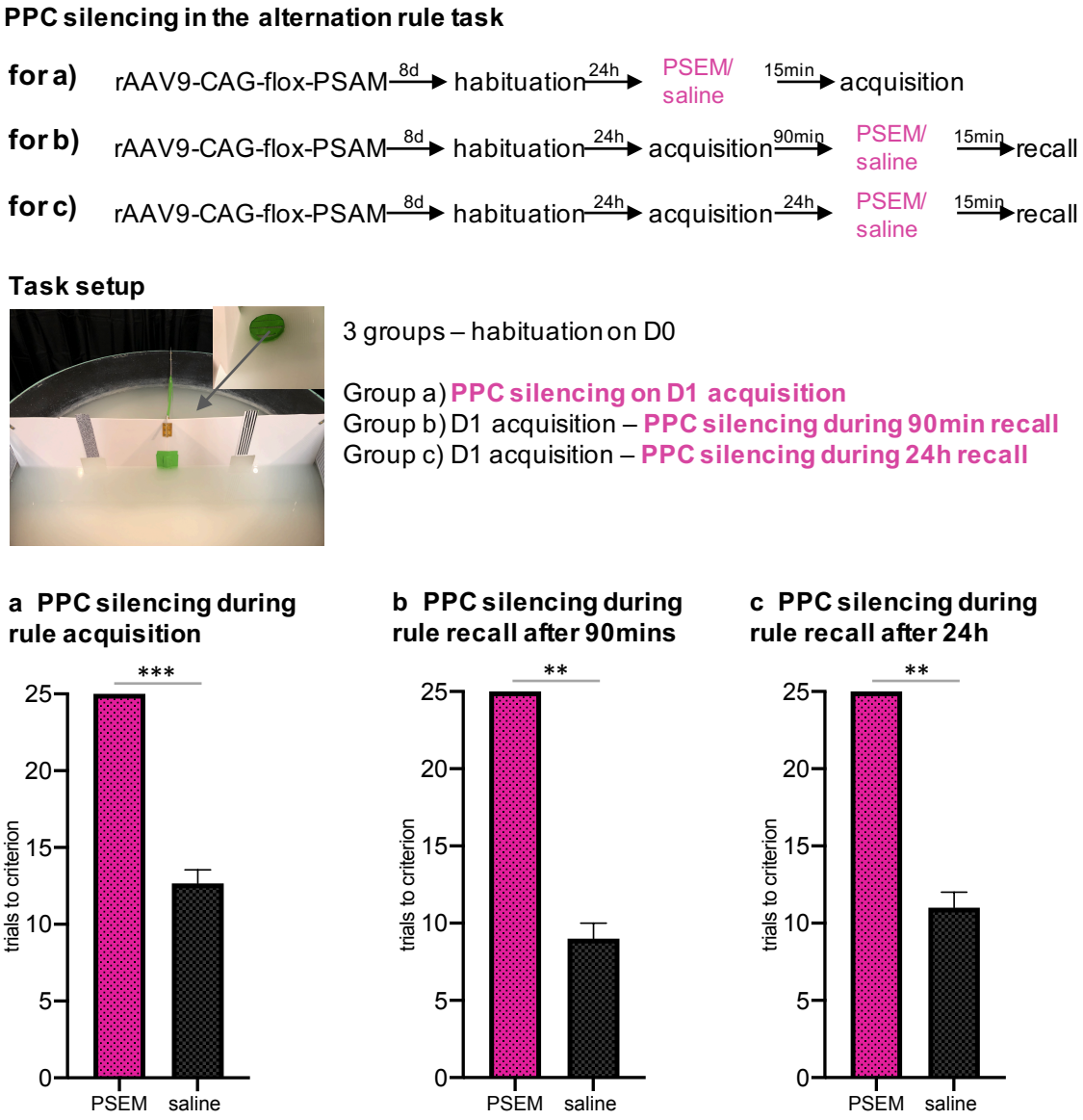


Fig.38 PPC silencing in the alternation rule (a) task setup with a pool filled with opaque water consisting of two chambers to chose from, one containing the visible platform. Mice were habituated prior to training to swim to the visible platform in an open pool. **(b)** PPC silencing impaired the learning of the alternation rule (unpaired t-test P=0,0002, n=3). **(c)** PPC silencing after normal rule acquisition impaired recall after 90min (unpaired t-test P=0,0039, n=2) **(d)** PPC silencing after normal rule acquisition impaired recall after 24h (unpaired t-test P=0,0051, n=2)

The recall of a previously learnt behaviour is not affected by PPC silencing, as long as it can be performed from memory entirely, such is the case in the recall of the 1-side rule. However, if mice need to take into account current information in order to

implement or change the rule provided from long-term memory, as is the case in recalling the alternation rule or chaning the 1-side rule, PPC silencing strongly impairs the ability to use short-term memory together with long-term memory to display correct behaviour.

Anterograde and retrograde tracing from PPC

In order to investigate the network within which PPC exerts its functions, anterograde and retrograde tracing was employed in order to identify brain regions projecting to PPC, and those that PPC projects to (Fig.39). AAV8-CamKIIa-mCherry-Cre was used together with SynaptophysinGFP (1:1) for anterograde tracing (Fig.39a,right), while RetroAAV-Cre-H2BGFP was used for retrograde tracing from PPC (Fig.39a,left).

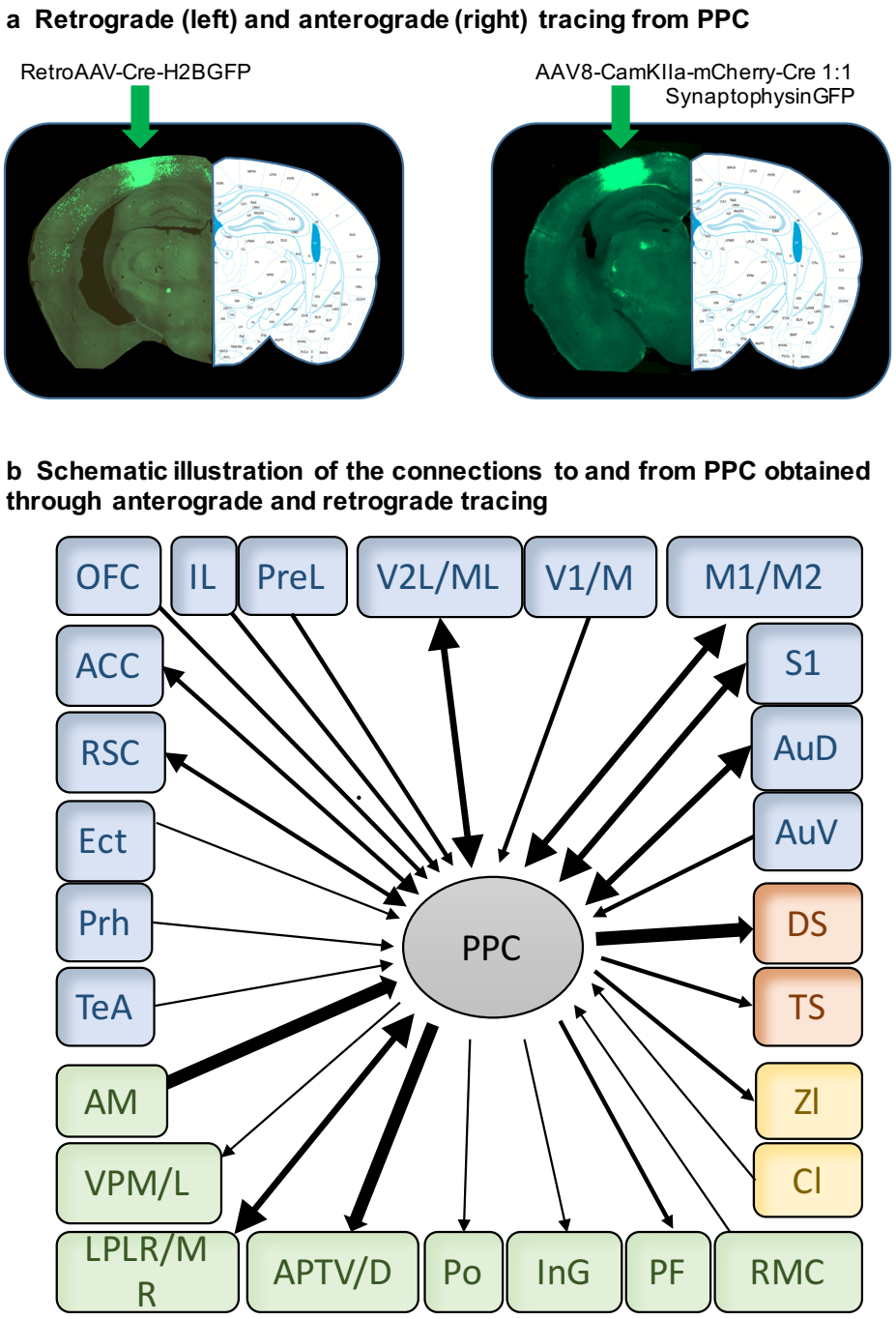


Fig.39 Retrograde and anterograde tracings from PPC (a) representative injection sites at PPC. Retrograde tracing employed RetroAAv-Cre-H2bGFP injected to PPC shown in the left panel. Anterograde tracing employed AAV8-CamKIIa-mCherry-Cre and SynaptophysinGFP

(1:1) injected to PPC shown in the right panel **(b)** Schematic illustration of the connections to and from PPC obtained through anterograde and retrograde tracing. Direction of arrow indicates whether PPC is receiving or sending projections. Reciprocal connections are indicated by bi-directional arrows. Thickness of arrows represents projection density. Abbreviations: ACC=anterior cingulate cortex; IL=infralimbic cortex; RSC=retrosplenial cortex; Ect=ectorhinal cortex; Prh=perirhinal cortex; TeA=temporal association cortex; AM=anteromedial thalamic nucleus; LPLR/MR=lateral posterior thalamic nucleus, laterorostral and mediorostral part; APTV/D=anterior pretectal nucleus, ventral and dorsal part; VPM/L=ventral posteromedial and posterolateral thalamic nucleus; PF=parafascicular thalamic nucleus; Po=posterior thalamic nuclear group; PreL=prelimbic cortex; InG=intermediate gray layer of the superior colliculus ; RMC=red nucleus ,agnocellular part; Cl=claustrum; ZI=zona incerta; DS=dorsal striatum; TS=tail of striatum; OFC=orbitofrontal cortex; AuV=secondary auditory cortex ventral area; AuD=secondary auditory cortex dorsal area; S1=primary somatosensory cortex;; M1/2=primary and secondary motor cortex; V2L=secondary visual cortex lateral area; V2ML=secondary visual cortex mediolateral area; V1/M=primary visual cortex, monocular part.

In line with previous findings (Hovde et al., 2018), the following regions were identified as shown in Fig.39b. Most cortical connections were dense and reciprocal as for ACC, RSC, secondary visual cortices, primary and secondary visual cortices, primary somatosensory and auditory cortices. Unidirectional projections to PPC were identified for ectorhinal, perirhinal and temporal association cortices, as well as for orbitofrontal and infralimbic cortices. The strongest thalamic projection originated from the anteromedial thalamic nucleus, with weaker but reciprocal projections originating from the lateral posterior thalamic nucleus. The claustrum as well as the red nucleus also projected weakly to PPC. Projections from PPC were found strongest in the dorsal striatum. The anterior pretectal nucleus also received dense projections from PPC. Less dense projections targeted the tail of the striatum, the parafascicular nucleus and the zona incerta. Weaker unidirectional projections were found to target the ventral posterior thalamic nuclei, the posterior thalamic nuclear group and the intermediate gray layer of the superior colliculus.

Abbreviations: ACC=anterior cingulate cortex; IL=infralimbic cortex; RSC=retrosplenial cortex; Ect=ectorhinal cortex; Prh=perirhinal cortex; TeA=temporal association cortex; AM=anteromedial thalamic nucleus; LPLR/MR=lateral posterior thalamic nucleus, laterorostral and mediorostral part; APTV/D=anterior pretectal nucleus, ventral and dorsal part; VPM/L=ventral posteromedial and posterolateral thalamic nucleus; PF=parafascicular thalamic nucleus; Po=posterior thalamic nuclear group; PreL=prelimbic cortex; InG=intermediate gray layer of the superior colliculus ; RMC=red nucleus ,agnocellular part; Cl=claustrum; ZI=zona incerta; DS=dorsal

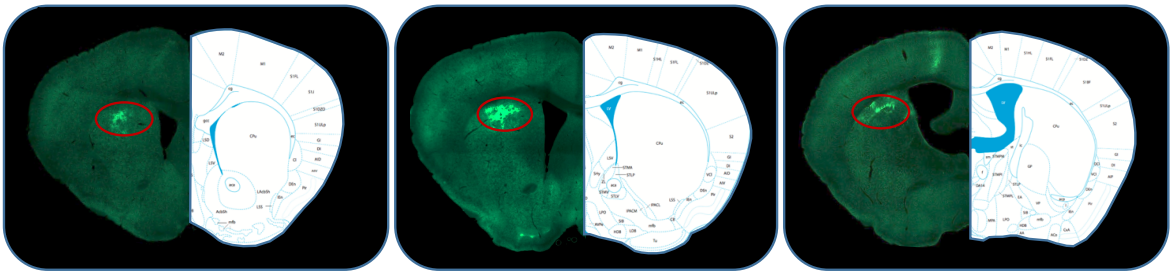
striatum; TS=tail of striatum; OFC=orbitofrontal cortex; AuV=secondary auditory cortex ventral area; AuD=secondary auditory cortex dorsal area; S1=primary somatosensory cortex;; M1/2=primary and secondary motor cortex; V2L=secondary visual cortex lateral area; V2ML=secondary visual cortex mediolateral area; V1/M=primary visual cortex, monocular part.

Projections from PPC to dorsal striatum

The dorsal striatum is known to play a role in flexible behaviour and decision-making (Baker and Ragozzino, 2014; Balleine, Delgado and Hikosaka, 2007). The previously discussed experiments suggest a role for PPC in enabling short-term memory to affect behaviour adaptively, a phenomenon that seems very similar to recent-history biases on action selection proposed by Hwang et al. 2017. Hwang and colleagues (2019) recently showed that the projections from PPC to dorsal striatum are necessary to implement recent-history dependent bias on action selection as discussed in the introduction. The conclusions published by Hwang complement the findings discussed in the next chapter, however were unpublished when the following experiments were carried out. Dorsal striatum was chosen for its proposed roles in decision-making, action selection and preparation, therefore posing a suitable downstream target of information flow from PPC to affect behavioural implementation (Pasupathy and Miller, 2005; Samejima et al., 2005; Delgado and Hikosaka, 2007).

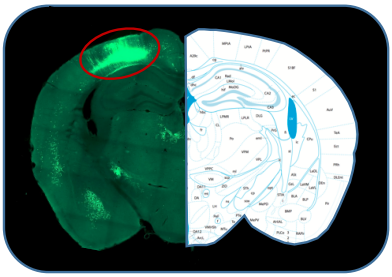
Projections from PPC to striatum target the most dorsal part of the striatum across the rostro-caudal axis of the brain, comprising parts of the dorsomedial, as well as the dorsolateral part of the striatum (Fig.40a). A detailed description of the projections was published previously in Hintiryan et al. (2016). Matteo Tripodi from our group has shown previously that PPC neurons projecting to DS target D1 and D2 receptor-expressing MSNs but not cholinergic interneurons (unpublished).
In order to investigate, whether specific projections were necessary for PPC functions observed across behavioural paradigms, a Cre-delivering retrovirus (RetroAAV-Cre-H2BGFP was used together with floxed PSAM-carrying AAV9 (inhibition: rAAV9-CBA-flox-PSAM(Leu141Phe,Tyr116Phe)GlyR-WPRE) targeting projections from PPC to dorsal striatum (DS). Fig.40b shows expression of the inhibitor virus at PPC and the retroviral injection site at DS respectively.

a Projections from PPC to dorsal striatum (DS)



b Silencing projections from PPC to DS

Inhibitor virus expression in PPC



Retroviral injection site in DS

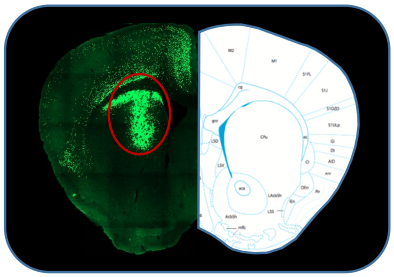


Fig. 40 Projections from PPC to dorsal striatum (DS) (a) projections across the rostro-caudal axis of the brain (left to right) in the most dorsal part of the striatum, comprising parts of dorsomedial and dorsolateral striatum (AAV8-CamKIIa-mCherry-Cre 1:1 SynaptophysinGFP) **(b)** inhibitor virus (rAAV9-CAG-flox-PSAM) expression in projection silencing experiments (left) retrovirus (RetroAAV-Cre-H2BGFP) injection site in the dorsal striatum (right)

I have decided to silence PPC-DS projections in three different behavioural paradigms to most accurately test their relevance for the PPC functions identified by silencing the whole PPC. These include PPC silencing on day 2 of the 3-day FOR, during cFC extinction and continuous silencing on subsequent days in the allocentric MWM from day 4. In each paradigm, I am comparing saline control mice with PSEM injected mice all of which were injected 15mins prior to start of each experiment.

Silencing PPC-DS projections on day 2 of the 3-day FOR

Silencing the projections from PPC to DS in the 3-day variant of the FOR task produced a phenotype very similar to the one observed with silencing the entire PPC through PV-positive interneuron activation (Fig.41). Object as well as context exploration were reduced, concomitant with an increased repetitive behaviour and abolished object discrimination on the day of silencing compared to saline controls (Fig.41a-c). As shown previously for general PPC silencing, learning about object identity during silencing was not affected as shown by a normal discrimination index displayed on day 3 (Fig41d). This experiment showed that the projections from PPC to DS are required for PPC function tested in this paradigm.

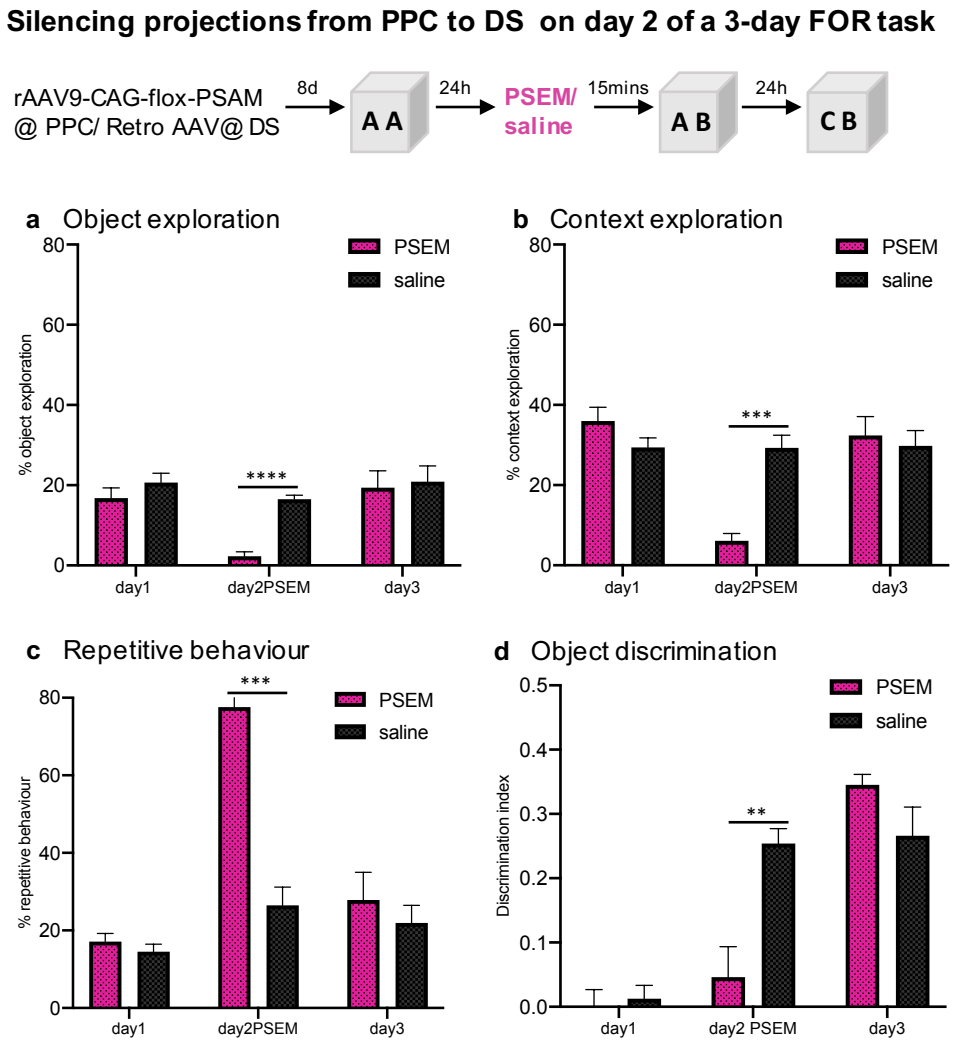


Fig.41 Silencing projections from PPC to DS in 3-day FOR (a) reduced object exploration on day 2 (2-way ANOVA with Sidak's multiple comparison, Interaction F(2,20)=3,773 P=0,0407; time F(1,422, 14,22)=11,26 P=0,0024; group F(1,10)=5,548 P=0,0403; multiple comparison: PSEM vs. saline day 1 p=0.6365; day 2 PSEM p<0.0001; day 3 p=0.9920, n=6)

(b) reduced context exploration on day 2 (2-way ANOVA with Sidak's multiple comparison, Interaction F(2,18)=15,23 P=0,0001; time F(1,628, 14,65)=15,82 P=0,0004; group F(1,9)=1,951 P=0,1959; multiple comparison: PSEM vs. saline , day 1 p=0.3950; day 2 PSEM p=0.0007; day 3 p=0.9662, n=6) **(c)** increased repetitive behaviour on day 2 (2-way ANOVA with Sidak's multiple comparison, Interaction F(2,20)=18,58 P<0,0001; time (F1,974, 19,74)=35,98 P<0,0001; group F(1,10)=15,98 P=0,0025; multiple comparison: PSEM vs. saline , day 1 p=0.7699; day 2 psem p=0.0007; day 3 p=0.8796, n=6) **(d)** reduced object discrimination on day 2 (2-way ANOVA with Sidak's multiple comparison, Interaction F(2,20)=7,246 P=0,0043; time F(1,741, 17,41)=32,97 P<0,0001; group F(1,10)=4,815 P=0,0529; multiple comparison: PSEM vs. saline , day 1 p=0.9090; day 2 PSEM p=0.0154; day 3 p=0.3709, n=6)

Silencing PPC-DS projections during cFC extinction

Silencing the projections from PPC to DS during extinction, 24h after cFC acquisition, prevented mice from reducing their freezing levels gradually during the 30 minutes compared to saline controls (Fig.42a). Thus mice showed no short-term memory of extinction. When freezing was tested during retention another 24h later, treated mice showed low freezing, demonstrating normal long-term extinction memory (Fig.42b). Thus, the projections from PPC to DS are necessary to implement PPC function tested in this paradigm.

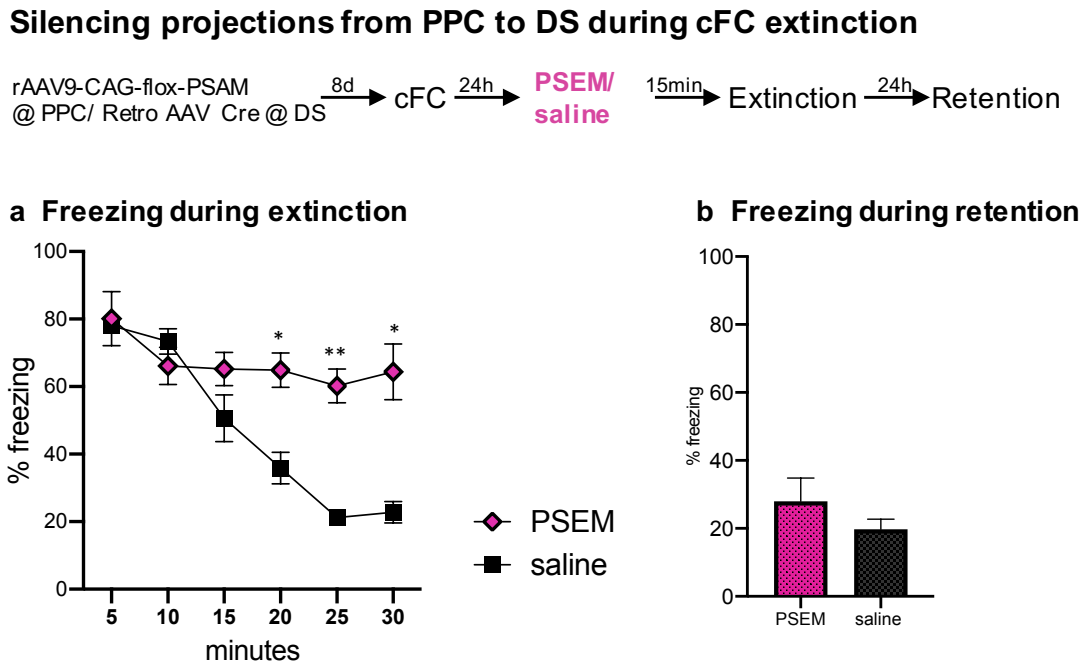


Fig.42 Silencing the projections from PPC to DS during cFC extinction (a) Freezing during extinction did not decrease (2-way ANOVA with Sidak's multiple comparison, Interaction F(5,50)=16,08 P<0,0001; time F(3,127, 31,27)=36,68 P<0,0001; group F(1,10)=10,67 P=0,0085; multiple comparison PSEM vs. saline, @20mins P=0,0113; @25mins P=0,0012; @30mins P=0,0166 n=6) **(b)** freezing during retention after 24h was unaffected (unpaired t-test p=0.2937, n=6)

Silencing PPC-DS projections from day 4 in the MWM

Silencing the projections from PPC to DS from day 4 onwards in the MWM caused a phenotype similar to the one induced by silencing PPC alone. Escape latencies during the days of silencing were higher than controls, although not significantly (Fig.43a). Examining the inter-trial behaviour on day 4 when the projections were silenced showed that mice failed to improve across trials compared to controls (Fig.43b). Strategy development during silencing was impaired compared to saline controls (Fig.43c,d).

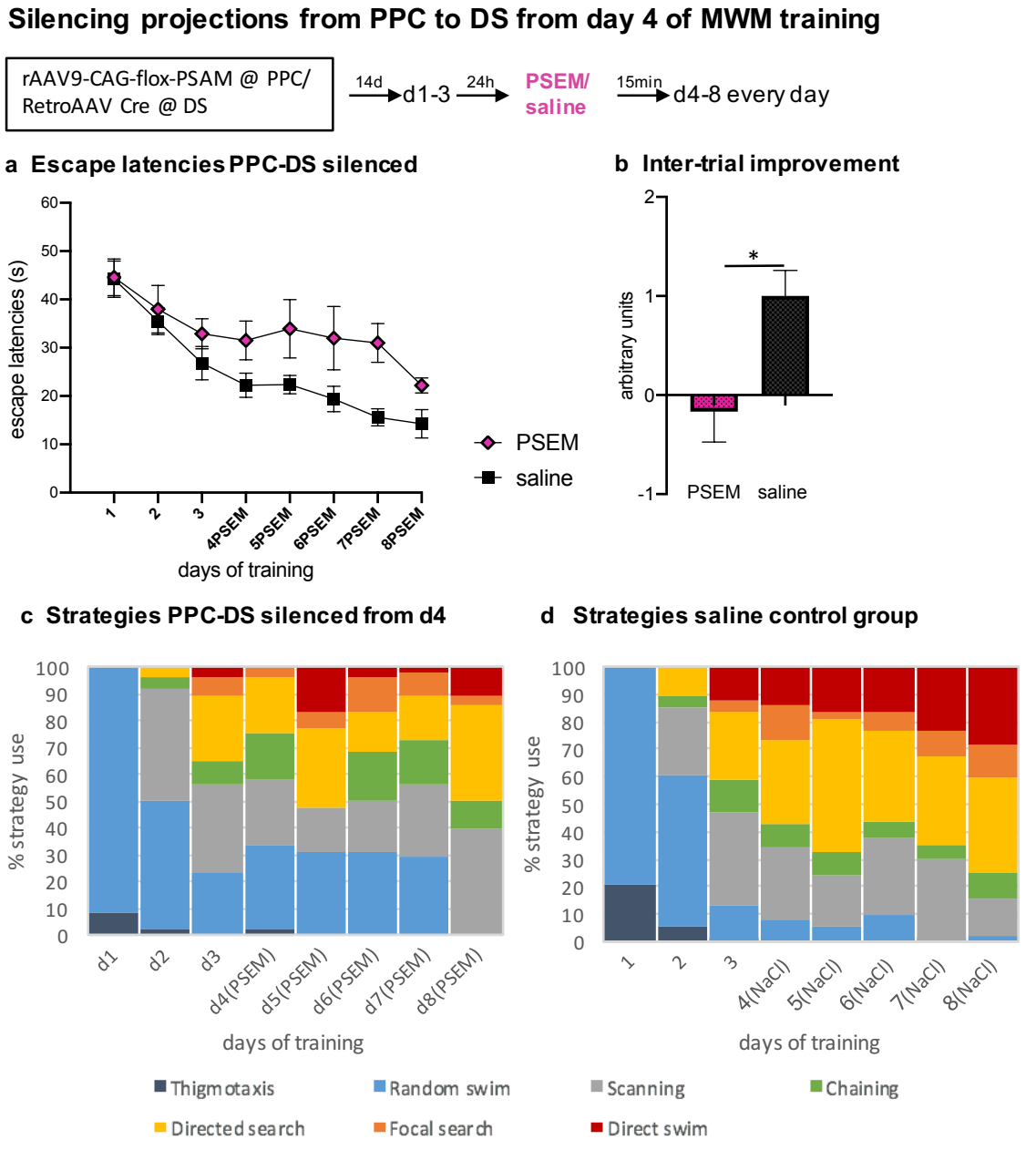


Fig.43 Silencing the projections from PPC to DS from day 4 in MWM (a) escape latencies remained higher under silencing conditions but not to statistical relevance (2-way ANOVA with

Sidak's multiple comparison, Interaction F(7,70)=1,423 P=0,2101; time F(3,991, 39,91)=14,55 P<0,0001; group F(1,10)=5,491 P=0,0411 n=6 **(b)** inter-trial improvement scores were significantly reduced (unpaired t-test P=0.0157 n=6) **(c,d)** strategy development under silencing conditions was impaired compared to saline controls. PSEM treated mice showed increased random swim behaviour, while they failed to develop their spatial strategies normally. Direct swim increased little with training and appeared intermittently. The use of chaining was elevated overall, however not used consistently either.

Mice failed to improve the spatial strategies learnt prior to the onset of silencing. Although mice still showed directed search corresponding roughly to pre-silencing levels, they failed to display focal and direct swim consistently. Instead, their behaviour was characterized by large proportions of random swim and scanning. Chaining was used to a larger extent in the PSEM treated group, however, also this strategy was not used consistenly across days. Taken together, silencing PPC-DS projections in the MWM impairs performance similar to the effects of silencing the entire PPC through PV-positive interneuron activation.

Overall, the projections from PPC to DS seem relevant to implement all the functions of PPC identified in these three paradigms. The ability to approach novel objects and behave normally in the presence of the latter seemed to depend strongly on PPC-DS projections. Similarly, the ability to adapt behaviourally during cFC extinction is dependent on these projections. Regarding the MWM, there is an impairment observed with silencing, however, not to a statistically significant extent regarding latencies. However, mice are strongly impaired in improving across trials within one training session and in consistently improving spatial strategies.

Silencing of projections from PPC to TS

Although the projections from PPC to the TS are relatively sparse, I decided to silence those using the same paradigms as for the PPC-DS projections in the previous paragraphs. As discussed in the introduction, TS is known to play a role in approaching novel objects (Menegas et al., 2018), a phenomenon that is similar to what we observe during PPC silencing, where the latter abolishes the ability of mice to interact with objects that they have not been familiarized with. Assuming that the lateral part of the SN influences TS via dopamine neurons regarding novelty and high intensity stimuli as discussed in the discussion, PPC could provide another input to regulate short-term memory-dependent behaviours regarding potential threat resulting from novelty or high intensity stimuli.

Silencing the projections from PPC to TS was done in the same way as described for the projections from PPC to DS using a Cre-delivering retrovirus (RetroAAV-Cre-H2BGFP together with floxed PSAM-carrying AAV9 (inhibition: rAAV9-CBA-flox-PSAM(Leu141Phe Tyr116Phe)GlyR-WPRE). Fig.40a shows a zoomed in image of the projections from PPC to TS obtained through anterograde tracing discussed earlier. Fig.44b and c show inhibitor virus expression at PPC (Fig.44b left) and the retroviral injection site (Fig.44b right) respectively.

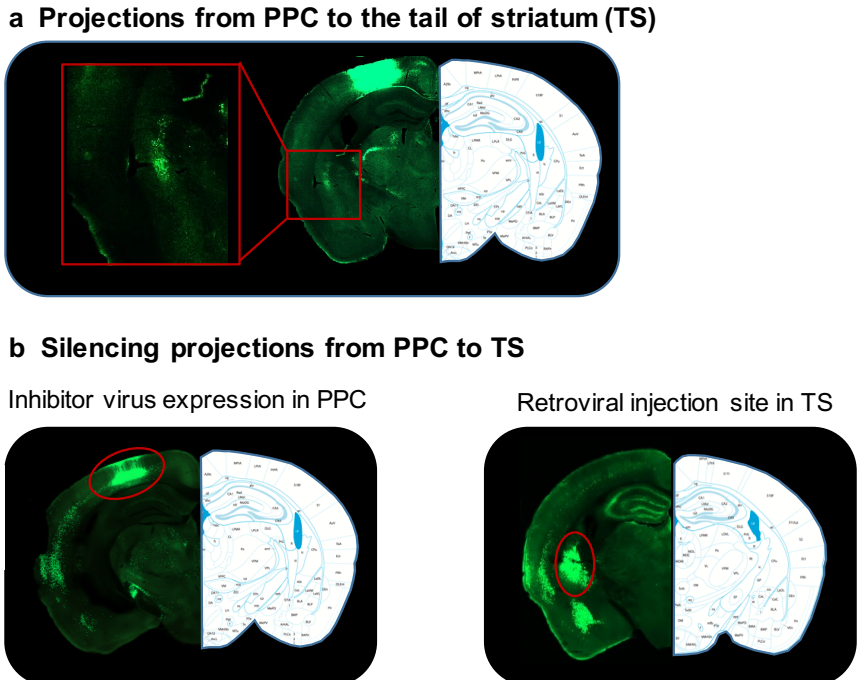


Fig.44 Projections from PPC to the tail of striatum (TS) (a) projections from PPC to the tail of the striatum (AAV8-CamKIIa-mCherry-Cre 1:1 SynaptophysinGFP) **(b)** inhibitor virus (rAAV9-CAG-flox-PSAM) expression in projection silencing experiments (left) retrovirus (RetroAAV-Cre-H2BGFP) injection site in the tail of striatum (right)

Silencing PPC-TS projections on day 2 of the 3-day FOR

In order to silence the projections from PPC to TS, PSEM or saline was injected 15min prior to testing on day 2, while there was no treatment carried out on day 1 and 3 for both the PSEM and saline groups. Silencing the projections from PPC to TS in the 3-day variant of the FOR task produced a phenotype very similar to the one observed with silencing the entire PPC through PV-positive interneuron activation. Compared to saline controls, object as well as context exploration were reduced (Fig.45a,b), concomitant with an increased repetitive behaviour (Fig.45c) and abolished object discrimination (Fig.45d) on the day of silencing that was not observed in saline controls. As shown previously for general PPC silencing, learning about object identity during silencing was not affected as shown by a normal discrimination index displayed on day 3.

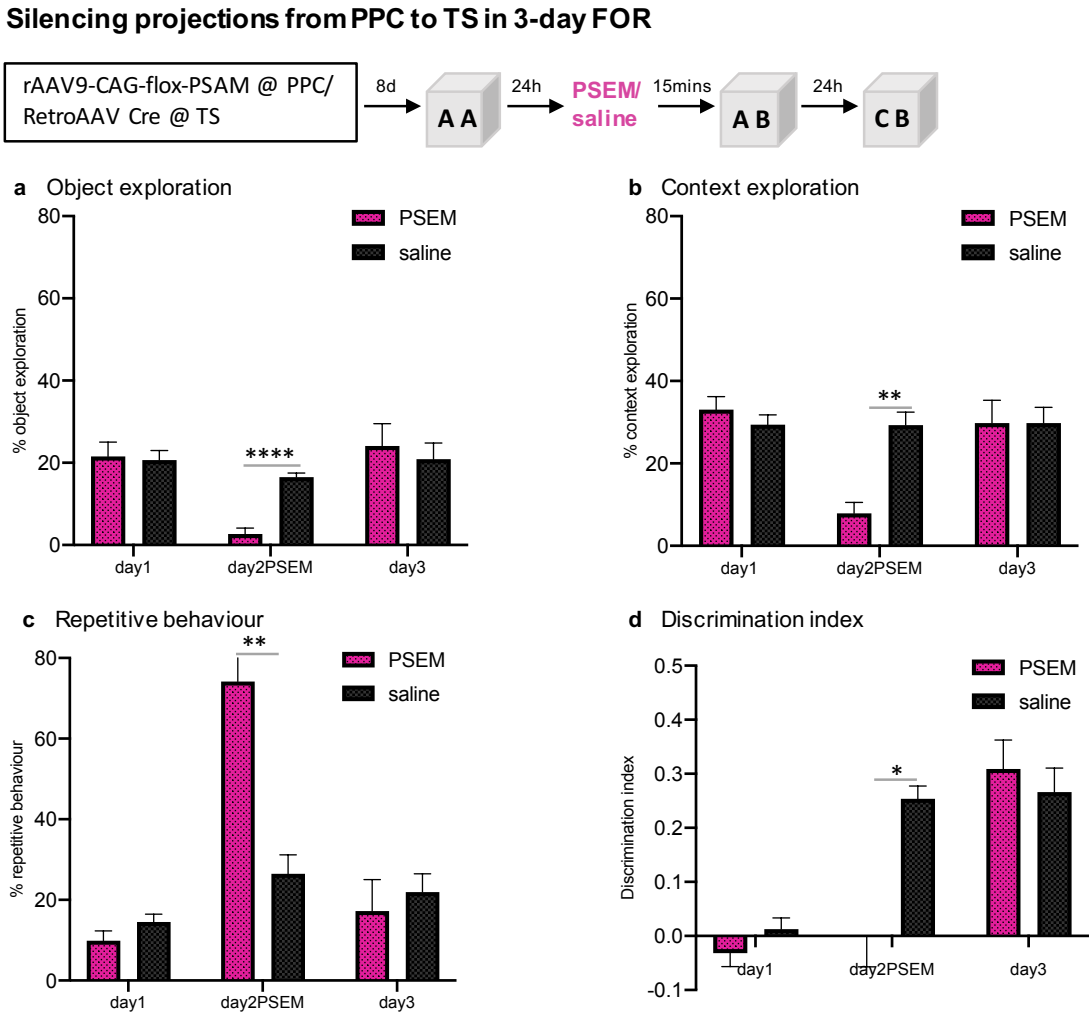


Fig.45 Silencing projections from PPC to TS in 3-day FOR (a) reduced object exploration on day 2 (2-way ANOVA with Sidak's multiple comparison, Interaction F(2,20)=6,271 P=0,0077; time F(1,846, 18,46)=14,75 P=0,0002; group F(1,10)=0,8301 P=0,3837; multiple comparison: PSEM vs. saline day 1 p=0,9956; day 2 PSEM p<0,0001; day 3 p=0,9536, n=6)

(b) reduced context exploration on day 2 (2-way ANOVA with Sidak's multiple comparison, Interaction F(2,20)=9,837 P=0,0011; time F(1,772, 17,72)=10,28 P=0,0015; group F(1,10)=2,556 P=0,1409; multiple comparison PSEM vs. saline , day 1 p=0,7592; day 2 PSEM p=0,0013; day 3 >0,9999, n=6) **(c)** increased repetitive behaviour on day 2 (2-way ANOVA with Sidak's multiple comparison, Interaction F(2,20)=26,15 P<0,0001; time F(1,869, 18,69)=46,88 P<0,0001; group F(1,10)=5,200 P=0,0458; multiple comparison: PSEM vs. saline , day 1 p=0.4305; day 2 psem p=0.0012; day 3 p=0.9431, n=6/6) **(d)** reduced object discrimination on day 2 (2-way ANOVA with Sidak's multiple comparison, Interaction F(2,20)=6,609 P=0,0063; time F(1,394, 13,94)=25,00 P<0,0001; group F(1,10)=8,543 P=0,0152; multiple comparison: PSEM vs. saline , day 1 p=0.4909; day 2 PSEM p=0.0139; day 3 p=0.9115, n=6)

Silencing PPC-TS projections during cFC extinction

In order to test whether silencing the projections from PPC to TS affected extinction, I injected PSEM or saline 15mins prior to the start of extinction on the day following cFC acquisition without treatment. Silencing the projections from PPC to TS during extinction prevented mice from reducing their freezing levels gradually during the 30 minutes while saline control mice extinguished normally (Fig.46a). When freezing was tested during retention another 24h later, treated mice showed comparably low freezing, demonstrating normal long-term extinction memory (Fig.46b).

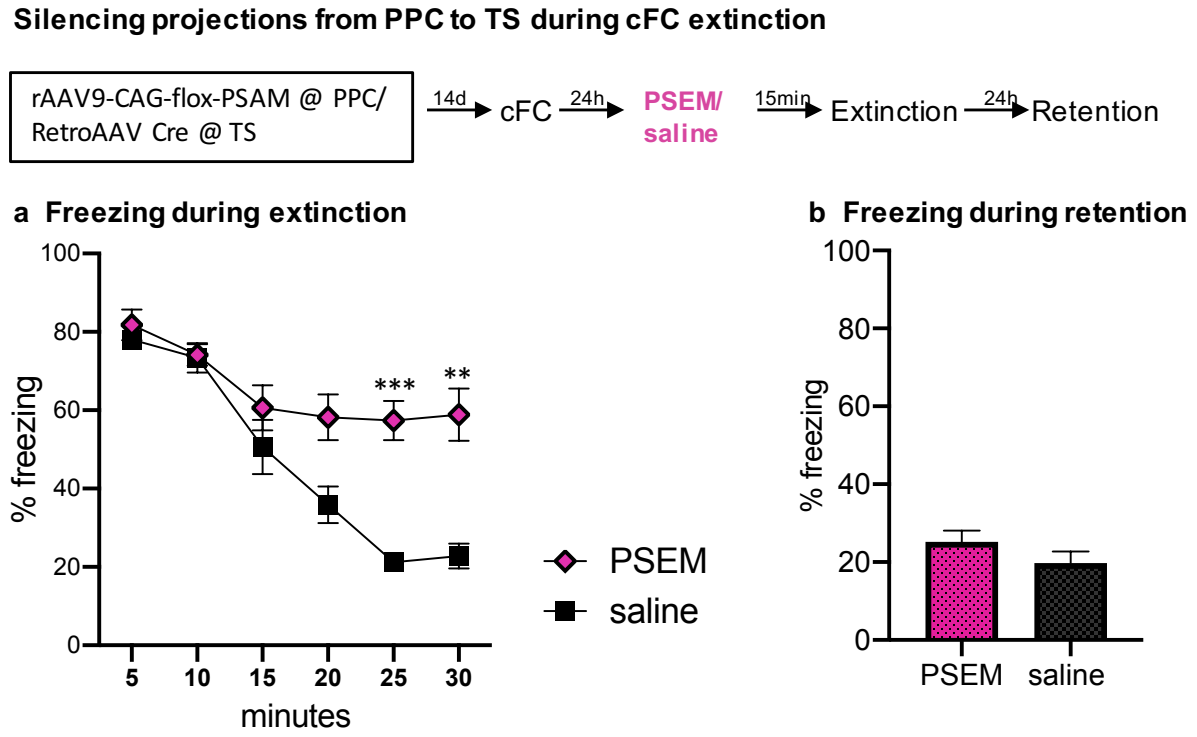


Fig.46 Silencing the projections from PPC to TS during cFC extinction (a) Freezing during extinction did not decrease (2-way ANOVA with Sidak's multiple comparison, Interaction F(5,65)=9,544 P<0,0001; time F(2,640, 34,32)=45,32 P<0,0001; group F(1,12)=10,61 P=0,0062; multiple comparison: PSEM vs. saline, @25mins P=0,0003; @30mins P=0,0029, n=9/6) **(b)** freezing during retention after 24h was unaffected (unpaired t-test p=0.2217, n=9/6)

Silencing PPC-TS projections from day 4 in the MWM

In the allocentric MWM, I tested the effects of silencing the projections from PPC to TS the same way as described before. I injected PSEM or saline respectively, 15mins prior to training on days 4-8. No treatment was done in either group on days 1-3. Silencing the projections from PPC to TS from day 4 onwards in the MWM caused no impairment in learning the task, both regarding escape latencies (Fig.47a) and also regarding strategies (Fig.47c,d) compared to saline controls. Inter-trial improvement was not impaired (Fig.47b). PSEM treated mice showed a slightly higher proportion of random swim compared to saline controls, which decreased towards the last days of training. Spatial strategies were used well despite PPC silencing.

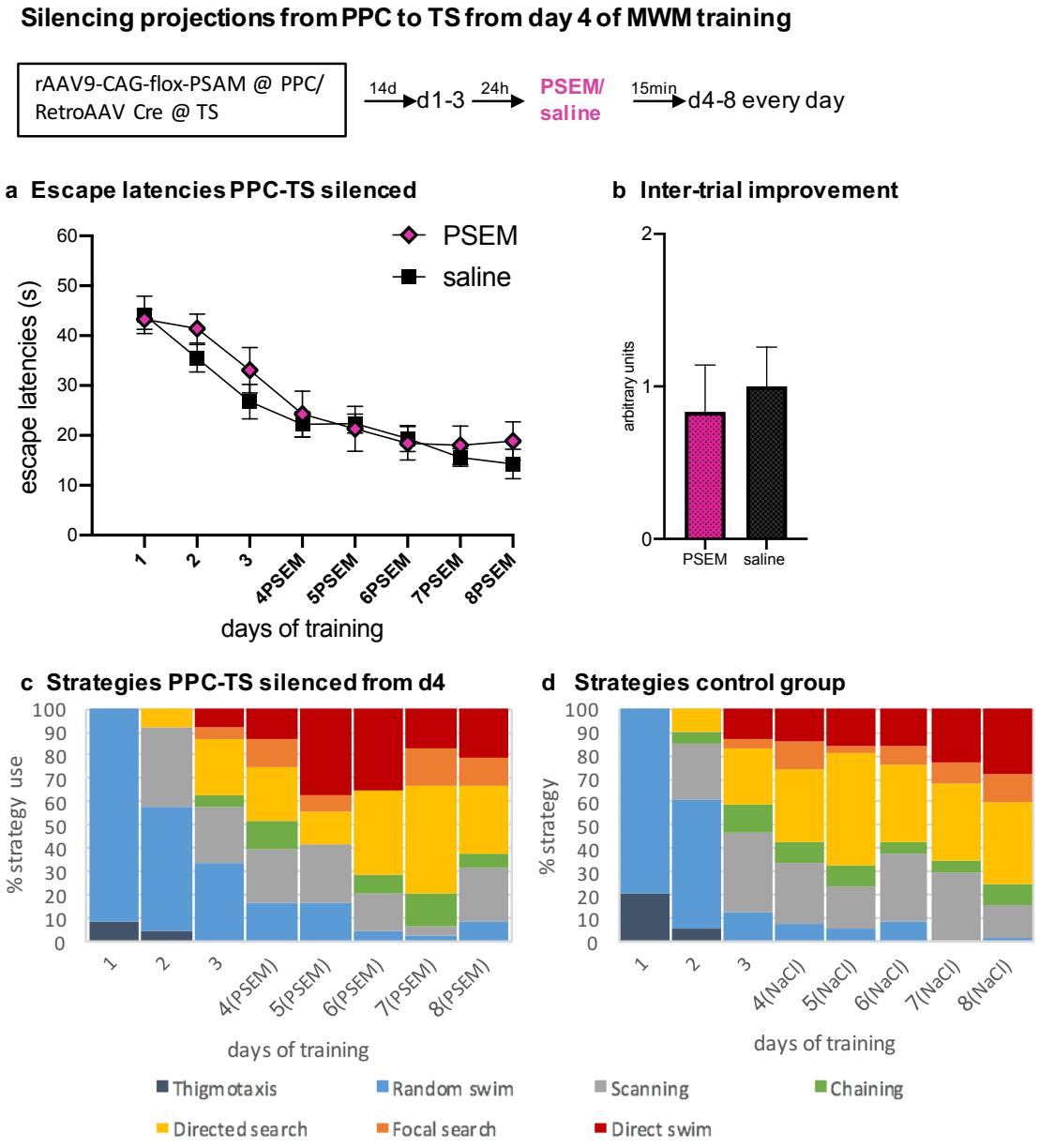


Fig.47 Silencing the projections from PPC to TS from day 4 in MWM (a) escape latencies were not affected under silencing consditions (2-way ANOVA with Sidak's multiple comparison, Interaction F(7,70)=0,6559 P=0,7082; time F(4,1444, 41,44)=28,57 P<0,0001; group F(1,10)=0,5600 P=0,4715 n=6) (b) inter-trial improvement scores were not affected (unpaired t-test P=0.6867 n=6) **(c,d)** strategy development under silencing conditions was not impaired compared to controls.

Overall, the projections from PPC to TS seem relevant to implement the function of PPC, much like the results for PPC-DS projections when it comes to short-term memory-dependent behaviour linked to caution. The ability to approach novel objects and behave normally in the presence of the latter, as well as the ability to adapt behaviourally during cFC extinction, seems to depend strongly on PPC-TS projections. However, silencing the projections in the water maze does not result in a change both in improving across days nor in improving across trials on the same day.

Taken together, these results suggest that the projections from PPC to TS may be specifically involved with behaviours that need responding to short-term memory-dependent actual or potential threat.

Discussion

The main goal of this thesis has been to increase our understanding of the role that PPC plays in relation to different aspects of memory. Previously published data had suggested multiple putative roles, but particularly a role in using recent sensory- and choice-outcome history to bias decision-making.

The experiments discussed in this thesis show that PPC function affects how short-term, but not long-term, memories impact decision-making and hence behaviour. The distinction between PPC-dependency of short- vs. long-term memories reflects the time span during which two distinct windows of memory consolidation take place (Karunakaran, 2016; Chowdhury, 2018).

Importantly, PPC function is not *per se* required for short-term memories to impact on decision-making. Rather decision-making is biased to caution through failure of the ability to ascribe appropriate value to short-term memories in the absence of PPC function. If long-term memories are available to guide decision-making, the latter dominate in the absence of PPC function as short-term memories fail to contribute appropriately.

Implementation of PPC function is critically achieved through projections to the dorsal striatum. For any decisions relating directly to the aspect of experienced or potential threat, projections from PPC to the tail of the striatum were also found to be required.

Strikingly, the work discussed in this thesis provides a framework to explain previously published data under a common premise, that is for example that the bias created through recent sensory and choice-outcome history follows the same mechanisms, that is to assign value to experiences, independent of whether their nature is a sensory *per se* or a rather complex cognitive one. This explains PPC function also within its nature in terms of connecting to a multitude of cortical and subcortical regions. The tracing and projection silencing data strongly suggest that the previously suggested bias on decision-making is indeed the ability to value experiences represented by short-term memories appropriately to influence decision-making and that this is a main function of PPC. Additionally, previous data confines the available insight to a time span of seconds to minutes, which has its own advantages as discussed in the introduction. However, by providing insight across a much larger time scale of hours to days, the work discussed in this thesis allowed us, on the one hand, to understand PPC function to an extent that we can explain its biological relevance to successful existence in changing environments. On the other hand, we concomitantly aquired

insight into the complex mechanisms by which decision-making is guided through long- and short-term memories differently.

PPC function relates to short-term, but not long-term, memories and dependent decision-making

PPC function does not affect the acquisition, consolidation or retrieval of long-term memories. Silencing PPC during cFC acquisition did not affect recall freezing tested 24h later. PPC silencing during fear memory recall did not affect freezing behaviour. Similarly, recall of extinction memory was unaffected by PPC silencing when the memory was aquired 24h earlier.

However, PPC function is critical in order for short-term memory to affect decision-making, independent of the influence of memories that were acquired 24h earlier. When PPC was silenced during extinction, mice were unable to immediately recall the newly acquired short-term extinction memory, despite being able to normally recall the memory after 24h.

A similar insight demonstrating that PPC guides the appropriate use of short-term memories on decision-making was provided by the finding that learning of the 1-side rule was impaired during PPC silencing. However, once the rule had been learnt, PPC silencing did not affect recall and performance on the following day.

The 3-day FOR experiment demonstrated that PPC function did not affect the acquisition of long-term memories. During PPC silencing, behaviour towards objects was changed but the resulting long-term memory about their physical appearances was unaffected. Additionally, the odour-FOR experiment showed that if PPC-dependent decision-making affected the experience and the associated limitations of information acquisition, PPC silencing affected long-term memories indirectly.

PPC function is not required for short-term memory recall *per se*

Nevertheless, PPC function does not generally affect the recall of short-term memories. Instead it affects decision-making based on short-term memories to be biased towards caution. This was demonstrated by silencing PPC during acquisition of cFC resulting in an increased immediate freezing response but normal long-term memory. When PPC was silenced during acquisition of cFC with either 1 or 5 foot

shocks, mice reacted to the shock(s) with excessive freezing instead of no freezing or a gradual increase as evidence for threat accumulated respectively. These effects on behaviour did not affect the long-term memory about the events tested after 24h or their subsequent modification (extinction). Thus, exaggerated freezing during acquisition could represent an inability to take into account the short-term memory that was acquired in relation to safety about the neutral context prior to experience of the shock. Instead mice display a stronger innate fear response independent of context-dependent short-term memory. Possibly, given the case that short-term memory-dependent interpretation of experience fails, innate behaviours dominate over otherwise complex behaviours. This would ensure the safety and potentially also the survival of the individual. Having the option to respond considerably to threat or aversive experiences could represent a comparably novel mechanism that is descriptive of higher order functions that allows to carefully weigh up contrasting evidence to reach optimal decisions. This explanation is supported by the finding that object familiarization changes the response to a foot shock in the presence of the object when PPC is silenced. In that case, not only the innate response i.e. freezing contributes to the response, but also long-term memories related to the object.

Another experiment demonstrating intact short-term memory recall, also on a sensory level only, was shown by the object familiarization. Object familiarization in the home cage and subsequent FOR with an object identical to the home cage object and a novel object abolished the effects of PPC silencing on object exploration without familiarization. Importantly, this experiment showed that the discrimination for the novel object was unaffected, thereby further demonstrating that short-term memory-dependent behaviour relying on sensory information *per se* was normal during PPC silencing (shocks in cFC, visual object information in FOR). This is concluded because in order to discriminate between objects in terms of exploration, an individual needs to keep track of recent sensory experiences and hence must be able to recall the associated short-term memory. However, ascribing complex meaning to sensory experiences (object safety in FOR), or the absence of such (cFC extinction), requires PPC function in order to act upon the meaning behaviourally while the memories are still not fully consolidated.

Absence of object exploration is due to excessive caution and not a result of a general phenotpe induced by PPC silencing

If PPC function was critical for short-term memory recall *per se,* mice should have reacted with less freezing to foot shocks instead of behaviong overcautiously. Rather, PPC function proved critical to ascribe meaning to short-term memories to influence decision-making adaptively. Thus in the absence of PPC function, short-term memories do not influence decision-making normally, but instead bias decisions towards caution thus avoiding risk-taking. As discussed previously, a role for PPC in decision-making has been widely suggested and there is particular evidence from functional magnetic resonance imaging (fMRI) work in humans, which demonstrates that PPC activity during decision-making is sensitive to the probability and variance of uncertain outcomes (Vickery and Jiang 2009; Symmonds et al. 2011; Studer et al. 2012). Another study invesitgating betting behaviour in patients with PPC lesions, found impairments in adjusting bets to the chances of winning. They found that patients selected higher bets than healthy controls when the chances of winning were unfavorable, but lower bets when the chances of winning were strongly favorable (Studer et al., 2015).

The FOR experiments demonstrate that the exploration of empty contexts and other mice follows different dynamics as exploring objects. PPC silencing only affects the latter, which may be due to objects exhibiting an intrinsic potential for threat. In order to overcome the resulting caution towards objects and the environment in which they are faced, mice must acquire information about safety of the objects. This is done in short bouts of exploration that become longer as safety evidence accumulates. When PPC is silenced, the value of the short-term memory relating to the object (cf. visual object information which is unaffected shown by DI after familiarization) cannot be used to affect behaviour adaptively. Instead, mice keep behaving excessively cautious despite learning about the object as was demonstrated by them discriminating well for previously seen objects tested on the following day without treatment. Familiarization, leading to knowledge about object safety, abolished all effects of PPC silencing, which points towards confirming that it is indeed an inherently associated potential threat concomitant with the inability to ascribe value to, or use the value of these memories constructively, that causes the decrease in object exploration.

Decision-making is shaped by innate behaviours, previous long- as well as newly acquired short-term memories, only the latter of which depends on PPC

Familiarization to an object in the home cage for 24h changed the behavioural response to an aversive foot shock when PPC was silenced. Instead of showing increased freezing compared to saline controls as a result of the foot shock, mice no longer showed a difference in freezing but increased object interaction compared to controls. This shows that although freezing is an innate response to an aversive foot shock, its exaggeration during PPC silencing can be abolished through long-term memory relevant to the situation within which the shock occurs. In conclusion, not only the integration of individual short-term memories is relevant for behaviour, but also the interplay of short- and long-term memories shapes innate responses differently. PPC function, however, only guides how short- but not long-term memories impact behaviour.

It becomes clear, beyond trying to understand PPC function itself, that innate fear responses seem to depend on a framework that is shaped by short- as well as long-term memory. This conclusion stems from the finding that in saline control mice, familiarization to an object in the home cage elevates the freezing response towards a foot shock. Assumingly, this may be a result of expecting safety in relation to the presence of the familiarized object that is considered safe. Thus, the experience of a foot shock is more unexpected than if mice had no previous memory about any components of the current situation. This finding is interesting thinking about the general framework guiding behaviour. Most likely under normal circumstances, behaviour is a well weighed interplay of innate responses and long-term memories, that are integrated with currently acquired short-term memory in order to allow for appropriate behaviour even under changing environmental conditions.

PPC function is critical for decision-making based on integration of short- with long-term memories

The influence of long-term memories on decision-making is independent of PPC function. However, if decisions require contributions from both long- as well as short-term memories, PPC function becomes critical for integration of the two in relation to decison making.

Looking at the allocentric MWM and the changes that occur from day 3 to day 4, which is the first day of silencing PPC, overall, mice performed worse on this day by increasing their percentage of random swim and chaining. Chaining was only increasingly used by those that had used it before. With days of silencing during training, it became apparent that those mice which had learnt to use spatial strategies efficiently prior to silencing onset were impaired less. In contrast, those mice that had used spatial strategies to a lesser extent, tended to increase their use of chaining across days. Thus, mice were able to learn about previously known strategies that were useful to them and succeeded to use them increasingly across days. However, random behaviour was prevalent regardless of proficiency level. Additionally, PPC silencing prevented mice from improving across trials within one day. These results point towards the conclusion that although mice seem able to use strategies available as long-term memories, they are unable to acquire them *de novo* under PPC silencing, as they would require short-term memory to be used constructively across trials within one training session in order to improve effectively. The MWM is special in terms of how improvement can only be achieved by trial and error. In other words, learning is dependent on ascribing value to previous behaviours that were carried out rather than to sensory experience alone. Thus, performing any specific behaviour during the water maze task, results in assignment of a retrospective value that relates to the success originating from the former on a short-term memory scale. This means that, as shown by saline control mice, success in a previous trial can be translated into following approaches on a short-term memory scale that is dependent on PPC. If we imagine PPC silencing in mice in the MWM on day 4, a certain repertoire of strategies is available from long-term memories of the previous days. Mice are able to perform the previously learnt spatial strategies, but they are not able to learn spatial strategies from scratch because for this to take place, they would be required to assign value to any of the behaviours that are part of reaching the goal as they perform it. Depending on whether a strategy led them to be sucessful or not. This is clearly shown by their inability to learn from success across trials within one day.

The alternation rule experiment demonstrated in a more straightforward way how value assignment to experience may be the reason why performance in this task fails with PPC silencing. In order to be able to use this memory for decision-making, mice are required to assign value to the trial preceeding the current trial in order to decide

appropriately which side to choose. Although having learnt and consolidated the alternation rule on the previous day, PPC silencing abolishes the ability to perform the rule. What is unique about this task is that there is no stable value attached to deciding for any of the two sides, rather it is dependent on the previous choice-outcome relationship and subject to re-evaluation following every new trial. Thus, although the rule itself can be recalled from long-term memory without PPC function, its implementation fails through the inability to assign value on a PPC-dependent short-term memory scale. In contrast, application of the 1-side rule is PPC-independent once it was learnt because the values ascribed to the choices of particular sides during learning remain constant during recall and hence do not need PPC contribution unless the rule is contradicted.

When looking at the egocentric MWM version, overall, mice were more impaired by PPC silencing. It was not possible to further classify the mice, as I did for the allocentric paradigm, because there were none that improved or remained relatively proficient. In order to extract further information about the ways PPC silencing impaired mice in this version of the MWM, I examined performance on an intra- and inter-trial level on individual days. Examining the behaviour within a trial, we can use the egocentric MWM to teach us more about the impairments mice show with PPC silencing. Because the egocentric MWM contains an identical trajectory with regards to pool entry and platform and we can use this trajectory to compare behaviours across groups and days. PPC silencing did not abolish the ability of mice to recall and perform the correct trajectory, especially during the first days of silencing. However, mice were less precise in successfully reaching the target and failed to compensate with the correct behaviour once the direct trajectory had failed. Instead of using a shuttling behaviour by returning to their entry point, mice turned to random swim, scanning or chaining. It becomes clear that mice were able to recall long-term memory and perform accordingly to the extent that was possible based on the latter. However, as soon as the scope of prediction from long-term memory failed, mice were unable to successfully adapt to changing circumstances by using currently acquired short-term memory. Shuttle search behaviour is key for interpreting the effects of PPC silencing in this version of the MWM. It seems that it is a strategy that cannot fully be performed from long-term memory. The reason for this may be that while the first part of shuttle search is long-term memory-dependent, the second part, the return to the entry and a new attempt,

is dependent on what value every previous attemt has been assigned. For example, if the platform was hit, the value would be positive driving mice to repeat this behaviour. If the platform was missed, the value would be negative, thus forcing mice to compensate, thereby initiating the second phase of what a shuttle search consists of. This would mean that shuttle search is not a uniform strategy *per* se, but in every trial, it represents a *de novo* generated sequence of behaviours attempted to find the hidden platform starting with a direct swim attempt that, if relevant, is followed by an attempt to compensate for the failed prediction of finding the platform. In other words, short-term memory-dependent value has to be ascribed to every direct swim attempt in order for it to result in a shuttle search behaviour rather than being a sequence of movements that is executed until the platform was found. Thus although the shuttle search was repeatedly executed before PPC silencing, it always depends on short-term memory-dependent value and hence requires PPC function. In untreated mice, the only reasonable decision is to return to the entry point as a reference and start over again. However, if mice cannot ascribe the appropriate value to the experience of prediction error and/or are unable to relate this new information to the available long-term memories, they fail to execute the returning part of the shuttle search as is the case when PPC is silenced.

These findings seem crucial for being able to interpret how PPC silencing affects the way short-term memories can be integrated with long-term memories in order to affect decision-making adaptively.

Decision-making and the associated behaviour does not necessarily reflect learning

Interestingly, continuously high freezing observed during extinction under PPC silencing, demonstrates that there is a disconnection between learning and behaviour. It is somewhat surprising that mice can learn extinction without experiencing to unfreeze in the conditioned context until next day recall. This result is important, as it demonstrates that behaviour does not necessarily reflect on the information that a mouse is acquiring or learning at a given time point and PPC seems to play a crucial role in ensuring that decision-making reflects learning on a short-term memory scale. However, this is only the case for learning that can be aquired passively, that is without having to display a particular behaviour in order to gain information. This was shown by the odour FOR, where mice were required to approach the objects as they were

only distinguishable by odour. In this case, mice were unable to learn under PPC silencing conditions as the latter prevented them from exploring the objects.

Decision-making is reliant on PPC function until two distinct windows of memory consolidation have completed

Learning encompasses the formation of a long-term memory. However, during learning i.e. while a new memory is consolidated, decision-making can be dissociated from learning through PPC silencing as discussed in the previous paragraph. The time span during which decision-making requires PPC function in order to implement the new memory seems to reflect the time span until two distinct windows of memory consolidation have taken place (Karunakaran, 2016; Chowdhury, 2018).
PPC silencing during extinction prevented a reduction of freezing, the inability to behaviourally implement the newly acquired memory. Similarly, after having undergone extinction, PPC silencing caused a reinstatement of freezing if retention was tested 11h or ealier after extinction. After 16h or more however, PPC silencing did not cause a reinstatement of freezing.
This suggests that behaviour resulting from long-term memories is guided differently, at least with respect to PPC involvement. PPC plays a role in guiding behaviour during the period of two windows of consolidation taking place, which are known to allow for modification of new memories, and the time in between these windows (Karunakaran, 2016; Chowdhury, 2018). Biologically, it seems plausible to ensure that not all environmental experiences that are transduced into neural signals affect behaviour the same way as consolidated long-term memories. It would be maladaptive if new experiences could easily overpower well established rules in the way they influence decision-making and behaviour. PPC function could represent part of a system functioning as quality control to ensure that not all new experiences are taken at face value and instead ensures that new memories are integrated with potentially existing long-term memories (extinction: fear memory compared to absence of predicted shocks) or other even new memories (acquisition: 1 shock compared to 299 seconds without shocks) in order to reach a reasonable evidence to base decision-making on. This seems to take place independently of ongoing consolidation of new memories, as is suggested by the finding that despite strong freezing reinstatement during PPC silencing post completed extinction at time points of 11h or less, the resulting long-

term extinction memories are unaffected. Once memories have been fully consolidated, they can guide behaviour without having to undergo quality control, independently of PPC function.

A central issue remains however, and that is how one would explain that although PPC silencing causes a failure to assign value to short-term memories, long-term memory-dependent value of i.e. extinction memory is unaffected. Possibly, this is due to decision-making taking place on point, rather than being a memory itself. Possibly, decisions are made *de novo* even if the same decision was previously made based on identical evidence. Hence 'value' might not be ascribed to memories but rather to experiences and, if applicable, outcomes of previous decision-making. This would mean that the value of any experience is not fixed, but rather depends on real-time circumstance and PPC function would only deal with assigning value to newly acquired information. Returning to the example of cFC extinction, recalling extinction memory after 24h would take place as follows in term of decision-making. There is long-term memory available, about the shocks during acquisition but also about the absence of shocks during a much longer period during extinction, thus the decision will be decided between these factual memories and hence lack the need for a *de novo* 'interpretation' leading to a value assigned by PPC. Hence long-term memories would not require interpretation and value assignmet because they have been proven to hold true at least to the extent that they have not been contradicted during the vulnerable period of consolidation. In contrast, newly acquired memories and the herein contained experiences are in need for a value assignment in order to ensure that they are not able to hijack the well-weighed interplay of innate behaviours and a lifetime of learning in order to optimize appropriate decision-making. Alternatively, any memory could be required to be assigned value when taken into account for decision-making, a case in which PPC would only take care of short-term memories. Long-term memories would be assigned value independent of PPC function.

To reconcile these conclusions with previously published work I will very briefly summarize the main findings again. Hwang et al. (2017) showed that PPC silencing abolished influence of recent choice-outcome history on behaviour, and instead increased the focus on learnt rules instead in a visually guided decision-making task in mice. Akrami et al., (2018) showed that in rats, PPC silencing abolished recent sensory-stimulus history and improved rats' performance to focus on trained rules

rather than recent trial history. Electrophysiological recordings showed that PPC neurons carried more information about recent sorsory-stimulus history than the current stimulus.
These results support the interpretation that PPC function is crucial for ascribing value to experiences on a short-term memory scale but lack to dissect how these findings relate to decision-making that is influenced by multiple factors such as innate behaviours and long-term memories. Thus, there is strong incentive to further investigate how decision-making is achieved in relation to the influencing factors discussed in this thesis.

PPC function is implemented by projections to the dorsal striatum

Through employing anterograde tracing, I could show that PPC projects strongly to the DS. This was also previously shown by various labs, most detailed to my knowledge by Hintiryan et al. 2006. For the well-accepted role of the striatum in controlling movement and behaviour, I have picked the DS as a first target to silence PPC projections. Silencing the projections replicated the whole set of phenotypes associated with silencing the entire PPC in the behavioural paradigms employed. My interpretation of this is that these projections could represent the main output of PPC to influence short-term memory dependent decision-making and associated behaviour. Hwang et al. (2019) have shown in the meantime, that the projections from PPC to DS are relevant for recent history-dependent information to affect future decisions. They optogenetically silenced PPC during the inter-trial interval preceeding the current stimulus and showed that this, but not silencing during stimulus presentation, abolished the effects of recent trials on decision-making. This provides further evidence that PPC controls how short-term memory allows for making adaptive choices, but that it is not required for making decision based on previously known information. Importantly, they showed that this role of PPC is conferred via projections to DS. Thus to conclude, PPC to DS projections are relevant for short-term memory dependent-decision-making but not for those decisions that can be based on long-term memory alone.

Projections from PPC to the tail of striatum are necessary for threat-related behaviours

Manipulating the projections from PPC to TS provided insight about the role of PPC for decision-making that is related to caution. Continuous freezing in extinction as well as avoidance of novel objects was induced by silencing these projections. Behaviour in the MWM was not significantly affected. The projections from PPC to TS could take on a specific role in providing short-term memory relating to real or potential threat for decision-making. This is reasonable as TS is known to control object novelty approach behaviour via dopamine neurons originating in the lateral part of SNc and that these dopamine neurons are activated by novel or high-intensity stimuli as discussed in the introduction (Menegas et al., 2019). However, cautious behaviour is not only relevant in the face of current threat, but also when threat is anticipated in a memory-dependent way, with more or less evidence depending on the situation. TS is well separated from the rest of the striatum and shows specific connectivity (Jiang et al., 2018). These include visual, auditory, somatosensory and insular cortical input, value-related inputs from SNc, thalamic and limbic inputs including perirhinal, entorhinal and amygdalar input, as well as multipsensory input from the PPC and claustrum. Thus, TS is well positioned to integrate a multitude of information regarding threat, and potentially other behaviour-relevant information to guide appropriate decision-making.

Matteo Tripodi from our lab has shown through rabies-tracing experiments, that cholinerigc interneurons in the dorsal striatum receive significant projections from the tail of the striatum. These projections could represent a way to integrate threat-related information with other behaviourally-relevant information. Firstly, because threat is such a vital component for survival, it is feasible to imagine a separate system to ensure correct behaviour. Second, because it is reasonable to assume that threat behaviour-related information would eventually converge on the same pathways for implementation as general decision-making. There is much scope for investigating the role cholinergic interneurons play in conferring the functions identified for PPC to TS projections (preliminary results in appendix). Striatal cholinergic interneurons are tonically active neurons (TANs) and it was suggested that the relationship between activity of the latter and dopamine variations achieved through phasic dopamine release induced through TAN firing pauses shape learning and motor adaptation in the striatum (Kim et a., 2019). Most likely, the pathway via TS is not the only pathway accounting for threat-related information, as suggests the finding that silencing PPC-

DS projections produces the full phenotype of behavioural impairments as observed for silencing the entire PPC. There is scope to investigate further how different inputs to TS affect different aspects of threat-related behaviour, both on a time-scale and regarding different types of stimuli leading to behavioural effects.

To summarize, I have shown in this thesis, that PPC plays a crucial role for decision-making that is dependent on the value of new memories on a short-term memory-dependent scale. The main output pathway for this function seems to be the DS. Projections from PPC to TS are relevant for the implementation of threat-related short-term memory-dependent behaviours.
These results suggest that there could be fundamentally distict mechanisms relating to the way newly acquired memories and those that have been consolidated affect decision-making respectively. Possibly, as sensory information is acquired, the extent to which it affects decision-making depends on the value these new experiences are endowed with. It is important for an individual not to take every new experience at face value, especially those contradicting previous long-term memories. Thus optimal behaviour takes into account previously acquired long-term memories and integrates those with new experiences, ensuring that the latter is judged within the appropriate framework of pre-existing information relevant to the situation. Importantly, even in the absence of available long-term memories, multiple experiences on a short-term memory scale also require integration in order to achieve reasonable decisions in the face of complex environmental experiences. Once new memories have undergone consolidation, they can affect decisions independently of PPC, provided that they are not challenged by new evidence against the former. In the latter case, decision-making would again require PPC-dependent value assignment to short-term memory in order to use the latter together with long-term memories as a basis for decision-making.

Materials and methods

Experimental mice

PV-Cre reporter mice (B6;129P2-Pvalb$^{tm1(cre)Arbr}$/J) were ordered from Jackson Laboratories. ChAT-Cre reporter mice (B6;129S6-Chat$^{tm2(cre)Lowl}$/J) were a kind gift from Silvia Arber (Friedrich Miescher Institute). Wild-type mice C57BL/6J were ordered from Janvier.

Mice were kept in temperature-controlled rooms at a constant 12h light/dark cycle with *ad libitum* access to food and water. Experiments were done in accordance with institutional guidelines and approved by the Cantonal Veterinary Office of Basel Stadt, Switzerland.

Behavioural experiments

Male mice of all genotypes were 2-3 months old at the start of each experiment. For cFC experiments, mice were transferred from group housing to single housed cages three days prior to the start of the experiments. For all other experiments, mice were group housed with siblings from weaning age onwards in group sizes of 2-4 mice per cage. Group-housed mice always received identical treatments and experimental conditions.

Contextual fear conditioning

A detailed description of the procedures was published previously by group members (Donato et al., 2013). Briefly, the rectangular conditioning chamber was cleaned with acetic acid (2%) prior to the onset of each session. During acquisition, mice were placed into the chamber, which they could explore freely for 2.5 minutes until they received their first foot shock (1 second duration and 0.8mA). A total of five foot shocks are delivered with an inter-shock interval of 30 second each unless stated otherwise. After 24h, fear memory recall was tested by re-exposing the mice to the conditioning chamber without foot shock delivery for 5 minutes. Memory recall was measured by freezing behaviour. For the fear extinction procedure, mice were not removed from the conditioning chamber after 5 minutes of recall, but instead remained inside for a total of 30 minutes. Gradually decreasing freezing behaviour was measured across the 30 minutes in chunks of 5 minutes. Recall of extinction memory was measured by re-introducing mice to the conditioning chamber 24h following extinction. Freezing

behaviour was defined as complete immobility excluding respiratory movements and quantified constantly except for the first minute of being placed within the chamber. All behaviours were recorded electronically and analysed manually.

Familiar object recognition

Mice were exposed to 2 identical objects in a rectangular arena approximately measuring 30x50cm and left to explore the objects freely for 10 minutes. After 24h, mice were re-introduced to the same context with exposure to one object from the previous day and another novel object. If stated, a third day was added to the procedure where mice were exposed to the novel object from day 2 (now familiar) and another novel object.

One version of this task, called the odour FOR, used only identical objects carrying different odours, in order to make sure that mice had to physically interact with the objects in order to form a memory about their differences. In yet another version of this task, called the social intruder test, one object was replaced with a younger mouse of the same gender under a small cage in order to test social interaction.

Object and context exploration, repetitive (sitting and grooming) behaviour, as well as object discrimination (discrimination index = $(t_{novel} - t_{familiar})/(t_{novel} + t_{familiar})$) were analyzed for each session. In between individual sessions, objects and contexts were cleaned thoroughly with 70% ethanol.

Behaviour was recorded with an overhead HD camera and analyzed using ANY-maze Behavioural tracking software (Stoelting Europe, Dublin, Ireland).

The 1-side and the alternation rule

The set-up for these two experiments was identical consisting of the MWM pool for which I built an insert made of plexiglas in order to divide the pool into three chambers, 1/2 and 2x 1/4 (image shown on page 94). The large chamber served as the starting chamber into which mice were placed facing the wall at the start of each trial. Mice were required to swim into either of the 1/4 chambers through openings of approximately 10x10cm. The correct chamber contained a visible platform from which mice were taken out of the pool at the end of each trial. The visible platform was positioned in a way that it could only be seen once mice had entered the respective chamber, but not from the starting chamber.

Mice were habituated to the pool and platform on the day prior to acquisition of the task. This was done in an open pool in which the platform was positioned randomly. Mice received 4 trials of a maximum of 60 seconds entering the pool from 4 different entry points. Mice were then required to swim to the visible platform to be taken out of the water from there. In the case that mice did not reach the platform in 60 seconds, they were guided towards it by hand and taken from the pool once they had climbed on it. On the day of rule acquisition, mice were placed into the starting chamber and left to explore the pool freely. Mice were allowed to make wrong choices throughout the experiment. Every trial lasted for a maximum of 60 seconds. If after this time, mice had not entered the correct chamber and climbed on the platform, they were taken out of the pool from their current position. However, this happened rarely. Thus, the incentive for a right choice to be made resulted from the shorter duration of the total time in the water. Behavioural rating was dependent on the first choice the mice made in each trial. If they chose the wrong chamber, it was rated as a missed trial although mice were subsequently allowed to swim into the correct chamber after returning to the starting chamber. Thereby mice learned quickly, that correct choices result in fast trials. Confirmation of learning the task was set at 80% trials to criterion, which means that mice had to perform 8 correct trials out of a sequence of 10 trials. The maximum number of trials per day was set at 25. If by then mice had not reached criterion, they were returned to their home cages receiving a score of 25, which was the worst possible in these experiments.

For the 1-side rule, the platform was positioned randomly in one of the chambers and remained there throughout the training and recall, unless the rule was switched.

For the alternation rule, the platform position was changed after every trial so that mice had to learn to alternate their choices.

Behaviour was recorded with a camera attached above the pool. Data collection and analysis was done using Viewer2 Software (Biobserve, Bonn, Germany).

Morris water maze

Our lab has previously published a detailed protocol of the procedures (Ruediger et al., 2012). In summary, the pool filled with opaque water measured 140cm and was surrounded by 3 visual reference cues and otherwise black curtains. The pool water was kept at a constant temperature of 23 degrees Celsius. A circular escape platform with a 10cm diameter was positioned approximately 0.5 centimetres below the water surface. On the first training day, the platform was always visible at approximately 0.5 centimetres above the water surface and highlighted with green tape. On this day, the position of the platform was at the opposite quadrant than during the subsequent training sessions. Mice were trained to navigate to the escape platform which was hidden from day 2 of training onwards by receiving 4 training sessions per day. Each session lasted for a maximum of 60 seconds, after which the mice were taken from the pool even if they have not found the platform. Inter-trial intervals were 5 minutes in order to allow for the mice to dry and warm up.

A modified version of the standard (allocentric) Morris water maze task was also employed. This version is referred to as the egocentric task and was introduced to force mice to employ egocentric navigation, which is not based on visual cues but instead relies on body movements in relation to pool entry and expected hidden platform position. In this setup, visual cues were removed wherever possible. To account for the fact that, despite greatest efforts to remove cues, mice might still be able to form a spatial map about the environment, the escape platform position was changed after every trial in accordance with a change of the entry position to the pool. By using this method, mice are only able to successfully navigate to the platform if they remember the trajectory between entry and platform position. Mice received 4 training sessions per day with a maximum duration of 60 seconds as described above.

For both tasks, latencies to find the escape platform as well as search strategies were used to rate overall task performance. Behaviour was recorded with a camera attached above the pool. Data collection and analysis was done using Viewer2 Software (Biobserve, Bonn, Germany).

Elevated plus maze

This task consisted of an elevated cross of which two opposing sides contained walls. The remaining sides had no walls. Mice were placed at the center and left to explore all four arms for a duration of 5 minutes. Times spent in open arms were compared to times spent in closed arms for the purpose of this experiment. Normally, this task is employed to investigate anxiety or anxiolytic compounds and protocols reported may vary considerable. For this experiment, the purpose was to test whether mice in which PPC is silenced, behaved differently from controls when left to explore the maze. Behaviour was recorded with an overhead high definition camera and the times mice spent in each of the arms were analyzed manually.

Two-chamber choice test

In this task, mice were placed into a plexiglass box containing two chambers that were connected through an opening. One of the chambers was filled with beddding material, while the other one was empty. Mice were always placed into the empty side and left to explore freely for 5 minutes. The times spent in each chamber were compared in order to test whether PPC silencing caused a preference for any one side of the box. Behaviour was recorded with an overhead HD camera and analyzed manually.

Surgical procedures

All surgeries were performed under aseptic conditions initially anesthetizing mice with 4% isoflurane using O2 as a carrier gas (Oxymat3), followed by 1.5% for maintenance throughout surgery. Body temperature was maintained with a heating pad placed under the mouse for the duration of surgery. Buprenorphine (Temgesic) (0.1 mg/kg) was applied subcutaneously as pre-emptive analgesia half an hour before initiation of surgery and another time within 6 hours post-surgery. After induction of anaesthesia, mice were injected subcutaneously with a local anaesthetic, which was a 1:1 mix of Lidocain: 10mg/kg and Ropivacain: 3mg/kg (Naropin, Astra Zeneca) in the area of the surgery. Meloxicam (Metacam, 5 mg/kg), was given at the day of surgery once mice were awake and on the two days following surgery with breaks not exceeding 24h.

Injections were made using a stereotaxic frame (Kopf instruments). Virus injections were done using glass capillaries (tip diameter 10– 20 µm) connected to a picospritzer (Parker Hannifin Corporation). Usually, 100nl of virus (or saline for controls animals) were injected per injection site. Depending on the region of interest, virus injections were carried out over a period of several minutes, with a 10 min delay period before extracting the capillary from the injection site for subcortical structures to avoid backflow to cortical areas. Stereotaxic coordinates were:

PPC: AP -2mm; ML ± 2.1mm; DV +1.2mm

DS: AP +1.2mm; ML ±1.4mm; DV +2.7mm

TS: AP -0.85mm; ML ±3.2mm; DV +2.8mm

After surgery, mice were left to fully wake up in their home cage, which was placed on a heating plate for approximately 20 minutes. Overall, mice were left to recover for at least 8 days after surgery before any behavioural procedures were carried out.

Anterograde and retrograde neuronal tracing

For anterograde tracing, approximately 100nl of AAV8-CamKIIa-mCherry-Cre were injected unilaterally together with SynaptophysinGFP (1:1) into PPC of wild type mice. For retrograde tracing from PPC, the same amount of RetroAAV-Cre-H2BGFP was injected unilaterally in the PPC of wild type mice. Mice were returned to their home cage for at least 14 days before perfusion to allow for virus expression.

In vivo pharmacogenetics

The procedures for silencing and activating neurons through non-endogenous PSAM-carrying AAV9 was previously described in detail by Magnus et al. (2011).

For silencing of specific projections, floxed PSAM-carrying AAV9 (inhibition: rAAV9-CBA-flox-PSAM(Leu141Phe,Tyr116Phe)GlyR-WPRE) was delivered bilaterally in the region of projection origin of wild type mice. During the same surgery, a cre-delivering retrovirus (retroAAVCre-H2BGFP) was bilaterally injected into the projection target regions. After a period of 14 days, acute silencing of projections was achieved through i.p. injection of the non-endogenous ligand PSEM308 (5mg/kg) 15 minutes before behavioural procedures in order to activate the PSAM channels. A similar approach was used for ChAT-cre mice, where floxed PSAM-carrying AAV9 (inhibition: rAAV9-CBA-flox-PSAM(Leu141Phe,Tyr116Phe)GlyR-WPRE) was injected bilaterally in order

to acutely silence ChAT-expressing neurons in the target region through PSEM308 injection no earlier than 8 days following surgery.

For silencing of the whole PPC by activation of PV-positive inhibitory interneurons, floxed PSAM-carrying AAV9 (excitation: rAAV9-CAG-flox- PSAM (Leu41Phe,Tyr116Phe)5HT3-WPRE) was injected bilaterally into PV-cre mice. After waiting for virus expression for at least 8 days, mice were also injected i.p. with PSEM308 (5mg/kg) 15 minutes before behavioural procedures.

Immunohistochemistry and image acquisition

After behavioural procedures, mice were transcardially perfused with chilled 4% paraformaldehyde (PFA) in PBS at pH 7.4. Brains were extracted and kept for a post-fixation period of 12h in same 4%PFA solution before being transferred to a 30% sucrose solution for at least 24h. Brains were sectioned at 40um thickness using a cryostat and kept in PBS at 4° Celsius until staining.

In order to detect the positions for virus injections and to control for spread, α-Bungarotoxin, Alexa 488 Conjugate (Molecular Probes, Life Technologies, B-13422) was used at a concentration of 1:500 for 2 hours in 3% BSA in PBS-T (0.3% Triton X-100 in PBS) to detect the expression of rAAV9-CAGflox-PSAM(L41FY116F)5HT3-WPRE. Images were taken at 10x magnification using the ZEISS axioscan.Z1 (TK instruments) automatic slide scanner.

Statistical analysis

The software GraphPad Prism 8.4.2 (published April, 7, 2020 by GraphPad software Inc.) was employed for all statistical analyses. Statistical analyses were performed using Student's t tests, two-way ANOVA (or mixed effects analysis for Fig.11) with Sidak's multiple comaprison test; Statisitcal significance was set at $P < 0.05$. Results are presented as mean ± s.e.m. Data distributions were assumed to be normal but this was not formally tested. Male mice of similar age were assigned randomly to experimental groups. The size of experimental groups is reported in the figure legends. Where group sizes differed, individual group sizes were reported as follows n=PSEM/saline.

Appendix

Exposure to novel and familiarized object carrying the smell from the home cage increases interaction with the latter but does not affect novel object interaction

I was curious to test how mice would behave if they were exposed to the known object from familiarization, together with a new one during the test. For this experiment, I picked a 24h familiarization window for technical reasons. After 24h familiarization, mice were injected with PSEM or saline respectively and exposed to the object from the home cage, together with a new one in a neutral context (Fig.I). Silencing PPC caused mice to spend significantly more time at the old object, hence shifting the discrimination index towards the old object (Fig.Ia). However, silencing did not affect the total time, which mice spent at the new object compared to controls, it is only so that they spent even more time at the old object, which is also reflected in an overall decreased context exploration of treated mice (Fig.Ib). Saline control mice discriminated well for the novel object and spent more time exploring the latter. Repetitive behaviour was not different from saline control mice (Fig.Ic).

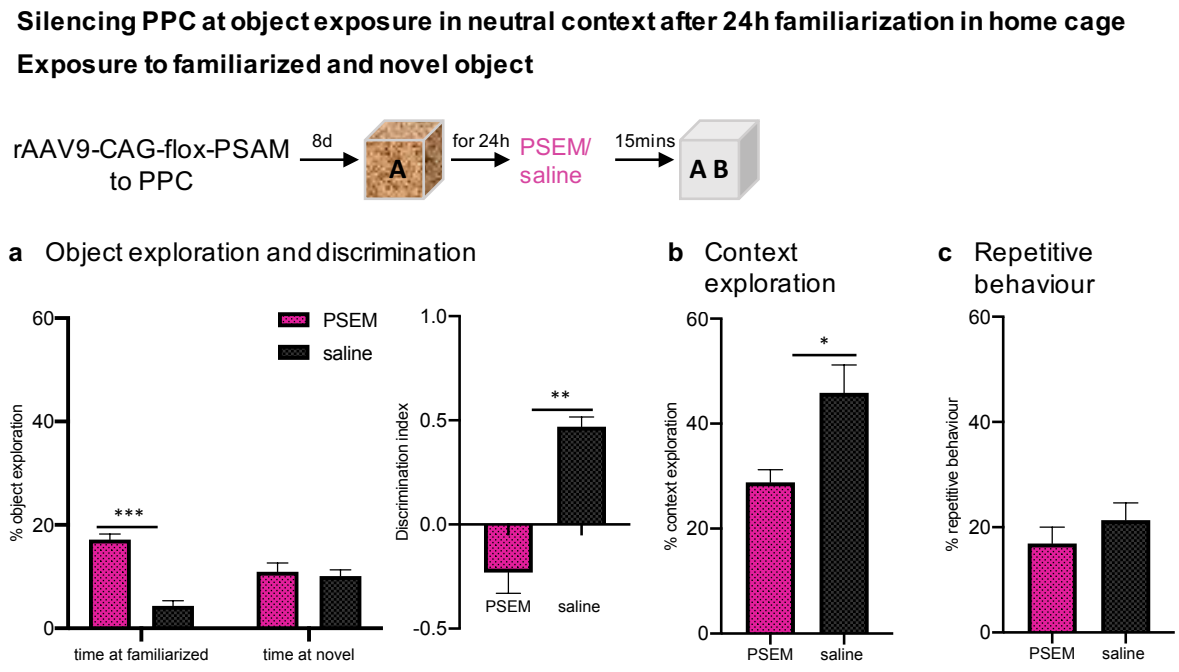


Fig.I (a-c) Silencing PPC during exposure to familiarized and novel object after 24h exposure PPC silencing **(a)** increased familiarized object exploration but did not affect novel object exploration (2-way ANOVA with Sidak's multiple comparison PSEM vs. saline: time at old P=0.0001; time at new P=0.8999 n=4/3) Object discrimination was shifted towards the familiar object in the treated group (unpaired T-test P=0.0024 n=4/3) **(b)** reduced context exploration (unpaired t-test P=0.0228 n=4/3) **(c)** did not affect repetitive behaviour (unpaired t-test P=0.3787 n=4/3)

Silencing of cholinergic interneurons in dorsal striatum

Because TS was found to target DS mainly at cholinergic interneurons (unpublished data from our lab from Matteo Tripodi), I was curious to see whether silencing the receiving population of TS input to DS would cause a behavioural effect in these experiments.

With the use of a Chat-Cre transgenic mouse line expressing cre recombinase in their cholinergic interneuron population, I could test the effects of silencing the cholinergic interneurons within the DS through bilateral delivery of floxed PSAM-carrying AAV9 (inhibition: rAAV9-CBA-flox-PSAM(Leu141Phe,Tyr116Phe)GlyR-WPRE). Fig.II shows sparse inhibitor virus expression in cholinergic neurons within the striatum.

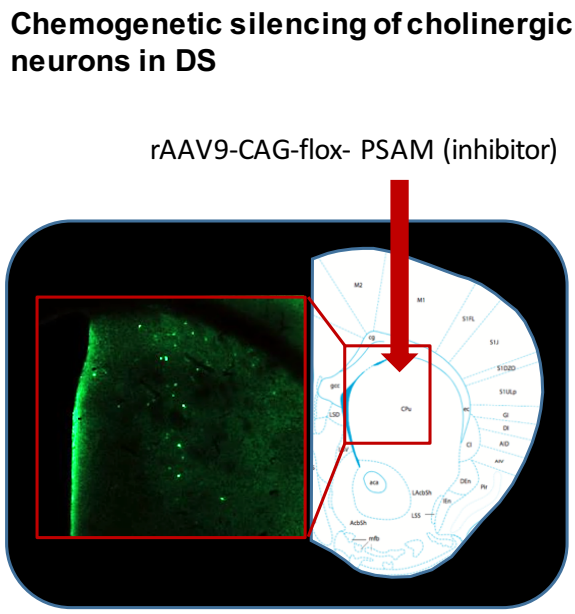


Fig.II Chemogenetic silencing of cholinergic interneurons in DS sparse expression of Cre-dependent AAV9 chemogenetic inhibitor virus in cholinergic neurons of Chat-Cre mice visualized with bungarotoxin staining (left) Schematic representation of DS location according to Paxinos mouse brain atlas (right)

Silencing cholinergic interneurons on day 2 of the 3-day FOR

In order to silence cholinergic interneurons in DS, I injected PSEM or saline respectively 15min prior to testing on day 2 of the 3-day FOR. On day 1 and 3, there was no treatment done in either group. Silencing cholinergic interneurons on day 2 of the 3-day FOR, the phenotype observed closely resembled the one induced through silencing either PPC itself or projections from PPC to DS or TS. The phenotype was pronounced regarding object exploration and discrimination on the day of silencing compared to saline controls (Fig.IIIa). Context exploration and repetitive behaviour however, was only affected mildly (Fig.IIIb,c).

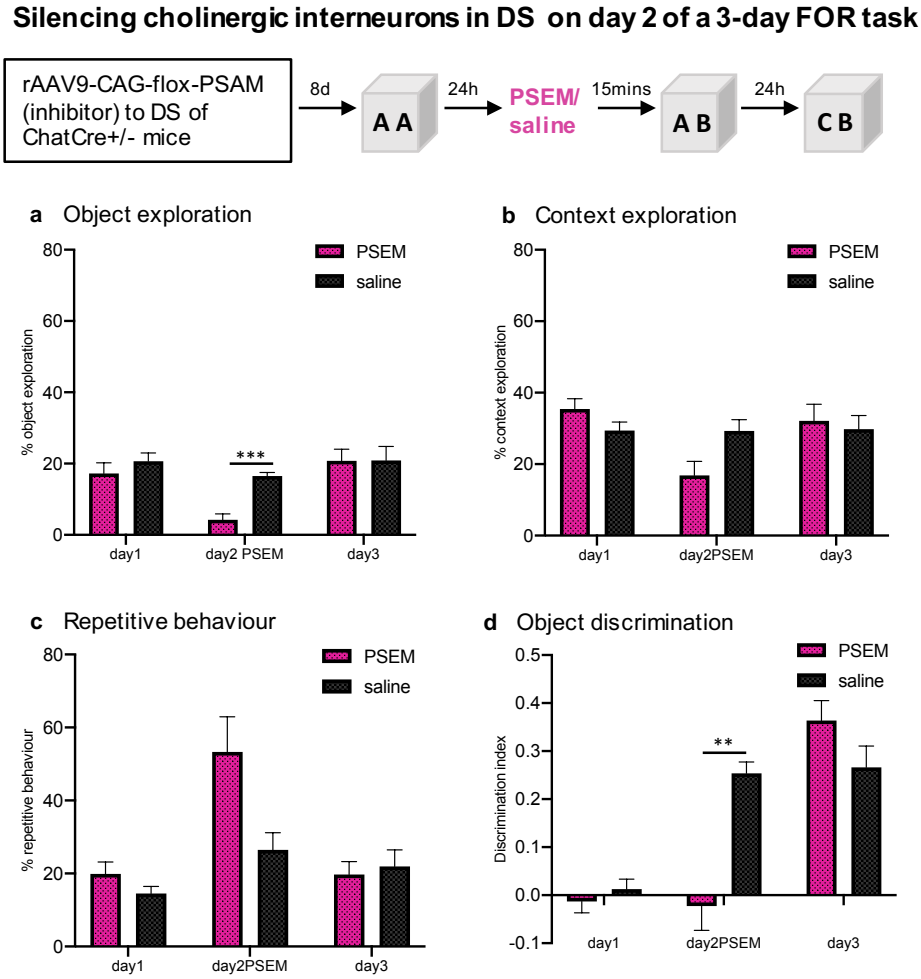


Fig.III Silencing cholinergic interneurons on day 2 of a 3-day FOR task (a) reduced object exploration (2-way ANOVA with Sidak's multiple comparisons test: Interaction F(2,24)=4,245 P=0.0264; time F(1,498,17,98)=13,39 P=0,0006; group F(1,12)=3,012 P=0,1082 multiple comparison PSEM vs. saline, day1 P=0,7651, day2PSEM P=0.0002, day3 P=0.1082 n=8/6) **(b)** mildly reduced context exploration (2-way ANOVA with Sidak's multiple comparison test: Interaction F(2,24)=8,438 P=0,0017; time F(1,469,17,62)=8,932 P=0,0040; group F(1,12)=0,08898 P=0,7706; multiple comparison PSEM vs. saline, day 1 P=0.3369, day2PSEM P=0.0870, day3 P=0.9739 n=8/6). **(c)** and mildly increased repetitive behaviour (2-

way ANOVA with Sidak's multiple comparison test: Interaction F(2,24)=5,087 P=0,0144; time F(1,380,16,56)=13,38 P=0,0009; group F(1,12)=2,935 P=0,1124; multiple comparison PSEM vs. saline, day 1 P=0.4594, day2PSEM P=0.0912, day3 P=0.9755 n=8/6). **(d)** abolished object discrimination on the day of silencing but not thereafter (2-way ANOVA with Sidak's multiple comparison test: Interaction F(2,24)=11,27 P=0,0004; time F(1,825, 21,89)=31,50 P<0,0001; group F(1,12)=5,902 P=0,0318; multiple comparison PSEM vs. saline day1 P=0.8130, day2PSEM P=0.0019, day3 P=0.3593 n=8/6).

Silencing cholinergic interneurons in cFC extinction

In order to test the effects of silencing cholinergic interneurons during extinction, I injected PSEM or saline respectively 15min prior to starting extinction 24h after initial cFC acquisition without treatment. Silencing cholinergic interneurons during cFC extinction did not affect mice during the initial decrease in freezing observed during the first 15 minutes compared to saline controls (Fig.IVa). However, for the remaining duration of extinction, there was no further decrease in freezing observed when cholinergic neurons in DS were silenced. In contrast, saline controls continued to decrease their freezing levels. Retention without any treatment done tested 24h after extinction was not different between the groups (Fig.IVb).

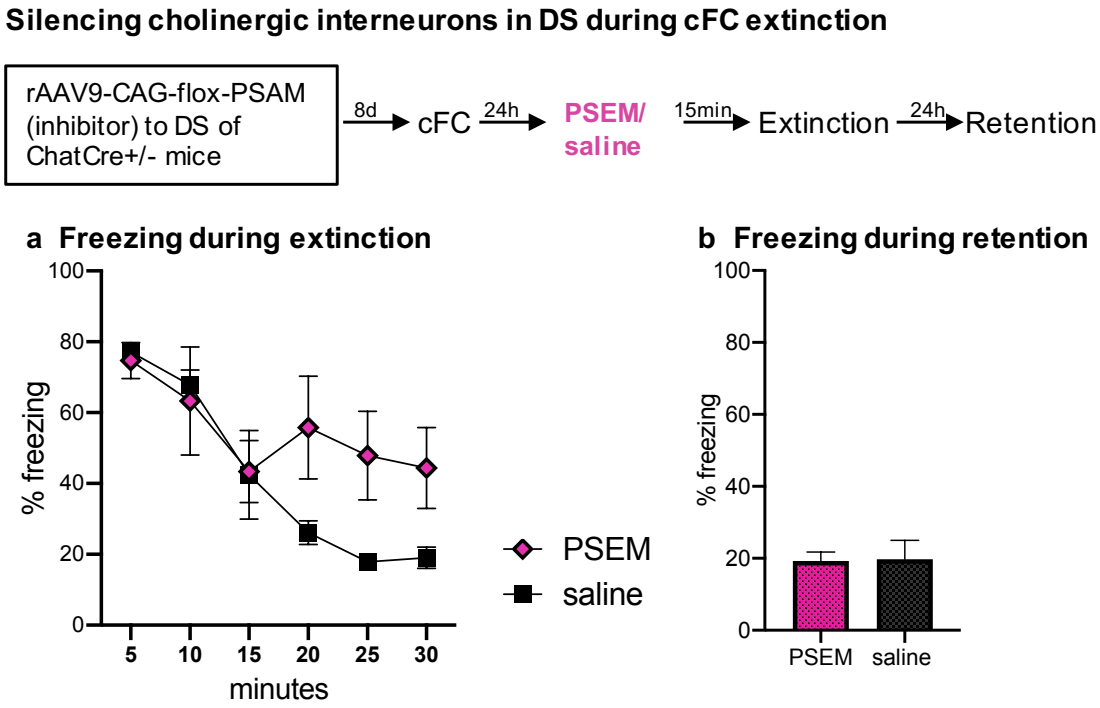


Fig.IV Silencing cholinergic neurons in DS during cFC extinction (a) prevented a reduction in freezing beyond 40% across the 30 minutes period but did not affect an initial decrease in freezing (2-way ANOVA with Sidak's multiple comparison; Interaction F(5,20)=4,464 P=0,0068; time F(2,175, 8,701)=21,05 P=0,0004; group F(1,4)=1,445 P=0,2956 n=3/3) **(b)** did not affect freezing during retention (unpaired t-test P=0,9292 n=3/3)

Silencing cholinergic interneurons from day 4 in the MWM

When the effects of silencing cholinergic neurons in DS were tested in the MWM, there was no effect on escape latency across the days of silencing (Fig.Va). Looking at inter-trial improvement, there was no difference between silenced mice and controls. Both groups were able to improve from previous success within one training session. Concerning swimming strategies, there was a very mild effect of silencing cholinergic interneurons from day 4 onwards (Fig.Vc,d). Mice showed some increase in the use of random swim from the onset of silencing. This seemed to occur at the expense of directed spatial search strategies used during those days.

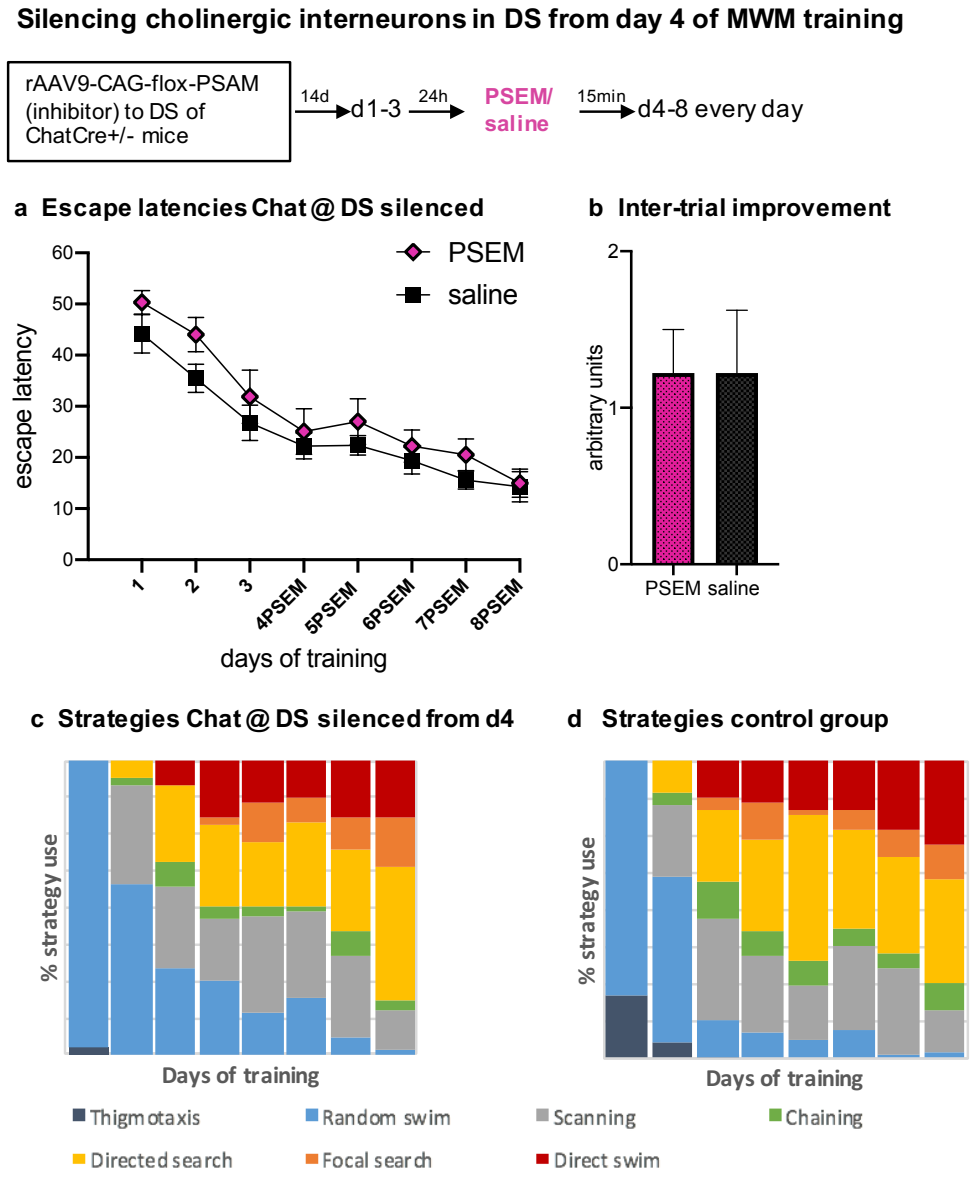


Fig.V Silencing cholinergic neurons in DS from day 4 in MWM (a) escape latencies remained unaffected (2-way ANOVA with Sidak's multiple comparison Interaction F(7,91)=0,2459 P=0,9723; time F(3,360, 43,67)=21,57 P<0,0001; group F(1,12)=3,141 P=0,0998 n=9/9) **(b)** inter-trial improvement scores were not affected (unpaired t-test

P>0,9999 n=9/9) **(c,d)** strategy development was only mildly affected by silencing. Random swim was slightly increased at the expense of spatial search in the PSEM treated group.

Overall, the effects of silencing cholinergic interneurons in DS were milder compared to silencing the projections from PPC to DS or TS regarding behaviour involving caution. The effects of silencing DS cholinergic interneurons in the water maze were restricted to a slightly increased use in global strategies. To conclude, cholinergic interneurons in DS seem relevant for conferring the functions PPC exerts via its projections to TS.

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