Cortical circuits underlying flexible learning

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Abbreviations

ACC anterior cingulate cortex
ACd dorsal agranular cingulate area
ACv ventral agranular cingulate area, dorsal and
AId1 dorsal agranular insular area, dorsal part
AIp posterior agranular insular area
Al agranular insular cortex
AM anteromedial thalamic nucleus
ASST attention set-shifting task
ATN anterior thalamic nuclei
BLA basolateral amygdaloid nucleus, anterior part
cFC contextual fear conditioning
CL centrolateral thalamic nucleus of thalamus
Cl Claustrum
CNO clozapine-N-oxide
(d)HP (dorsal) hippocampus
DI dysgranular insular area
DLS dorsolateral striatum
DMS dorsomedial striatum
dPreL dorsal prelimbic cortex
DTT dorsal tenia tecta
Ect ectorhinal cortex
Fr2 frontal area 2
iAD interanterodorsal thalamic nucleus
iAM interanteromedial thalamic nucleus
IL infralimbic area
IL infralimbic cortex
LD lateral dorsal nucleus of thalamus
LDDM laterodorsal thalamic nucleus, dorsomedial part
LDVL laterodorsal thalamic nucleus, ventrolateral part
LO lateral orbital cortex
LPMR lateral posterior thalamic nucleus, mediorostral part
LPtA lateral parietal association cortex
M2 secondary motor cortex
MCC midcingulate cortex
MD mediodorsal thalamic nucleus
MDL mediodorsal thalamic nucleus, lateral part
MO medial orbital area
mPFC medial prefrontal cortex
MPtA medial parietal association cortex
PaF parafascicular thalamic nucleus
PC paracentral thalamic nucleus
PCC posterior cingulate cortex
PFC Prefrontal cortex
PL Prelimbic area
PRh perirhinal cortex
PSub postsubiculum

PTSD post-traumatic stress disorder
RSA agranular retrosplenial cortex
RSC retrosplenial cortex
RSG granular retrosplenial cortex
S1 primary somatosensory cortex
V1 primary visual cortex
V2MM secondary visual cortex, mediomedial are
VA ventral anterior thalamic nucleus
VL ventrolateral thalamic nucleus
VLO ventrolateral orbital area
VLOp posterior ventrolateral orbital area
VM ventromedial thalamic nucleus
VO ventral orbital area
ZI zona incerta

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Preface

Animals survive and compete in their environment by making adaptive memories of the situations they have encountered. Flexible learning then allows to adjust to the great variety of possible environmental changes. This requires comparison of past and present values of rewards and costs associated with behaviour in order to make a decision whether the default course of behaviour needs to be adjusted to the new circumstances. This complex behaviour involves a variety of brain functions, such as detection of the salience of stimuli and its changes, memory of the history of reinforcement, and cognitive control of behaviour. These functions have most consistently been associated with the subdivisions of medium prefrontal cortex and limbic areas of the brain. Within this context, the anterior cingulate cortex (ACC), a prefrontal area, is of special interest due to its specific position within prefrontal and limbic brain systems. ACC has recently been a focus of extensive research in humans, primates and rodents. However, despite a wealth of descriptive data, and numerous theories about the role of ACC in sensory, motor and cognitive processes, it has not yet been possible to combine current views on the function of ACC in cognition into a coherent model.

In my thesis, I explore the role of mouse ACC in flexible learning. I use chemogenetic silencing to locally interfere with the acquisition and consolidation of memory in order to investigate the role of ACC in Pavlovian and non-Pavlovian forms of learning. First, I address the role of ACC in attention set-shifting tasks, which represent a close analogue to the foraging paradigms that have mainly been explored in monkeys. Second, I compare the function of ACC in acquisition and consolidation of single-trial and multi-trial versions of contextual fear conditioning (cFC) learning. By utilising newly available genetic tools, which allow us to selectively silence the group of cells projecting to the area of interest and further manipulate it, I then proceed to a more in-depth study of ACC function within the wider brain network. To this end, I describe in detail the connectivity of ACC with other brain areas, and then address the role of those ACC-based networks in acquisition, consolidation and modification of learning. My results reveal how the function of ACC in supporting flexible learning is embedded dynamically within a specific network of systems, within which specific areas are associated with different forms of subsequent learning. This study provides a

comprehensive view of how ACC and the structures monosynaptically connected to it are implicated in the formation of adjustable memories.

1.1 Introduction

Structure and function of the prefrontal cortex

The prefrontal cortex of the brain (PFC) is one of the most lately developed cortices both phylogenetically and in its ontogenesis. In primates, it reaches the highest relative growth compared to all other species. PFC is an association cortex comprised of several Brodmann areas, located on the frontal lobe of the brain. Those areas include, in humans: the frontal eye fields (area 8), dorsolateral prefrontal cortex (area 9), anterior prefrontal cortex (area 10), orbitofrontal area (area 11), orbitofrontal area (area 12), insular cortex (13), ventral anterior cingulate cortex (24), dorsal anterior cingulate cortex (32), dorsolateral prefrontal cortex (46), pars orbitalis, part of the inferior frontal gyrus (47) (Brodmann 1909; Jerison 1994). Although human PFC has the highest level of development among all animal species, some shared homology still could be identified between the major subdivisions of human and rodent PFC. For instance, rodent infralimbic area (IL) can be related to Brodmann area 25, while prelimbic (PL) and anterior cingulate area (ACC) in rodents share homology with areas 32 and 24 in humans respectively (Bicks et al., 2015)

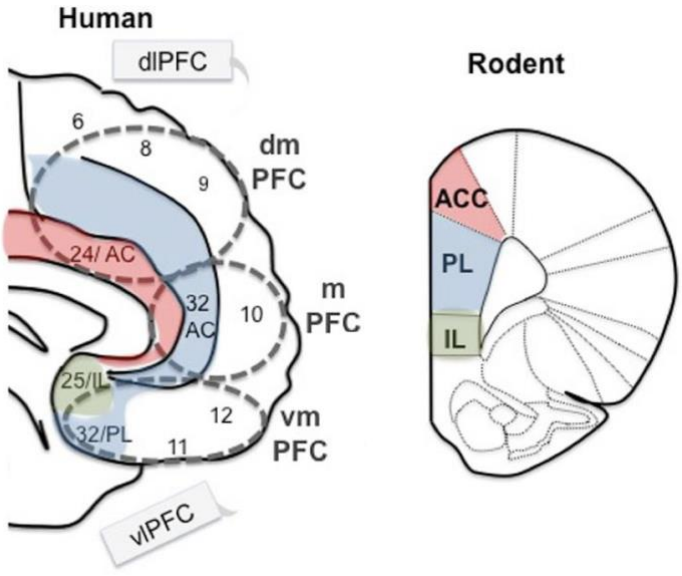


Fig 1.1 Homology of different areas of human and rodent PFC
AC – anterior cingulate, dlPFC – dorsolateral prefrontal cortex, dmPFC – dorsomedial prefrontal cortex, IL – infralimbic, mPFC – medial prefrontal cortex, PL – prelimbic, vlPFC – ventrolateral prefrontal cortex, vmPFC – ventromedial prefrontal cortex.
Adapted from Bicks et al., 2015

Current research recognizes the fundamental role of PFC in cognitive control and decision making. These functions are necessary for adapting behaviour to adjust to changing circumstances, select actions based on expected costs and benefits, as well as exerting top-down inhibition and control of undesirable actions. PFC is thought to execute in part its critical role in flexile decision making through its modulatory influence on affective and reward systems (Beauregard et al., 2001; Hare et al., 2009; Knoch et al., 2006). Research on the neural basis of adaptive behaviour describes functions of its lateral (Brass et al., 2005; Egner and Hirsch, 2005) and dorsomedial (Botvinick et al., 2004; Rushworth et al., 2004; Badre et al., 2009) aspects.

The medial prefrontal cortex (mPFC) constitutes the highest level of the cortical hierarchy dedicated to representation and execution of actions. Anterior and posterior areas of mPFC are functionally different. More anterior areas are thought to guide more abstract representations, while more posterior areas encode more concrete representations, important for specific actions (Badre et al., 2009; Koechlin et al., 2003). The medial region of the PFC, which includes the most anterior portion of the cingulate gyrus, appears to be generally involved in motility, attention, and emotion. Lesions of this region commonly lead to loss of spontaneity and difficulty in the initiation of movements and speech (Verfaellie and Heilman, 1987; Cummings, 1993). In humans with a large lateral prefrontal damage, the most common disorder is the inability to formulate and to carry out plans and sequences of actions. Ventromedial prefrontal cortex has a role in coding outcomes, establishing stimulus-outcome associations and encoding reward value of a stimulus (Kennerley and Wallis, 2009; Rushworth et al., 2011; Bartra et al., 2013). At the same time, the anterior cingulate cortex (ACC) has a complementary role in reward prediction and error prediction coding (Kennerley et al., 2011; Silvetti et al., 2013).

The lateral prefrontal cortex (lPFC) is implicated in self-control, which has a distinctive representation in subareas of lPFC. Dorsolateral PFC engages flexible cognitive control through interactions with dorsomedial PFC and perceptual cortex (Egner and Hirsch, 2005; Kerns et al., 2004). Ventral and posterior lPFC support inhibitory control and response stopping via circuit interactions with the pre-supplementary motor area and subthalamic nucleus (Aron and Poldrack, 2006; Nachev et al., 2007). Rostrolateral PFC is thought to support self-control implemented via highly abstract goals, intentions, and strategies (Ariely and Wertenbroch, 2002).

The PFC is connected with other association cortices, but not with primary sensory or motor cortices. Within PFC dorsal areas are heavily connected with sensorimotor and association neocortical areas, whereas ventral areas virtually lack such connections but have extensive connections with the amygdala and temporal, limbic association cortices, and strongly project to the septum, the medial preoptic and hypothalamic areas and the brainstem (to primarily monoaminergic cell groups) (Heidbredera and Groenewegen, 2003).

Frontal memory network and posterior network are required during novel and complex behaviour and are not engaged during routine, automatic, or overlearned behavioural sequences (Grafton et al., 1992; MacLeod et al., 1998; Phillips et al., 2019). As the memory networks of posterior cortex acquire associations with action, they extend into the PFC to shape the networks of executive memory (Fuster, 2001).

Overall, the PFC plays a crucial role in the two major aspects of flexible behaviour. The PFC inhibits undesired actions as the result of favouring one particular behavioural response over the others. At the same time, it evaluates the result of the chosen actions to compare the expected reward value with the actual received value. Those functions depend on distinctive areas of the PFC, which process different aspects of cognitive control and decision making.

Anterior cingulate cortex

The function of the dorsal anterior cingulate cortex is a yet unsolved puzzle in cognitive and systems neuroscience. It was linked by Papez to the limbic system and emotional processing. However, in the late 1990s imaging studies revealed a novel role for dACC in cognitive functions, while neuroanatomical methods established a close connectivity between dACC and motor areas. Despite extensive data produced by neuroimaging, anatomical and electroencephalographic studies, these do not lead to a coherent view of dACC function. The main challenge remains in bringing together the views and theories produced by the various experimental methods, including what each of them is able to firmly contribute. Such challenges need to be addressed before a unifying theory of dACC function can be attempted.

Anatomy of ACC

One of the most widely accepted parcellations of cingulate cortex was completed by Vogt (Vogt et al. 2005, Vogt and Gabriel 1993), who described cingulate cortex as comprised of four major subdivisions: the anterior cingulate cortex (ACC, very rostral part of the cingulate cortex, consisting in non-human primates of Broadmann areas 24, 25, 32), the middle and caudal parts of the cingulate cortex, which are designated as midcingulate cortex (MCC, Broadmann areas 24a´, 24b´), the ventral and dorsal parts – designated as posterior cingulate cortex (PCC, Broadmann areas 23 and 31), and finally the most caudal part of the cingulate cortex, which is constituted by the retrosplenial cortex (RSC, Broadmann areas 29 and 30).

The dACC in humans is located dorsal to the genu of the corpus callosum, between frontopolar cortex (rostrally) and posterior cingulate cortex (caudally). Due to the substantial evolutionary gap between them, primate and rodent PFC exhibit a number of differences, which have to be taken into account in studies aiming to translate rodent research into primate models or human studies. Since primate and especially human prefrontal cortex is characterised by the highest developmental level among the animal species, there is no exact equivalent of primate dACC in rodents. However, there is a number of important similarities between the anatomy of the primate area 24, of which dACC is a part, and area 24 in rodent ACC. While rodent–primate ACC correspondences are not precise, they are stronger than those for many other prefrontal areas (Passingham and Wise, 2012). Particularly, in their study (Vogt and Paxinos, 2014) Vogt and Paxinos conclude that rodents and primates, including humans, possess MCC, along with homologies in ACC and retrosplenial cortices, which in turn permit scientists to test hypotheses on rodent models of the respective human diseases (Fig 1.2).

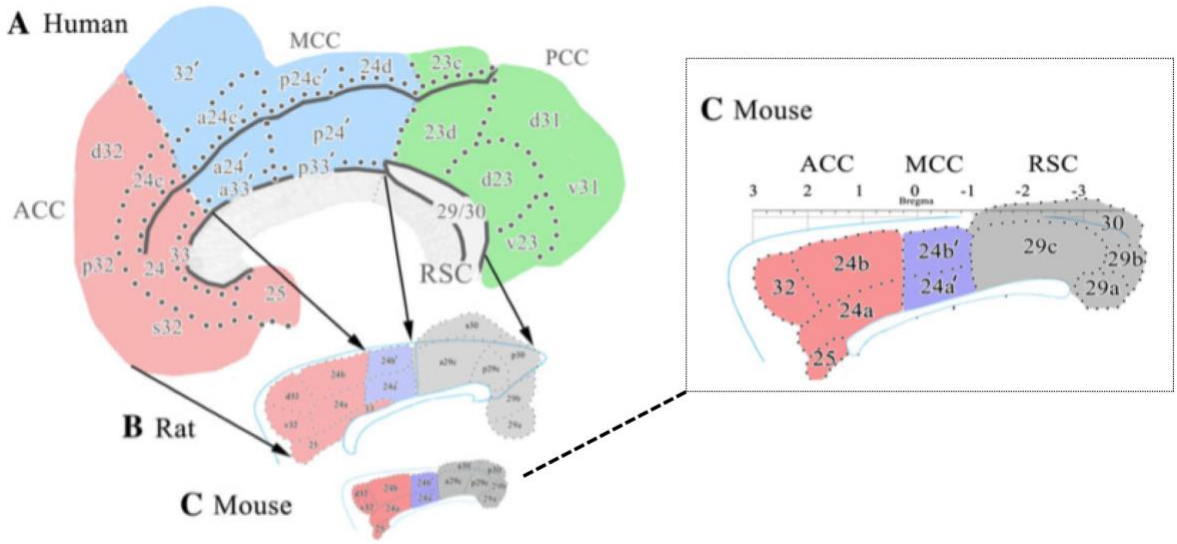


Fig 1.2 Comparison of rodent and human brain maps of cingulate cortex.
Labelling with similar colours and arrows between human and rat maps emphasize homologous relations between ACC, MCC and RSC (adapted from Vogt and Paxinos, 2014)

Overview of major theories concerning dACC function

Since dACC was first described in literature, theories on its function kept growing in numbers as more neuroscience techniques emerged. Numerous studies have reported a great variety of cognitive functions and psychological variables to be dependent on dACC. However, those studies remain mostly fragmented and interpretation of the neurophysiological data differs according to research priorities of the separate study groups, leading sometimes to conflicting hypotheses.

One group of theories describes the role of dACC in the process of new learning. According to these studies, in human and in animals dACC is considered as part of a larger network underlying early stages of learning, when a subject is gaining insights into the nature of a task. At this stage, control and flexibility are important for successful task performance. dACC was shown to be sensitive to the probabilistic occurrence of the condition stimulus, and active during reversal learning. In this context, dACC exherts cognitive control as part of a more general start and stop system, made up of the anterior and posterior cingulate, the subiculum, the anterior ventral and mediodorsal nucleus of the thalamus (Bussey, T.J. et al. 1996; Gabriel, M. 1990; Brooks, V.B., 1986; Gemba, H. et al.,1986).

A different group of theories belongs to the Oxford view and concentrates on behavioural adaptation and persistence. This interpretation of dACC function refers to its role in decision-making, rather than cognitive control. At the basis of this functionality lies the ability of dACC to encode values of choices and outcomes, signalling the value and costs of behavioural shifts while regulating behavioural adaptation and being possibly implicated in the value comparisons. The activity of dACC is highest during sequential decision-making tasks, when research subjects reject an offer or a default behavioural choice and switch to an alternative. Within this framework, dACC is proposed to have broad effects on subsequent behaviour, changing one's estimate of the environment, adjusting learning rates or altering high-level strategies (Kolling et al. 2016; Boorman et al., 2013; Hare et al., 2011)

A further view on dACC function is known as "Expected value of control", or the Princeton view. (Kolling et al., 2016; Shenhav et al., 2016; Shenhav et al., 2013). The modern version of this theory shares the concept of dACC being able to increase top-down control when it detects the need for it, such as in a situation of conflicting choices or reversal learning. At the same time, dACC also computes the value of control required by the task. In this case, signal strength in dACC gradually reflects the effort required to exert behavioural control.

These theories agree on two major roles of dACC. First, it is active when a particular response is not performed (suppressed or rejected) in favour of a different one, particularly when one of the potential responses represents a default choice. And second, dACC signals values and costs of alternative behavioural strategies, ultimately setting strategy choices one or several trials into the future (Ebitz and Hayden, 2016). Decision making processes also require other functions, which are as well attributed to ACC. For example, ACC has a role in guiding motor behaviour using reward information (Paus, 2001), it monitors and adjusts behaviour (Rushworth et al., 2004), it tracks the balance of evidence in favour of one of the available options for further adjustments of action strategy (Shima and Tanji, 1998), and it estimates the opportunity cost of the next best alternative.

A further development of studies of flexible learning describes the role of ACC in schema formation. For flexible learning, humans form coherent frameworks of knowledge called "mental schemas" (Bransford 1979; van Kesteren et al. 2010). Schemas can serve for advanced organisation of information in the brain, representing

a framework for storing and organisation of information during learning. This, in turn, facilitates recall of acquired information, as well as fast and efficient incorporation of new information. Rodents were reported to be able to form schemas, as was shown by their ability to learn concurrently multiple flavour–place paired associations and form strategies that allow for rapid future learning (Tse et al. 2007). Importantly in this context, one of the recent studies (Wang, et al., 2012) provided evidence that ACC is implicated in schema formation and storage.

Thus, the role of ACC in flexible learning involves several major functions, such as cognitive control, decision making and schema formation. In particular, ACC can use information about the expected reward of available options to plan the behavioural strategy of future trials, and decide upon staying or switching from the default option. Indeed, some studies suggest that cingulum can be considered the fifth lobe of the brain, with a unique role that distinguishes it from adjacent frontal cortex regions, and with similarities to pregenual and subgenual cingulate as well as PCC (Ebitz and Hayden, 2016).

The connectivity of dACC

dACC exhibits a broad range of connections (Barbas and Pandya 1989, Morecraft and Van Hoesen 1998, Vogt and Pandya 1987). These include prominent projections to and from the major brain systems that are associated with emotion (amygdala, hypothalamus, ventromedial prefrontal cortex (vmPFC), insula, ventral striatum), cognition and executive control (dorsal prefrontal cortex, ventrolateral prefrontal cortex, frontal pole, parietal cortex), and motor control (motor cortex, premotor cortex, spinal cord). The three major sets of connections (emotional, cognitive and motor) have served as foundations for theories of dACC function (Morecraft and Van Hoesen 1998; Paus 2001, Rushworth et al. 2011).

Of relevance to this study, ACC receives strong input from subareas of the RSC (Shibata et al. 2004), but the functional role of this input was not yet established. Papez first suggested that the cingulate cortex (comprised of the anterior cingulate and retrosplenial cortices) was involved in processing limbic information. The modern view describes the general role of the Papez circuit as being implicated in a large variety of

functions, including emotional regulation and memory formation (Van Groen and Wyss, 1990; Van Groen and Wyss,1992a Van Groen et al., 2002).

The Hippocampal–diencephalic–cingulate (extrahippocampal) network

Overview of the extrahippocampal network

The Papez circuit was initially described as several highly interconnected areas and their interconnecting pathways, namely: the hippocampus, which is linked with the mammillary bodies of the hypothalamus, the anterior nucleus of the thalamus and the cingulate gyrus, which in turn project back to hippocampus; it is vital in particular for episodic memory (Aggleton and Brown, 2006; Vann and Nelson, 2015).

However, other areas of the cerebral cortex are as well recruited into the various functions associated with the Papez circuit, largely through the connections of the cingulate gyrus (Haines and Mihailoff, 2017). Understanding the functional contribution of each of the additional components of this circuit, therefore, represents one of the key challenges in memory research.

The Papez circuit together with the limbic cortex of Broca is called the limbic system of the brain (Paul MacLean, 1949, 1952), which plays a key role in regulating emotions, memory, personality, spatial function and navigation and at the same time, when disrupted, is linked to the numerous disorders including schizophrenia, autism, depression, obsessive-compulsive disorders, amnesia, mild cognitive impairment, and Alzheimer's disease (Aggleton and Brown, 1999; Dalgleish, 2004. This brain network is known as the larger limbic system (Catani et al., 2013; Livingston and Escobar, 1971; Rolls, 2015).

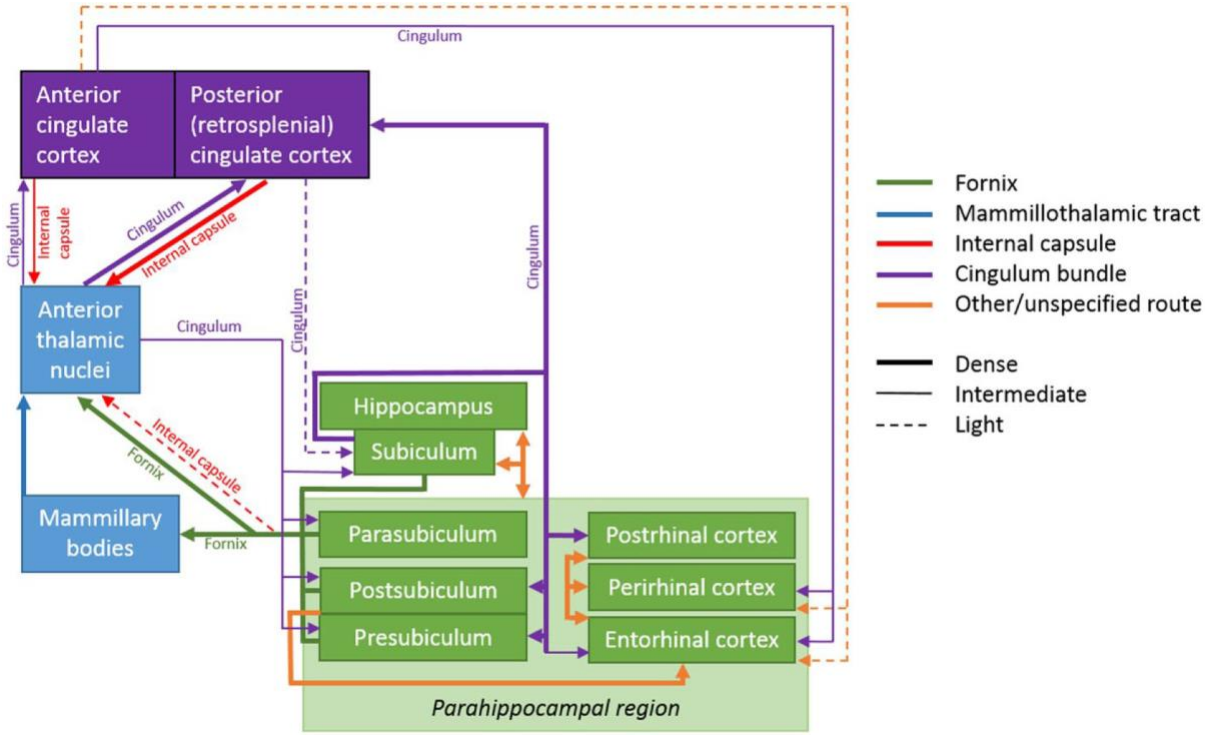


Figure 1.3 The rat hippocampal–diencephalic–cingulate network.
Line thickness reflects the strength of the connections. Most of the structures within the circuit are densely interconnected with several more structures (except mammillary bodies, which project only to ATN). Note how the various connections within the circuit are topographically organized, creating parallel pathways that presumably reflect multiple functions (Bubb, E. J. et al.,2017).

The hippocampal–diencephalic–cingulate network (Fig 1.3) is represented by dorsal and ventral twin routes, which connect the hippocampal and the parahippocampal regions to the cingulate cortices and the thalamus. Such parallel projections will as a rule protect against the full effects of a damage to an individual brain area beyond the hippocampus, as this damage would lead only to incomplete disconnections (for instance lesions of the involved cortical regions can be mitigated by other parallel hippocampal pathways) (White N.M. and Robert McDonald J., 2002). Thus, lesions made to the extrahippocampal system do not lead, as a rule, to strong deficits at memory recall. Instead they lead to other, more subtle kinds of memory impairment.

The anterior thalamic nuclei complex

Thalamic structures, in particular the anterior thalamic nuclei (ATN), are a part of the extrahippocampal memory circuit. The ATN are connected to the amygdala and the cingulate cortex; there are as well dense, direct projections to the anterior thalamic nuclei from the subiculum, presubiculum, postsubiculum, and parasubiculum (Meibach and Siegel, 1977; Swanson and Cowan, 1977; Van Groen and Wyss, 1990). Beside this, a number of reciprocal temporal-diencephalic pathways connect both directly and indirectly the anterior thalamic nuclei and the hippocampal formation. These extended pathways also involve the mammillary bodies, the retrosplenial cortex and parts of the prefrontal cortex (Aggleton et al., 2010; Vann et al., 2009). The ATN are implicated in memory consolidation. In the case of fear memory, the ATN are specifically implicated in contextual, but not tone FC. Damage or lesions of the ATN are not leading to a complete memory loss, but instead they lead to a faster extinction and impaired cFC recovery, while damage to the ATN in humans is associated with anterograde amnesia (Marchand et al., 2014). The ATN receive convergent inputs from the hippocampus, the mammillary bodies, and the cingulate cortices (ACC and RSC) and are vital for hippocampus-dependent learning, including episodic and event memory in human and rodents (Bubb et al., 2017). Furthermore, increased activity in the striatum and the anterodorsal thalamic nuclei is linked to spatial memory retrieval (Méndez-Couz et al., 2015).

The retrosplenial cortex

The cingulate cortex is a major part of the hippocampal–diencephalic–cingulate network and belongs to the limbic system of the brain. The most anterior part of it is represented by ACC, while the most caudal portion of the posterior cingulate in humans and non-human primates is represented by RSC (in rodents it occupies the whole area of the posterior cingulate cortex). Early studies of cingulate cortex postulated a fundamental dichotomy between the functions of the anterior and posterior cingulate cortices. The anterior cortex was implicated primarily in executive functions related to the emotional control of visceral, skeletal, and endocrine outflow.

The posterior cortex, in turn, was implicated in evaluative functions such as monitoring sensory events and the organism's own behaviour in order to support spatial orientation and memory (Vogt et al., 1992).

The position of retrosplenial cortex between the hippocampal formation and the neocortex underlys its key role in hippocampal processing. RSC is able to integrate interoceptive head-direction information (for instance, from the anterior dorsal thalamic nuclei) with exteroceptive information that uses both allocentric and egocentric frames of reference (Byrne et al., 2007; Vann et al., 2009). Both the main inputs and the main outputs of the RSC are to areas in the limbic cortex and thalamus. Thus, RSC is reciprocally interconnected with ACC and the subiculum (Bassett and Berger, 1982; Vogt and Miller, 1983). Furthermore, RSC projects to and receives differential projections from the ATN (Van Groen and Wyss, 1990).

Numerous recent studies performed on the RSC have suggested a role in regulating cognitive function, context representation and action planning (Byrne et al., 2007; Burgass et al., 2001). In addition to it, a number of imaging studies suggest that the retrosplenial cortex has a role in the interface between emotion and episodic memory (Maddock, 1999; von Zerssen et al., 2001). In both humans and animals, RSC forms an integral part of the limbic system. It receives and sends dense projections to the anterior cingulate cortex, which in turn constitutes another part of the limbic system and is involved in the extrahippocampal memory circuit. Notably in the context of this study, although a large number of studies address the function of the connection between RSC and the hippocampal formation or RSC and the ATN, the specific role of its connection with ACC was barely studied.

1.2 Aim and rationale of the thesis

Despite numerous theories aimed at explaining the function of ACC, its specific role has remained unclear, and no unifying theory exists to address its role in learning. Several studies have suggested that ACC is essential for translating high-level strategies into action and action selection via its connections to areas such as dorso-medial striatum and the claustrum. By contrast, the role of ACC in the formation of adjustable memory traces was hardly addressed.

In my thesis, I address the role of mouse ACC in the formation of adjustable memory for adaptive learning. To this end, I combine immunohistochemical analysis, reversible chemogenetic manipulations of principal cells in defined brain areas and selected neuronal-ensembles, behavioural tests, and viral tracing techniques to locally analyse circuit mechanisms, underlying acquisition and consolidation of memory as well as subsequent learning. Furthermore, I investigate ACC function within its connectivity context by analysing its input and output connections.

In the supplementary part of my thesis I enclose more detailed data on the expression of the immediate early gene cFos within ACC, and present experiments addressing the nature of relearning.

2. Results

2.1 Role of ACC in memory formation during multi-step foraging learning

The majority of the studies addressing the function of ACC concentrate on its role during behaviour either by silencing the area at the time when behaviour is performed, or by monitoring neuronal activity in ACC during behaviour. Given that ACC is thought to have a major role in guiding decision making, and under the assumption that ACC memories guide those processes, I have investigated the function of mouse ACC by reversibly silencing this structure during memory consolidation, and then investigating the consequences of these manipulations in subsequent behavioral settings resembling decision making during foraging.

To investigate the function of ACC I focused initially on the attention set shifting task (ASST) (Bissonette et al., 2008; Garner et al., 2006; Brady and Floresco, 2015). Thus, ASST resembles a foraging situation, during which the animal needs to explore the environment, identify the sources of reward (such as food), develop the strategy appropriate for obtaining reward and, when reward is depleted, switch to the next foraging option. The ASST paradigm allows us to test for several important aspects of flexible learning, including rule learning, integration of the history of reinforcement, and decisions involving switching to an alternative goal versus staying with the current goal. One of the best-established ways in which ACC addresses flexible learning is in keeping track of the values and costs of alternative options during multiple learning sessions. These computations include estimating changes in the value of reward, and switching behaviour when the value of reward becomes low compared to the costs of behaviour, needed to obtain the reward (Shenhav et al., 2014). ASST tests in mice consist of a sequence of several learning sub-stages, distributed over several days. This allowed me to specifically study ACC silencing effects at separate stages of this complex paradigm and to make first assessments of ACC function in flexible learning.

The setup for ASST is comprised of a starting compartment, where a mouse is placed at the beginning of every trial, and two initially closed compartments containing bowls, infused with two different odours, of which one is rewarded with food pellet and another one is empty (Fig 2.2 A). The food is covered by a digging medium, and cannot be sensed by the mouse from afar; this forces the mouse to examine specifically every bowl, recognize its odour, and dig at the correct odour. The correct choice is rewarded by a food pellet obtained upon digging in the bowl, and the wrong choice results in

restarting of the trial. Thus, by making a wrong choice the mouse will be deprived of the reward until the next (successful) attempt. To increase the animal´s motivation to obtain the reward, mice are kept under food restriction 24h before the trial. In this protocol, the animals are fed only once a day, immediately upon completion of the training session. The successful completion of the training session is defined as a series of 8 out of 10 correct decisions within a rolling window. The number of trials required to reach each learning criterium is calculated for every mouse during the learning sessions.

Separate learning sub-stages of the protocol are performed over consequent days. After an initial habituation to the training context performed over 3 days, the follow-up training consists of the standard discrimination phase (SD), complex discrimination (CD), intradimensional shift (IDS), intradimensional shift reversal (IDSre), and then optionally extradimensional shift (EDS) or repetition of intradimensional shift (IDS2). During those stages, the animal acquires the specific rules assigned to each task (SD, EDS), generalises or strengthens the existing rule (CD, IDS), or reverses the stimulus-reward association (IDSre, IDS2). These stages will be further described in detail.

To address the role of ACC in consolidation of each of the learning stages, I have performed ACC silencing during memory consolidation of each sub-stage, and tested the consequences during the next sub-stage of the ASST protocol. Silencing was done by chemogenetic hyperpolarization of principal neurons through combined injection of two viral constructs locally to ACC: Cre-delivering AAV8-Camk2a-Cre, and Cre-dependent PSAM channel (an inhibitor construct introduced by Scott Sternson, which is selectively activated by PSEM ligand). To interfere with memory consolidation, PSEM ligand was injected 15 minutes after the successful completion of the training session. Under these experimental conditions, PSEM has been shown to activate its target inhibitory channel within 1-2 min, and during 60-90 min. The next session was performed 24h later, and followed by the same silencing scheme (Fig 2.1, 2.2, A).

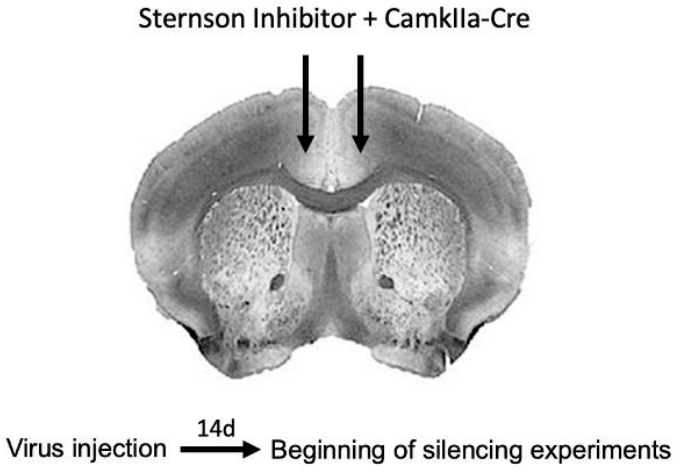


Fig 2.1 Illustration of ACC silencing procedure. Arrows indicate virus injection sites.

During the first sub-stage (SD) mice learn the rule that reward is associated with one particular odour, but not with the other one. Bowls were infused with two different odours and their respective position in the left or in the right compartment was randomly changed between the trials to avoid association with the right or left side of the test box. This learning phase took on average about 15 trials to complete (Fig2.2, B).

During the second sub-stage of ASST (CD) mice had a choice between the same two odours, thus the learning process included recall of the previous rule, but this time textures were introduced as another dimension of variance. Mice had to choose the exemplar associated with the odour rewarded during SD, and ignore the texture. Initially, mice noticed and explored the change to the bowls by biting and sniffing the textures. However, they stayed with the previously enforced rule (odours) and completed the learning sessions with less trials then when learning SD. Silencing of ACC at consolidation of SD did affect performance at CD, resulting in the same amount of trials to reach criterion both in the control and the experimental groups (Fig2.2, B).

In IDS, mice encounter a pair of new odours and have to generalise the previously learned rule to efficiently complete the training session. Initially, mice explored both

odours a few times before choosing one of the alternative options, which was followed by mice consistently associating reward with the bowl scented with the correct odour, and avoiding digging in the other bowl. It took the same amount of trials for the control and the experimental group to reach the learning criterium in IDS (Fig 2.2, B), showing that the memory trace in the ACC is not required to effectively generalize the odour rule.

In the next sub-stage of the task (IDSre), the reward-odour association from the previous session (IDS) was reversed, forcing the mice to switch from the previously correct exemplar to the alternative one. In order to achieve this switch, mice have to overcome the previously learnt reward association in order to abandon the default option, and switch to the alternative one. The switching behaviour therefore consists of two processes – the choice of the previously correct exemplar has to be suppressed, and the exploration by active digging of the alternative one has to be initiated. Mice require more trials to learn to reverse the rule than to learn the rule anew (Fig2.2 A, B).

Saline-treated mice required multiple trials to start digging in the alternative (previously incorrect) exemplar, instead sticking to the default option (previously correct exemplar) as seen on the graph representing the number of errors (Fig2.2 C). This stage was then followed by a period when active exploration was initiated in the alternative exemplar, but the default exemplar was not yet suppressed consistently. This stage of the learning process is represented by equal preference for both odours. At the final stage of IDSre, mice consistently shifted from the default option to the alternative, as shown by full suppression of exploration of the previously correct exemplar, and stable preference for the alternative one.

In contrast to saline-treated controls, the experimental group in which ACC was silenced 15 minutes after SD, CD and after IDS, required dramatically less trials to switch from the default option to the alternative one (Fig2.2 B). It took them about 2 wrong choices to initiate the immediate switch to the alternative exemplar and inhibit choosing of the previously baited one (Fig 2.2 C). This result suggested consolidation of SD, CD and IDS memories in ACC is necessary in order to prevent instant switch to the alternative, when the default option is no longer rewarded during reversal

learning. With other words, memory consolidation processes in ACC are necessary after rule learning in order to prevent immediate abandonment of one specific option associated with the learned rule, and switch to the alternative option. Accordingly, the finding suggests that memory processes in ACC might be important to initially stay with the previously reinforced option, as opposed to abandoning that option at the first contradiction. This result is reminiscent of foraging decisions, where the implications of new evidence need to be weighed against consistent evidence to the contrary in previous sessions.

Since ACC silencing at memory consolidation specifically affected the reversal sub-stage of the ASST protocol, I next asked whether interference with memory consolidation in ACC upon IDS might be sufficient to accelerate reversal learning in IDSre (Fig 2.2 D,E). Indeed, and like ACC silencing after CD-IDS, interfering with consolidation of memory in ACC after IDS led to a faster switch to the alternative exemplar during IDSre, although the effect was less pronounced than upon interference after SD, CD and IDS. As is shown by the rate of errors within blocks of trials (1-10, 11-20) in the initial block of 10 trials the error rate between the saline control group and the experimental group was the same, indicating that the initial (10 trials) preference for the default option was not altered by the treatment (a rule consolidated already in CD). However, this was followed by a near instant switch to the alternative in the experimental group in the next block of 10 trials, while the saline control had not yet developed a preference for the alternative option over the default one in the block of 10-20 trials (Fig 2.2 E). As a result, mice in which memory consolidation in ACC was interfered with specifically after IDS, performed in IDSRe as well as when first encountering IDS, i.e. they were not slowed down by previous reward of the alternative option.

I then asked whether IDSre might have led to the formation of a new reward-relation between two options, which can be again flexibly adjusted notwithstanding ACC silencing upon memory consolidation after IDS. We therefore introduced an additional IDS2 sub-stage, in which the reward-odour relations were again reversed and identical to those during the IDS stage (Fig 2.2 D, E).

The number of trials in IDS2 in the control group was undistinguishable to that in IDSre, indicating that the reward-odour associations learnt during IDSre required a comparable amount of evidence and effort for switching back to the original odor. With other words, switching between two alternatives seems to be equally challenging the first and the second time. Mice in which ACC was silenced at consolidation of IDS required slightly more trials compared to the control group to reach the learning criterium in IDS2, and exhibited a higher rate of incorrect responses during trials 10-20 (Fig2.2 E). This suggests that the memory trace in ACC which was formed at IDS has a facilitating effect at IDS2, possibly because it helps define the IDS-IDSRe-IDS2 process as an alternative between two options.

Taken together, these results suggest that memory traces in ACC are not required for learning and application of binary choice rules. Instead they are required to prevent a rapid switch from the default option to the alternative upon withdrawal of the reward from the previously correct option and rewarding of the alternative choice.

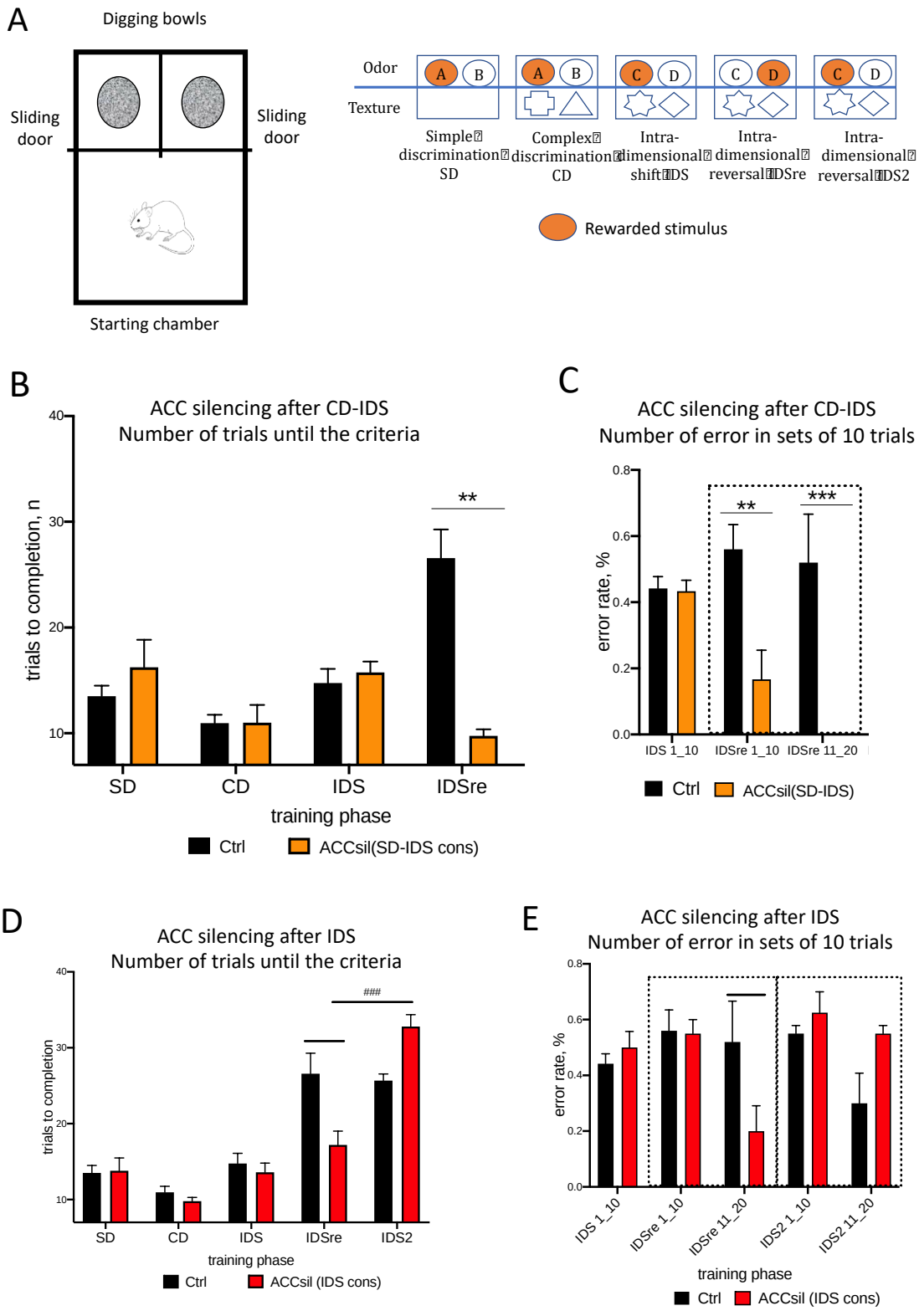


Fig 2.2 In set shifting learning, ACC is required at memory consolidation to learn the history of reinforcement, and specifically control choices between opposite alternatives.
(A) Scheme of the ASST protocol and the experimental set-up. (B) Silencing ACC at memory consolidation of SD, CD and IDS greatly accelerates shift during IDSre, but does not affect rule generalization in IDS. (C) The accelerated shift is characterised by significantly reduced number of incorrect trials in IDSre starting from the first block of 10 trials. Incorrect trials in IDSre represented by two blocks of 10 trials each are highlighted in red. N= 4-5 each. (D)

Silencing ACC at consolidation of IDS facilitates shift during IDSre, and increases persistence during IDS2. (E) The accelerated shift in IDSre is characterised by the significantly reduced number of incorrect trials in IDSre in the second block of 10 trials, whereas increased persistence during IDS2 is characterised by the increased number of errors in the second block of 10 trials. Incorrect trials are represented by two blocks of 10 trials each and are highlighted in red (IDSre) and yellow (IDS2). N = 4-5 each.
*Statistical analysis – two-way ANOVA followed by multiple comparisons test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001*

2.2. Memory processes in ACC are required for subsequent learning of alternative values within the same context

The results obtained in ASST demonstrated that ACC activity is crucial during consolidation of the preceding substages to regulate flexible behaviour in the situation of binary choice between two alternatives. Memory in ACC appears to be specifically required during alterative learning to increase adherence to the previously reinforced option and prevent instant switch to the alternative, once the new evidence enforces the alternative choice. I aimed to find a complementary learning paradigm, which allows to test flexible behaviour in the situation when external contingencies change and the animal needs to adapt accordingly. For this I have chosen to investigate whether the memory trace in ACC is required to regulate adaptive behaviour in non-instrumental Pavlovian forms of learning, namely contextual fear conditioning paradigm (cFC).

cFC produces stable long-lasting fear memory from a single learning session. This memory can be subsequently extinguished by exposing animals to the training context for 30 minutes. Extinction of cFC is considered a form of alternative learning, which does not replace the previously learned fear memory, but instead provides an alternative value (safety) to the same context (Sierra-Mercado et al., 2011; Courtin et al., 2013; Lacagnina et al., 2019; Martínez-Canabal, A. et al., 2019).Thus, similar to IDSre, extinction can be described as a choice between two alternative behavioural strategies, corresponding to the amount of evidence obtained in the favour of the context being safe or dangerous.

Extinction of fear memory was carried out on the next day after cFC acquisition by exposing the animals to the training context for 30 minutes in the absence of foot shocks (Fig 2.3. A). During this prolonged time the mice slowly decreased time spent freezing. Retention of extinction learning and spontaneous recovery of fear memory were measured on the next day, and 12 days after extinction learning respectively. As was shown by the previous findings in the lab (Karunakaran et al., 2016), memory consolidation is characterized by two windows of elevated cFos activity, interference with which can dramatically affect the memory. In order to test ACC involvement in both stages of the fear memory consolidation, silencing of ACC was carried out during the onset of the 1st or the 2nd consolidation window in two different groups of mice (i.e. either 15 minutes, or 12 hours after acquisition of cFC).

Silencing ACC during consolidation of cFC memory did not affect fear memory recall 24h later, which indicates that the memory in ACC is not required to successfully learn or retrieve fear memory in the training context. However, and in contrast to saline-treated controls, the experimental group showed rapid extinction of the fear memory, with a strongest effect when blocking at the onset of the 1st cFos peak (Fig 2.3 B, C, D).

In order to further characterise how extinction learning is affected by ACC silencing during consolodation of cFC memory, I monitored spontaneous fear recovery (Lacagnina et al., 2019; Martínez-Canabal, A. et al., 2019). In contrast to extinction, erasure of cFC memory prevents spontaneous fear recovery and is characterised by an altered learning mechanism. This fact can be used in order to analyse whether the rapidly acquired memory is still the memory of an alternative behavioural strategy, or it affects the initial memory trace instead (Clem and Huganir, 2010; Gogolla et al., 2009; Quirk et al., 2010). Remarkably, ACC silencing at consolidation of cFC resulted in the loss of spontaneous fear memory recovery 12 days after extinction. This result indicated that ACC memory from the preceding cFC learning is required not only to maintain adherence to the default behavioural strategy during the extinction protocol, but also to acquire extinction learning as an alternative memory trace (Fig 2.2 D).

To further strengthen these findings, I have applied the same experimental protocol to older mice (8,5 months), which do not normally extinguish fear. Remarkably, ACC silencing at cFC consolidation also dramatically increased the rate of freezing reduction in older mice (Suppl. 1). Thus, the mechanism of fear memory erasure upon ACC silencing at cFC consolidation is not age-dependent.

I next determined whether activity in ACC might be required during extinction learning. I silenced ACC during extinction (PSEM was injected 15 minutes prior to the learning) and tested for retention and recovery of fear. Silencing ACC during extinction learning (Fig. 2.3 E) did not affect recall of fear memory (represented by the first 5 minutes of extinction) and it did not alter the gradual reduction of freezing during learning (Fig 2.3 F, G).

Taken together, these results indicate that, similar to the results obtained in ASST, ACC is required at consolidation of cFC in order to prevent the rapid switch from the default behavioural response to the threatening context (freezing) to the alternative one (reduction of freezing) during subsequent extinction learning. Notably, alternative learning upon perturbation of memory consolidation in ACC was replaced by re-learning which is indicated by the absence of fear recovery 12 days post extinction. This finding suggests a role for ACC in the formation of the initial memory trace, which can be flexibly adjusted and maintained as the alternative behavioural choice in the same context.

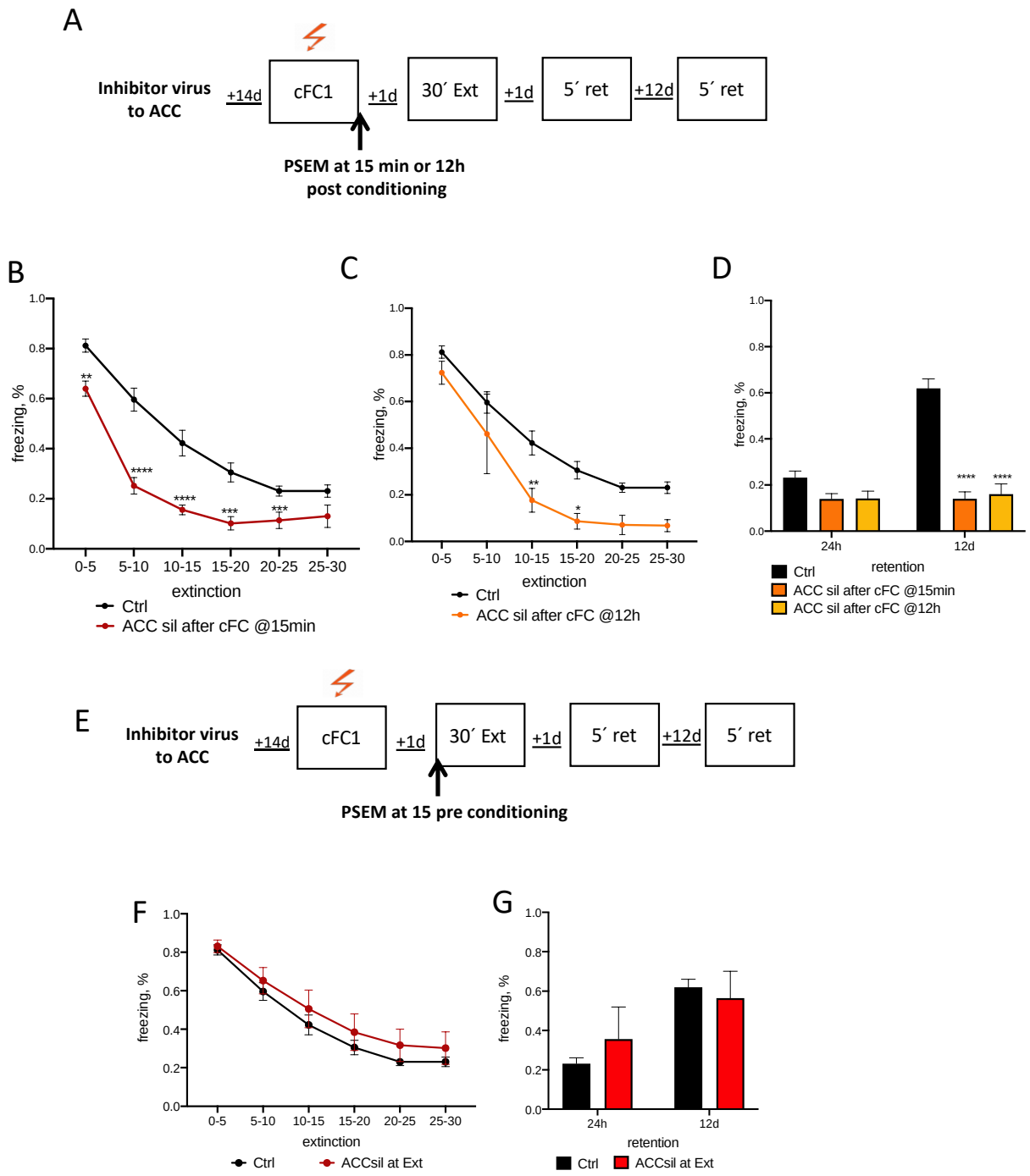


Fig2.3 Silencing of ACC at consolidation of cFC accelerates extinction learning, and leads to erasure.
(A) Schematic of the experiment for silencing at consolidation of initial learning. Silencing of ACC done 15 minutes (B) or 12 hours (C) after acquisition of cFC facilitates switching behaviour during subsequent learning and leads to erasure instead of alternative learning (D). N = 5-8 each.
(E) Schematic of the experiment for silencing during alternative learning. Silencing ACC during extinction of cFC does not affect extinction learning (F, G), nor spontaneous fear recovery 12 days later (G). N = 4-8 each.
*Statistical analysis – two-way ANOVA followed by multiple comparisons test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001*

2.3 ACC is required during cFC acquisition to enable subsequent learning

In the previous set of experiments I demonstrated a dramatic effect of ACC silencing during memory consolidation on subsequent flexible learning. Silencing ACC at consolidation adresses the role of ACC specifically as the learning is completed and the memory trace produced by the learning situation is being formed offline. However, it does not adress the role of ACC during the acquisition of the initial memory. It seemed possible that the role of ACC is most prominent in the initial learning, which establishes the default behavioral reaction and provides the basis for subsequent reversal learning.

To study the contribution of ACC activity during initial learning, I have silenced ACC during the acquisition of cFC. Injection of PSEM was done 15-20 minutes before the animal was placed inside the learning context, so that the silencing effect would last at least through the following 5-minute learning session. On the next day, the flexibility of the memory trace was assessed by utilizing the extinction protocol, which was followed by the retention and recovery tests 24 hours and 12 days later (Fig2.4 A).

Activity in ACC was not required during cFC acquisition to form and recall fear memory in the training context (first 5 minutes of extinction, Fig 2.4 B). In contrast to the results obtained by interfering with ACC activity during memory consolidation, ACC silencing during cFC learning did not alter the rate of freezing reduction at extinction, and prevented extinction learning, as determined at 24h retention (Fig 2.4 C).

This result demonstrates the remarkable and previously unaddressed role of ACC at acquisition of cFC – the resulting freezing response can be reduced during prolonged contextual exposure, but there is no evidence of extinction learning.

These results suggested that activity in ACC is required during the initial learning to produce a memory that can be modified during subsequent alternative learning.

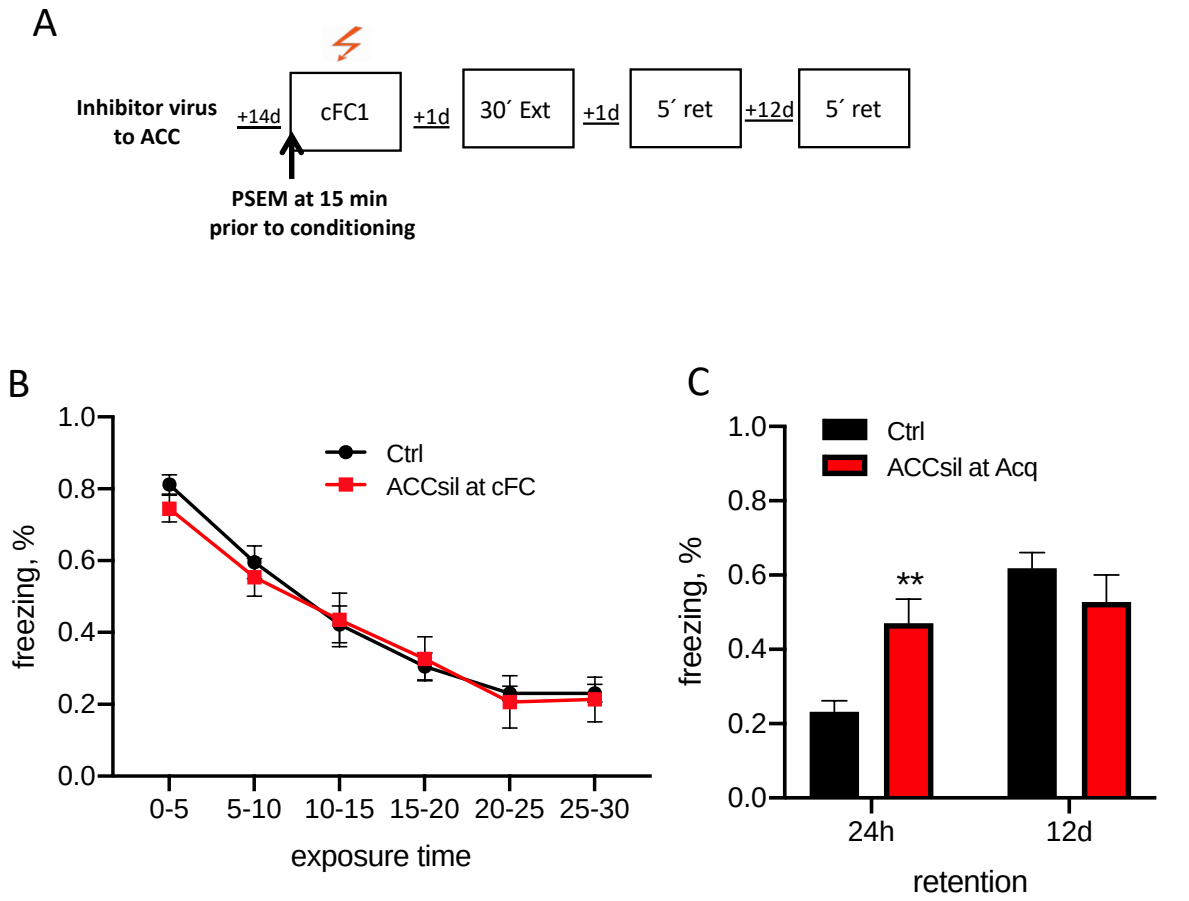


Fig2.4 ACC is needed at acquisition of cFC for subsequent extinction learning.
(A) Schematic of the experiment for ACC silencing at acquisition of cFC. (B) Silencing ACC at acquisition of cFC results in normal reduction of freezing during extinction learning, but absence of extinction as tested at retention (C). N = 3-6 each.
*Statistical analysis – two-way ANOVA followed by multiple comparisons test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001*

2.4 Role of ACC during repeated learning

In the previous set of experiments, I have determined that the ACC is required during acquisition of the initial cFC memory to learn subsequent extinction. To better understand this finding, I turned to multi-stage learning paradigms, and determined whether ACC activity during acquisition of the preceding sub-stage might affect learning of the subsequent sub-stage, and thus the integration of repeated learning episodes.

Typical foraging situations consist of sequences of learning events, including multiple encounters with the food source or the danger, which triggers re-evaluation of the

acquired reinforcement depending on its past and current value, thereby influencing decision making. Apart from ASST, another form of multi-stage learning involves repeated instances of cFC (Fig 2.5 A). In this learning protocol, the second cFC episode is integrated with the negative reinforcement from the first cFC, leading to a marked impairment in subsequent extinction learning (Fig 2.5 B).

I silenced ACC during acquisition of the first cFC; this was followed 5d later by a second episode of cFC, which was in turn followed by extinction learning 24h later. Notably, and in contrast to saline-treated controls, the experimental group readily extinguished the fear memory, which was followed by return of fear 12 days later (Fig 2.5 B-C).

To further investigate the role of ACC in multi-stage learning paradigms, I silenced ACC during ASST sub-stages, from SD on, and until IDS (Fig 2.5 D). Successful unperturbed learning of the task would here include rule learning for the rewarded dimension, rule generalisation, and learning of reinforcement relations between two particular alternative options (at IDS). Silencing ACC during SD and IDS did not significantly affect the number of trials the experimental group needed to reach the learning criteria compared to saline-treated controls. Remarkably, however, during the subsequent reversal stage (IDSre), animals in the experimental group only half the number of trials to make the shift compared to controls (Fig 2.5 E). Subsequent IDS2 was not significantly affected. This result provided evidence that ACC is required during the acquisition of SD and IDS to prevent a rapid switch to the alternative option during IDSRe.

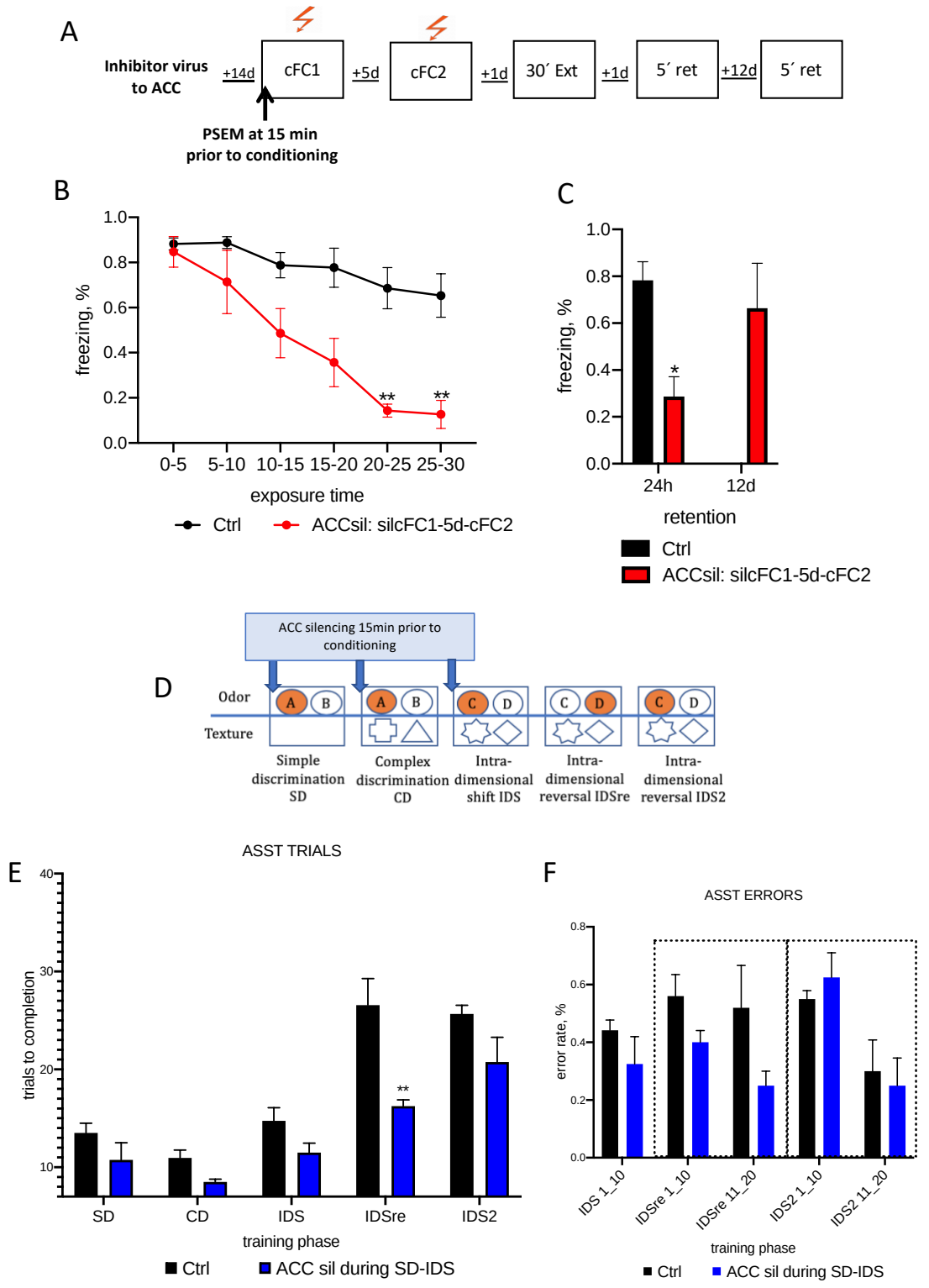


Fig 2.5 ACC is required to integrate repeated reinforcement sessions.
(A) Schematic of repeated cFC acquisition protocol. (B) ACC is required during acquisition of the 1st cFC to integrate repeated reinforcement of cFC and produce a memory trace which cannot be extinguished during one extinction session (C). N = 3-5 each.
(D) Schematic of ACC silencing during acquisition of sub-stages of the ASST protocol. (E) ACC is required during acquisition of SD, CD and IDS to prevent a rapid shift at IDSre. (F) Accelerated shift in IDSre is characterised by significantly reduced number of incorrect trials

in IDSre in the second block of 10 trials. Incorrect trials are represented by two blocks of 10 trials each and are highlighted in red (IDSre) and yellow (IDS2). N = 4-5 each.
Statistical analysis – two-way ANOVA followed by multiple comparisons test (B, E, F); Unpaired t-test (C). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

2.5 Connections from and to ACC

I have provided evidence that activity in ACC is important both during acquisition and memory consolidation for subsequent flexible learning, but that the consequences of silencing ACC at acquisition or at consolidation are dramatically different. To investigate how ACC might effect such distinct roles, I focused on its role within brain networks. As a prerequisite to address this question, I mapped anatomical connectivity from and to ACC. I applied retrograde and anterograde tracing approaches, by injecting ACC with pAAV-pCAG-iCRE-2A-H2BGFP-WPRE and pAAV-pCAG-Floxed-SynGFPreverse-WPRE respectively. The viruses were injected in separate animals unilaterally in ACC at +0.75 mm and +0.35 mm from Bregma (mediolateral 0.35, dorsoventral 1.4 and 1.5 respectively). The data were then acquired by Axioscan, manually analysed in Zen Blue software, and the green fluorescent signal was matched to the corresponding areas in The Mouse Brain Atlas (Franklin and Paxinos, 2019). The strength of connections was subjectively estimated by the relative intensity of the green fluorescent signal per surface of each of the brain areas (represented by 2D scanned images, acquired from 80 µm-thick slices). The results of the tracing are summarized in Fig 2.6, and sample coronal sections with selected areas are presented on Fig 2.7 and Fig 2.8 (anterograde and retrograde tracing respectively).

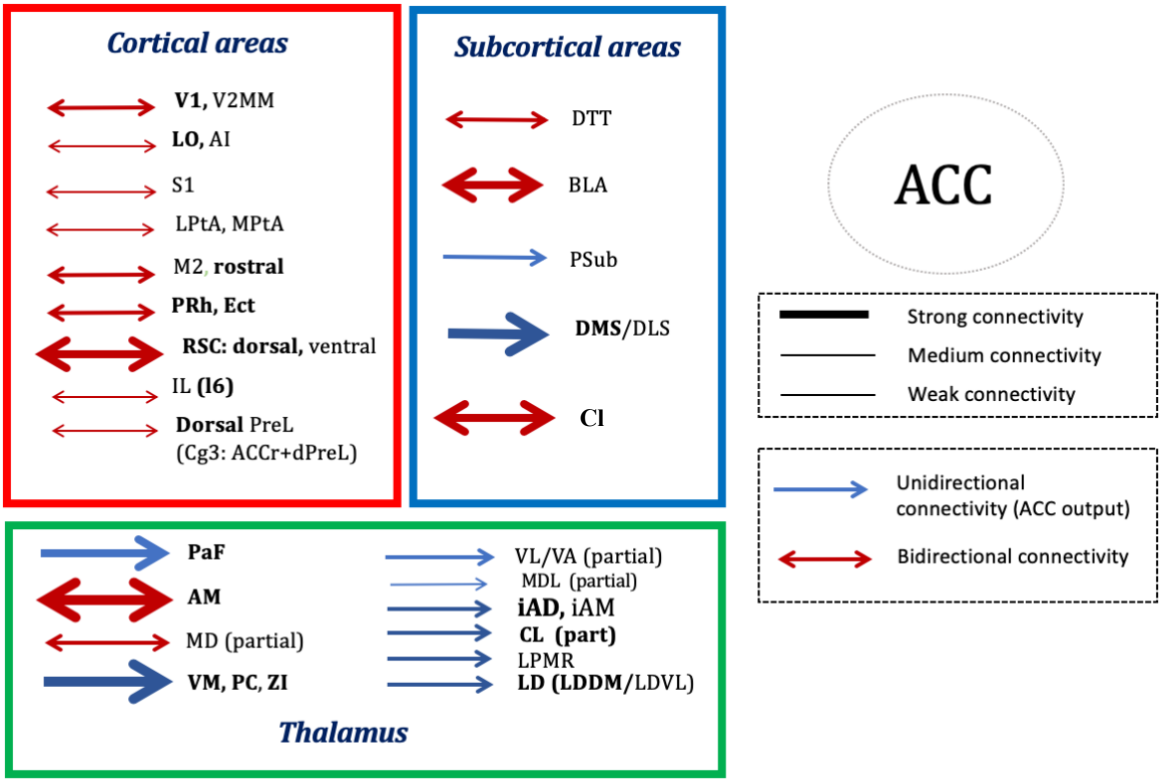


Fig 2.6 Summary of ACC connectivity

Bidirectional connections are indicated by double arrows and red colour, unidirectional (emerging from ACC) in single pointed arrows in blue. The thickness of the arrows illustrates estimated relative densities of connections (by subjective estimation of the amount of fluorescent signal per surface area of the brain region; the data was acquired from 80 µm-thick brain slices using Axioscan and is represented by 2D images).

AI - agranular insular cortex, AM - anteromedial thalamic nucleus BLA - basolateral amygdaloid nucleus, anterior part, Cl – Claustrum, CL - centrolateral thalamic nucleus of thalamus, DLS – dorsolateral striatum, DMS – dorsomedial striatum, dPreL – dorsal prelimbic cortex, DTT - dorsal tenia tecta, Ect - ectorhinal cortex, iAD - interanterodorsal thalamic nucleus, iAM - interanteromedial thalamic nucleus, IL – infralimbic cortex, LD – lateral dorsal nucleus of thalamus, LDDM - laterodorsal thalamic nucleus, dorsomedial part, LDVL - laterodorsal thalamic nucleus, ventrolateral part, LO - lateral orbital cortex, LPMR - lateral posterior thalamic nucleus, mediorostral part, LPtA - lateral parietal association cortex, M2 - secondary motor cortex, MD - mediodorsal thalamic nucleus, MDL - mediodorsal thalamic nucleus, lateral part, MPtA - medial parietal association cortex, PaF - parafascicular thalamic nucleus, PC - paracentral thalamic nucleus, PRh - perirhinal cortex, PSub - postsubiculum, RSC – retrosplenial cortex, S1 - primary somatosensory cortex, V1- primary visual cortex, V2MM- secondary visual cortex, mediomedial area, VA - ventral anterior thalamic nucleus, VM - ventromedial thalamic nucleus, VL – ventrolateral thalamic nucleus, ZI - zona incerta

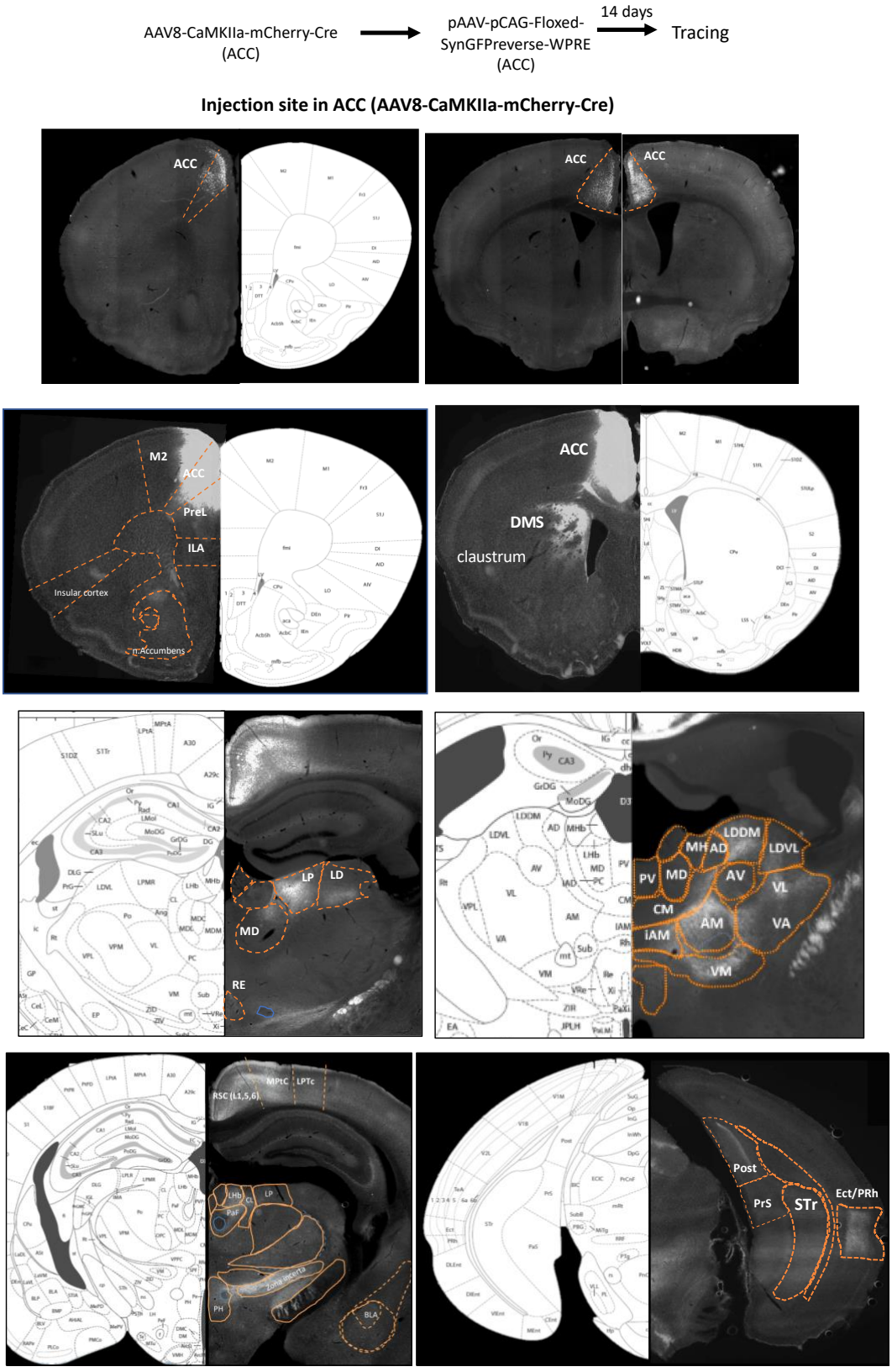


Fig 2.7 Anterograde tracing from ACC
Images of mouse brain coronal slices with detected GFP signal from unilateral anterograde tracing. The brain areas were matched to the Paxinos and Franklin mouse brain atlas (Franklin and Paxinos 2019). Sample areas with the highest visible signal are labelled according to the brain atlas.

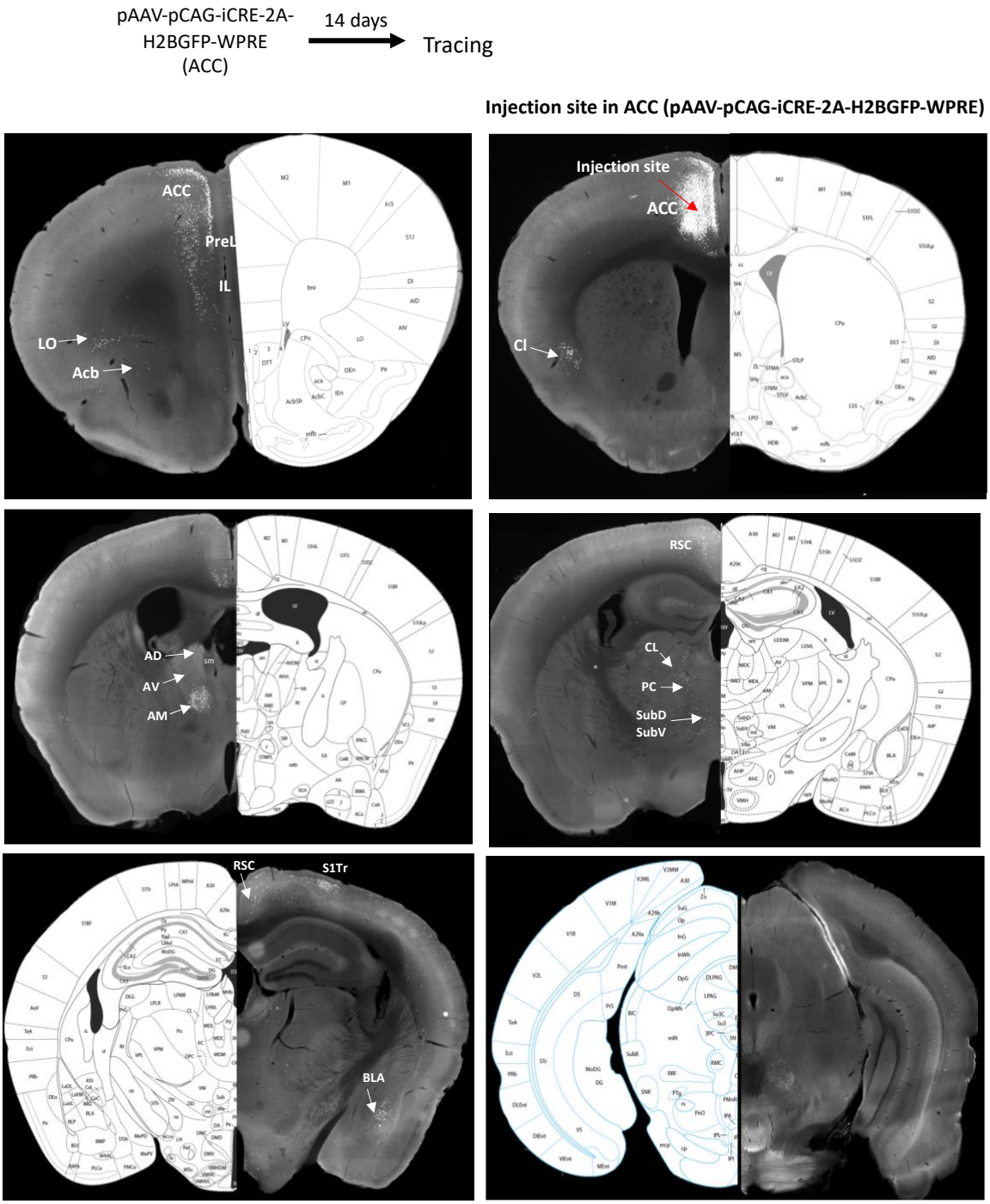


Fig 2.8 Retrograde tracing from ACC
Images of mouse brain coronal slices with detected GFP signal from unilateral retrograde tracing. The brain areas were matched to the Paxinos and Franklin mouse brain atlas (Franklin and Paxinos 2019). Sample areas with the highest visible signal are labelled according to the brain atlas.

According to these tracing findings, ACC bidirectionally connects with a number of cortical areas, such as primary sensory areas (primary somatosensory cortex and

primary visual cortex), PFC (dorsal prelimbic cortex and layer 6 of infralimbic cortex, lateral orbital cortex), agranular insular cortex, perirhinal cortex, ectorhinal cortex, lateral and medial parietal association cortex, secondary motor cortex. However, among all cortical areas, the strongest connectivity of and by ACC was with RSC.

Among subcortical areas, I found the strongest signal in the following areas: DMS (predominantly) and DLS, which receive ACC output, and the BLA and claustrum, which are connected with ACC bidirectionally. Other subcortical targets with relatively weaker fluorescent signal included the postsubiculum (receive ACC output) and the dorsal tenia tecta (bidirectionally connected).

I found that several thalamic areas receive ACC projections. Most of these connections were unidirectional outputs from ACC, with the exception of anteromedial nucleus and mediodorsal thalamic nucleus, which both received and sent projections to ACC. I have summarised my observations according to the major thalamic groups; marked in bold are the areas, in which I have observed the strongest signal:

1. Anterior group of the dorsal thalamus: anteromedial nucleus (AM), **interanterodorsal nucleus of the thalamus (iAD)**, interanteromedial thalamic nucleus (iAM), **lateral dorsal nucleus of thalamus (LD – predominantly to its dorsomedial part (LDDM))**
2. Medial group of the dorsal thalamus: mediodorsal thalamic nucleus (MD)
3. Ventral group of the dorsal thalamus: ventral anterior thalamic nucleus (VA), **ventromedial thalamic nucleus (VM)**, ventrolateral thalamic nucleus (VL)
4. Intralaminar nuclei of the dorsal thalamus: **centrolateral thalamic nucleus of thalamus (CL)**, **parafascicular neucleus (PaF)**, **paracentral nucleus (PC)**
5. Lateral group of the dorsal thalamus: lateral posterior thalamic nucleus, mediorostral part (LPMR)

I have found no direct connections between ACC and hippocampal structures. However, ACC was connected to the extrahippocampal system (for example postsubiculum, perirhinal cortex and the nuclei of the anterior group of the dorsal thalamus (ATN)). Remarkably, ATN lesions lead to a faster extinction of cFC and an

impaired fear recovery (Marchand et al., 2014); the ATN receive convergent inputs from the hippocampus, the mammillary bodies, and the cingulate cortices and are vital for hippocampus-dependent learning (Bubb et al., 2017).

Notably, apart from parahippocampal areas, ACC is also connected to the other two major areas, comprising independent memory systems – the amygdala and the DMS (White et al., 2002*).* All of these systems are thought to have an equal access to the information from learning processes, but are each specialized to represent a different kind of relationship among the elements of a learning experience (stimulus events, responses, reinforcers) that flows through it.

Thus, DMS has a major role in supporting of planning and execution of strategies and behaviour that is required for achieving complex goals, for instance, in learning action-outcome contingencies that subserve goal-directed action (Balleine and O'Doherty, 2010). DMS is also required for acquisition, expression and flexibility of goal-directed action (Yin and Knowlton, 2006; Balleine and O'Doherty, 2010). In addition, DMS has been reported to be part of the flexible learning circuit together with the perifascicular nucleus (Brown et al., 2010). By contrast, the caudate nucleus is part of the dorsal striatum memory system working in parallel with the hippocampal memory system (White et al., 2002). Neurons in mPFC and ACC project to dorsomedial striatum (DMS) as part of a corticostriatal circuit with putative roles in learning and other cognitive functions. There are indications that DMS interacts with the other telencephalic structures to control spatial-cognitive functions (Pooters et al., 2015; Devinsky et al., 1995; Reep et al., 2013; Pan et al., 2010; Oh et al., 2014).

Another group of structures studied extensively in connection to flexible learning, are the intralaminar nuclei of thalamus, in particular the centralaminar nucleus and the parafascicular nucleus. Thalamostriatal neurons are crucial for frontostriatal circuit functioning, they specifically regulate action selection in the performance phase of learning and control behavioural switching in response to changes in the environment (Rikhye et al., 2018).

Combining my connectivity data with evidence from the literature, I have selected several brain areas for further analysis of which brain systems might be important for acquisition and consolidation of memory with a focus on the ACC connections which are implicated in memory formation and flexible learning.

2.6 Involvement of ACC in subsequent and alternative learning

So far, I have shown that ACC activity is crucial during acquisition and consolidation of cFC for subsequent adaptive behaviour (Fig 2.9). The deficits, observed as the result of interference with ACC activity, can be described as two different cases. Depending on whether interference with ACC activity was carried out at acquisition or at consolidation of the initial learning, these deficits can be described as suppression of subsequent learning (acquisition) or suppression of alternative learning. These two aspects of flexible learning likely each depend on activity within wider networks of brain structures. In order to investigate brain networks involved in flexible learning, together with ACC, I silenced selected brain areas as identified through the connectivity considerations mentioned in the previous section.

I first focused on RSC due to studies conducted in our lab (by Melissa Serrano, Maria Spolidoro and Lisa Kaefer) showing that it is required for flexible learning. I then also focused on brain area sharing connectivity with ACC and RSC, and implicated in flexible learning, i.e. on DMS and PaF. Finally, I also included dorsal hippocampus due its role in secondary learning (as shown in the work of Maria Lahr from our lab).

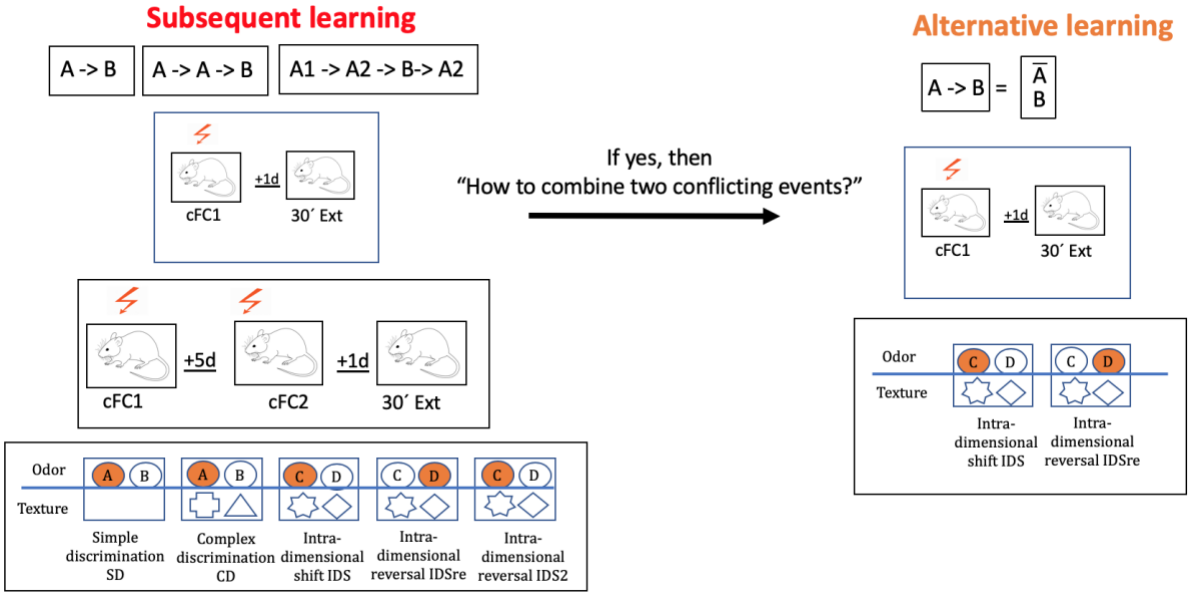
I define flexible learning as the more general phenomenon, which includes subsequent and alternative learning. A schematic representation of examples of subsequent and alternative learning is presented in Fig2.9 A, B. Generally, subsequent learning is characterized by recall of the previously learned experience, and revision of this experience in the light of a new evidence. An example of this form of learning would be repeated fear conditioning, when the second cFC session is delivered in the same context as the first one, rendering the fear memory resistant to subsequent extinction.

I define alternative learning as a particular case of subsequent learning. It also involves recall of previous experience, but the evidence provided now favors the alternative behavioural strategy. An example of alternative learning is extinction of cFC learning or IDSre during ASST.

An illustration of these concepts is provided on Fig 2.9 B. According to the model, prevention of subsequent learning by silencing ACC during acquisition of cFC leads to failure to learn extinction despite the fact, that the animals reduced freezing during prolonged contextual exposure in the absence of foot shocks. By contrast, instead of preventing subsequent learning, silencing ACC at consolidation abolishes alternative learning, leading instead to erasure of the initial memory.

The model proposes that if subsequent learning is prevented, neither alternative learning nor relearning can occur. I therefore assume, that disruption of both circuits simultaneously might be seen during behaviour as identical to only interference with the network for subsequent learning.

A. Flexible learning overview



B. Flexible learning exemplified by cFC paradigm

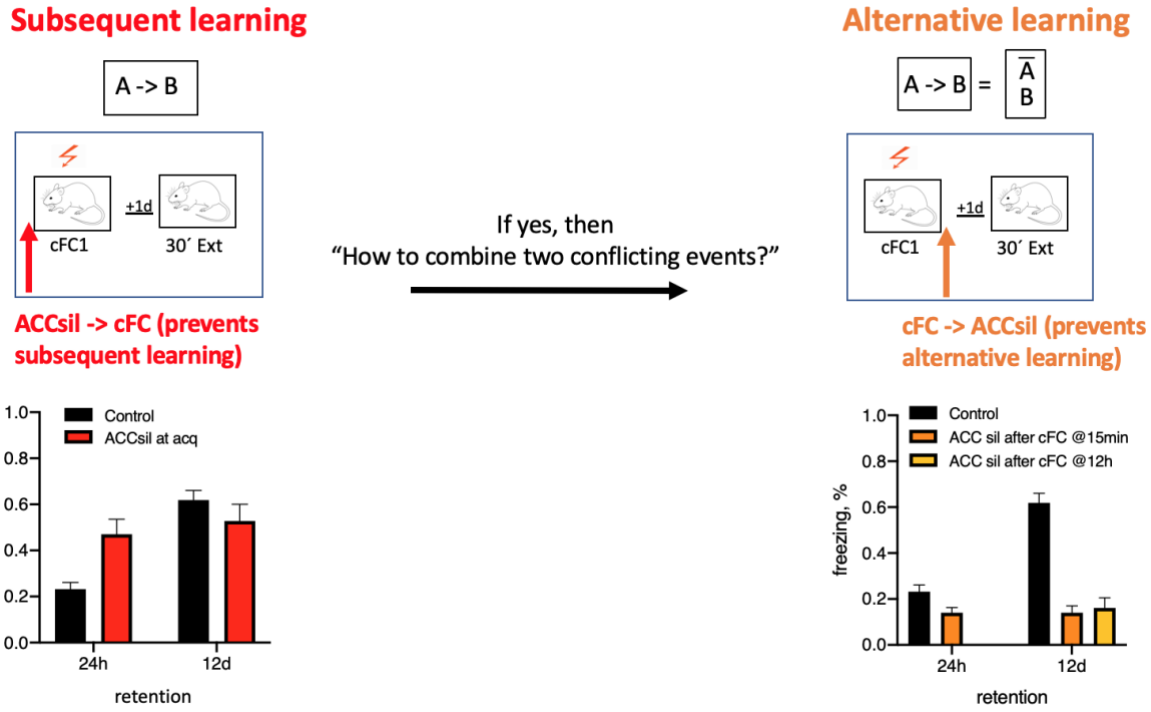


Fig 2.9 Schematic representation of subsequent and alternative learning protocols and their dependence on activity in ACC.
(A) Examples of subsequent and alternative learning paradigms involving different kinds of reinforcement. (B) Example of flexible learning dependent on ACC based on cFC paradigm.

2.6.1 Brain network implicated in flexible learning during acquisition of cFC

In the next part of my thesis I aimed to test the hypothesis, according to which both aspects of flexible learning, schematically represented on Fig 2.9A, might depend on specific parts of the network of interconnected structures, consisting of ACC and its direct connections. I have subdivided the analysis of brain networks implicated in aspects of flexible learning involving ACC into two steps. First, I analyze which role separate areas and their connections have during acquisition of cFC. In a separate cohort of mice, I then performed the same analysis for consolidation of cFC memory.

The analysis of interconnected areas underlying flexible learning is performed by utilizing the general silencing protocol, which is schematically represented in Fig.2.10A. To interfere with memory acquisition, PSEM was injected 15-20 minutes before cFC acquisition. Flexible learning was then assessed during the subsequent extinction protocol 24 hours later, followed by testing for extinction learning 24 hours later, and for spontaneous recovery of fear 12 days later. As an exception, DMS was silenced not by Sternson inhibitor, but by DREADD coupled with Gi due to the lack of strong effect of Sternson inhibitor in DMS, observed in my silencing experiments of the whole DMS area during extinction learning or during consolidation of cFC. In this case injection of the ligand to activate inhibitory DREADD channel is done 25-30 minutes before acquisition of cFC.

Silencing of circuits specifically involving projections from one area to the next was achieved by combining injections of two viruses according to the following protocol (Fig 2.10, B, C):

1. retroAAV carrying the genetic sequence for Cre-recombinase (pAAV-pCAG-iCRE-2A-H2BGFP-WPRE) was injected into the first area (area A), receiving projections from the second area of interest (area B); it was delivered retrogradely via the axons to the cell bodies of the projecting area (area B) and was expressed under CamkIIa promotor

2. AAV carrying the genetic sequence for PSAM-dependent inhibitor channel (rAAV9-CBA-flox-PSAM(Leu141Phe,Tyr116Phe)GlyR-WPRE) was injected into the projecting area of interest (area B) at the same time; its expression depended on Cre-recombinase.

For the protocol where Sternson could not be used and which required the use of DREADD, the second step had to include instead the injection of AAV carrying the genetic sequence for DREADD coupled with Gi, which further allows to cause chemogenetic inhibition of selected neurons by injecting mice with CNO (clozapine-N-oxide, the ligand with high selective affinity to DREADD channel).

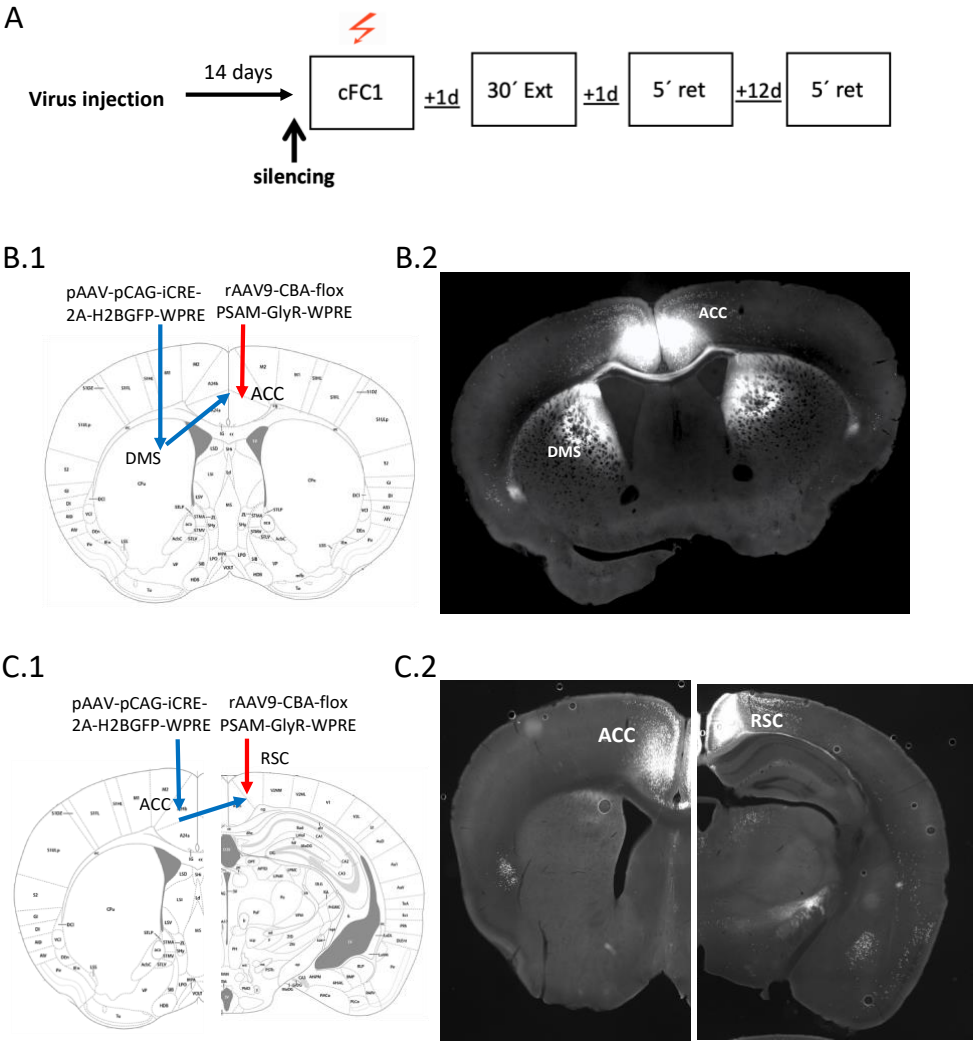


Fig 2.10 Brain networks of flexible learning involved during acquisition of cFC.
(A) Schematic of the experimental protocol. (B.1) Sample injection sites to study projections from ACC to DMS; retroAAV carrying the genetic sequence for Cre-recombinase is injected into DMS and is expressed under the CamkIIa promotor; AAV carrying genetic sequence for an inhibitor channel (Sternson inhibitor) is injected into ACC at the same time; (B.2) combined fluorescent signal from both sites of viral infection, driving expression of GFP fluorophore. (C.1, C.2) Sample injection to study projections from RSC to ACC, utilising the same approach as described above.

First, I probed the projection from RSC to ACC (Fig 2.11). Silencing ACC during acquisition of cFC, as I have shown above, leads to failure to learn extinction learning, although freezing did decline during the extinction protocol (Fig. 2.11, A). Similarly, silencing RSC->ACC projection neurons suppresses extinction learning, while only mildly affecting reduction of freezing during the extinction protocol (Fig. 2.11, B). Thus, ACC relies on the input it receives from RSC during acquisition of cFC to enable subsequent learning.

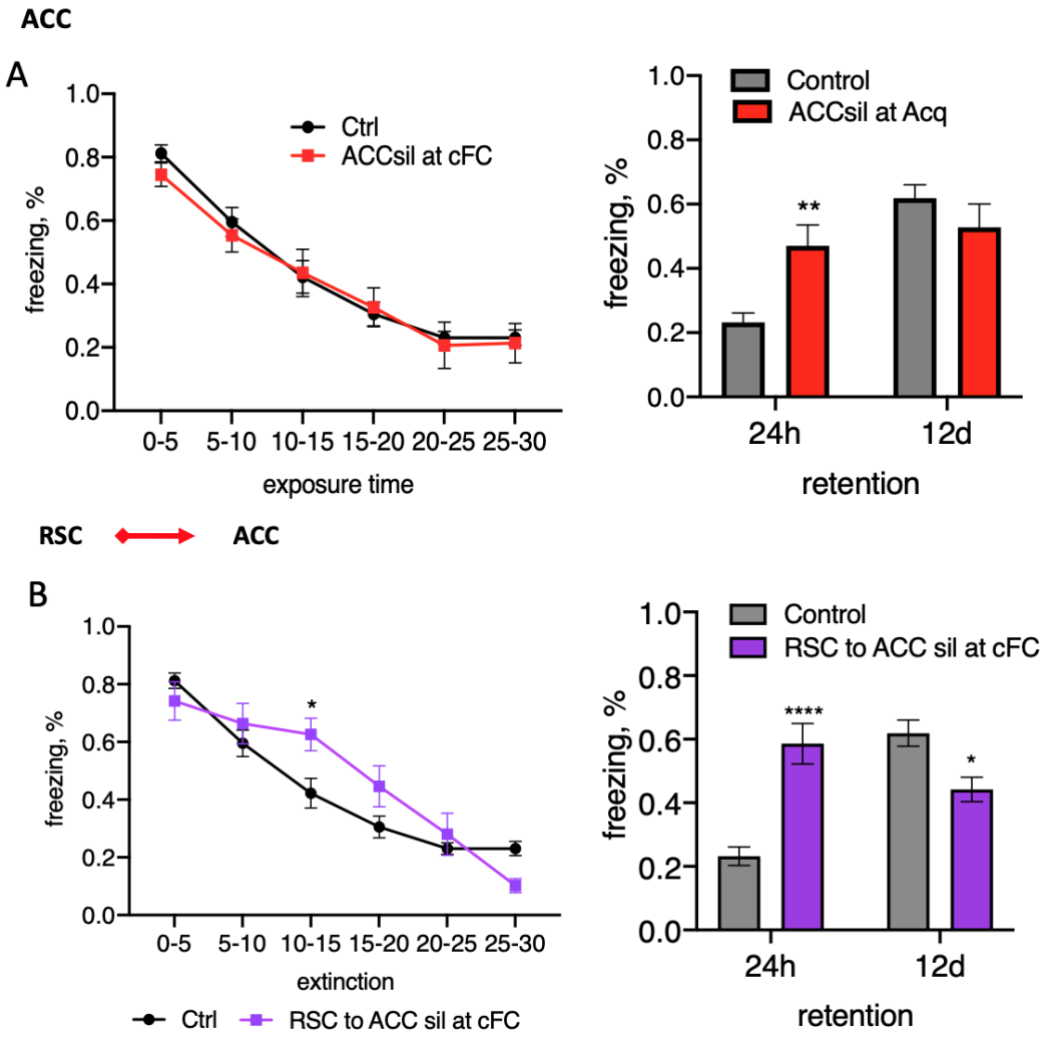


Fig 2.11 Effects of silencing ACC and the input it receives from RSC during acquisition of cFC.
(A) ACC is required during acquisition of cFC to form an adjustable memory trace. N = 3-6 each. (B) Input from RSC to ACC is required during acquisition of cFC for the formation of the memory trace, which can be adjusted during subsequent learning. N = 5 each.
*Statistical analysis – two-way ANOVA followed by multiple comparisons test *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001*

Then, I applied the same protocol to analyse the role of RSC itself during cFC acquisition (Fig 2.12 A). Remarkably, inhibiting RSC during acquisition resulted in very rapid loss of freezing during the extinction protocol, and no freezing either 24h or 12d later (Fig 2.12A). I obtained comparable results when silencing projection neurons from ACC to RSC (Fig, 2.12B). Therefore, activity in RSC is essential during acquisition of cFC for subsequent alternative learning (leading to erasure instead of extinction), and to perform this function it requires the input from the ACC.

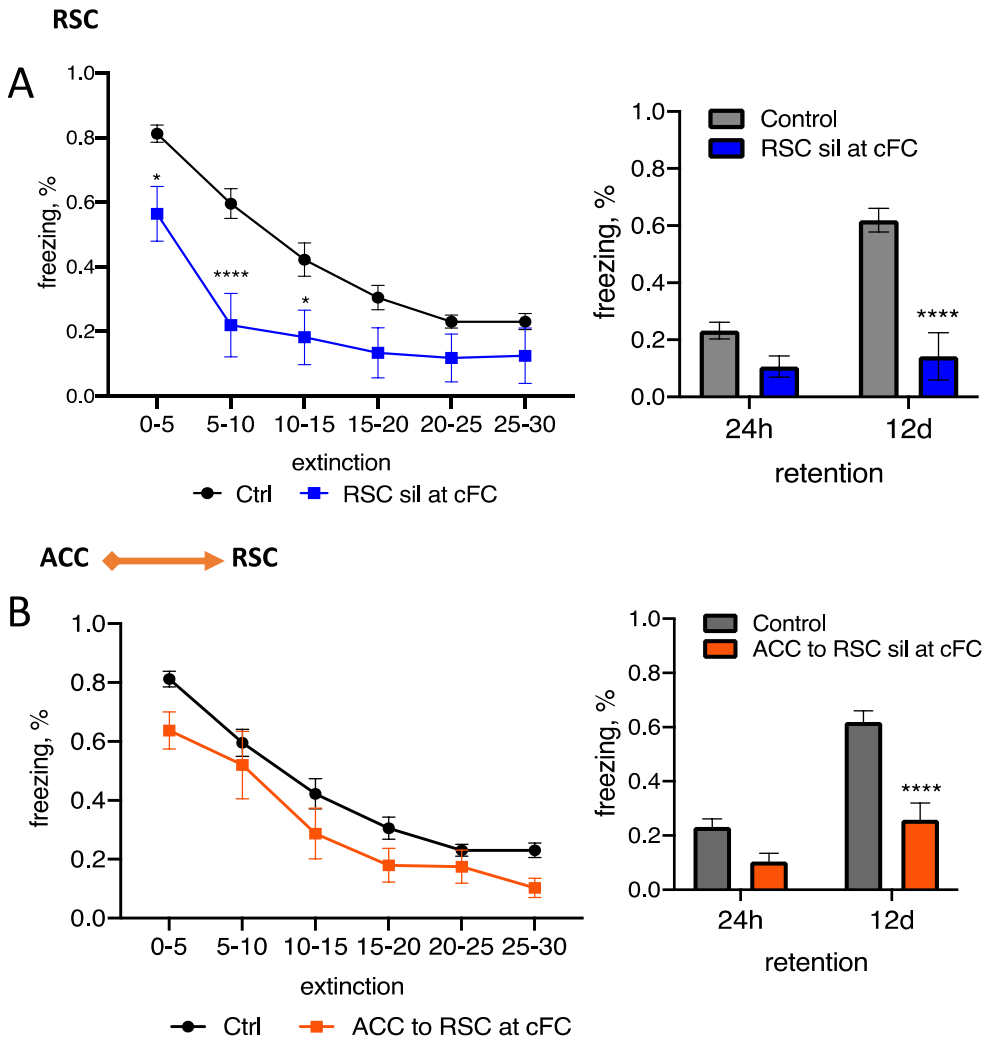


Fig 2.12 Effects of silencing RSC and the input it receives from ACC during acquisition of cFC.
(A) RSC silencing during cFC acquisition leads to subsequent relearning instead of extinction. N = 5 each. (B) Silencing connection from ACC to RSC prevents subsequent alternative learning.N = 3-5 each.
*Statistical analysis – two-way ANOVA followed by multiple comparisons test *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001*

Next, I probed downstream targets, which receive inputs from ACC and RSC, and are involved in flexible learning, according to published reports as well results from our lab. First, I silenced DMS during acquisition of cFC (Fig 2.13A). This again led to erasure instead of extinction, although reduction of freezing during the extinction protocol was not noticeably accelerated (Fig. 2.13A). I made comparable observations when inhibiting ACC->DMS (Fig. 2.13B), RSC->DMS (Fig. 2.13C), ACC->PF (Fig. 2.14A), or RSC->PF (Fig. 2.14B) connectivity during acquisition of cFC.

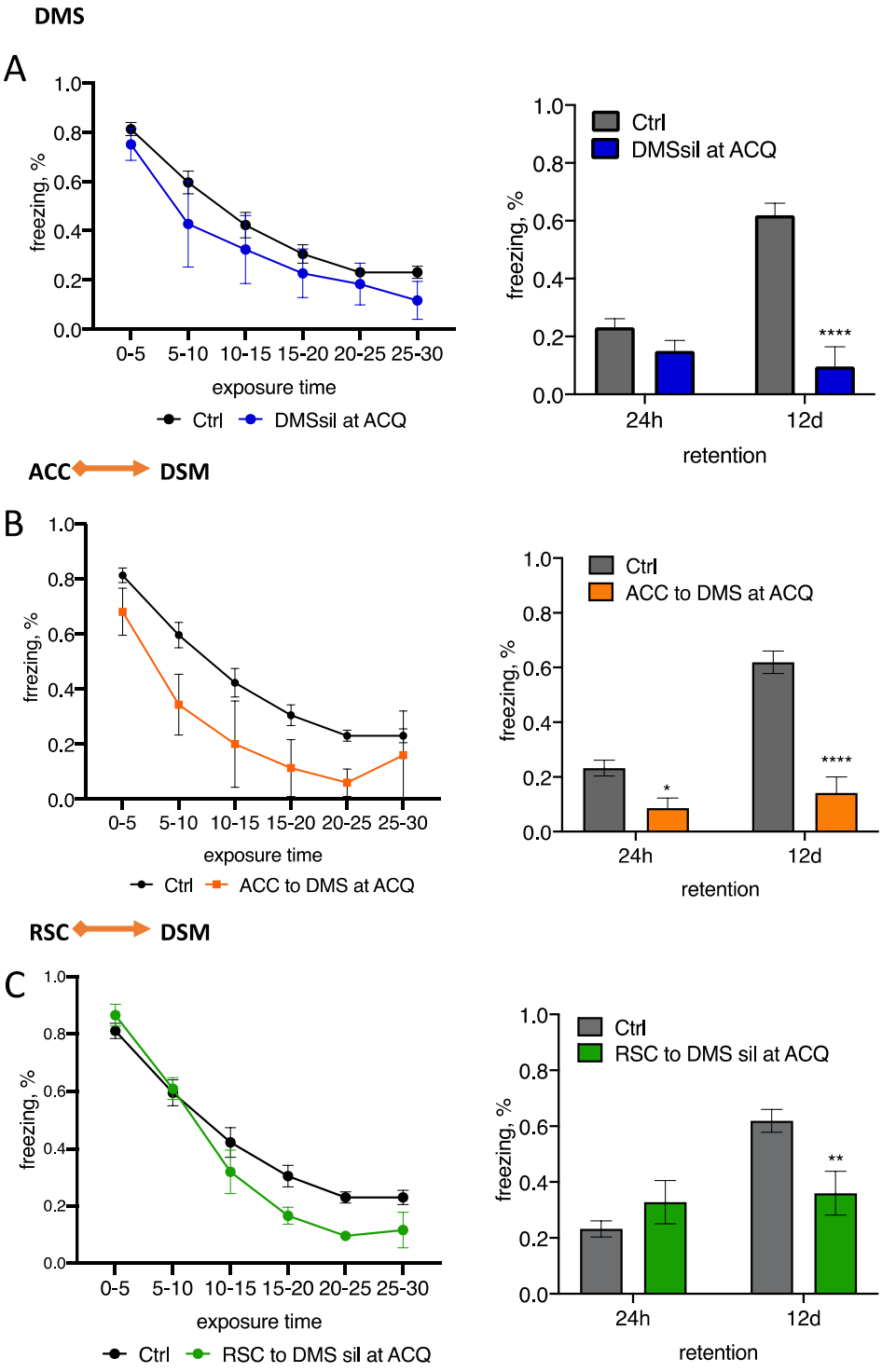


Fig 2.13 Effects of silencing DMS and the input it receives from RSC and ACC during acquisition of cFC.

(A) DMS is required at acquisition of cFC for alternative learning. N = 3-5 each. Connections from ACC to DMS (B) and RSC to DMS (C) are required during acquisition of cFC to enable alternative learning. N = 3-5 each.

*Statistical analysis – two-way ANOVA followed by multiple comparisons test *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001*

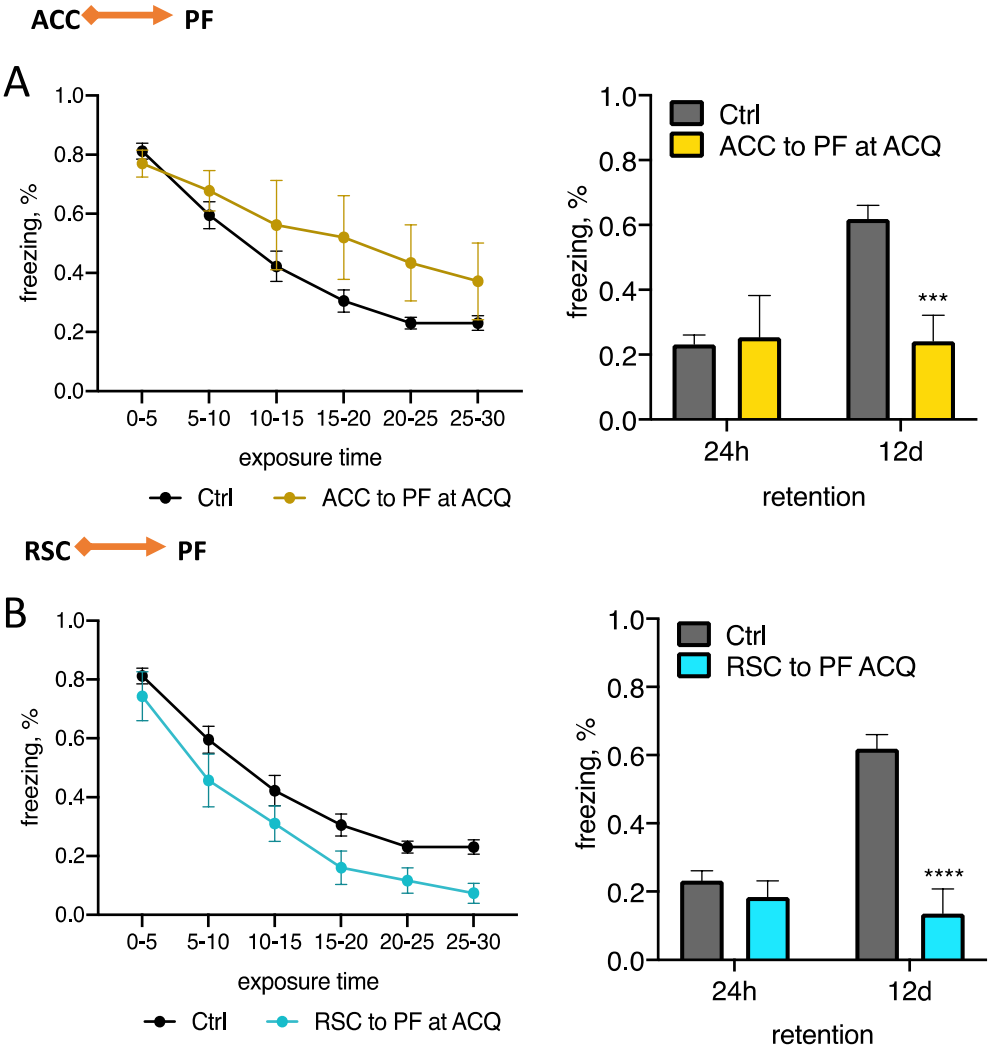


Fig 2.14 Effects of silencing ACC to PF and RSC to PF connectivity during acquisition of cFC.
Connections from ACC to PF (A) and RSC to PF (B) are required during acquisition of cFC to enable alternative learning. N = 3-5 each.
*Statistical analysis – two-way ANOVA followed by multiple comparisons test *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001*

Taken together, these findings provided evidence that activity in RSC, DMS, as well as in ACC->RSC, RSC->DMS, ACC->DMS, RSC->PF and ACC->PF connectivity during acquisition of cFC is critically important to prevent erasure during extinction of cFC (Fig 2.10, 2.11, Fig 2.12, Fig 2.13, Fig 2.14). By contrast, activity in ACC and in RSC->ACC connectivity is necessary during acquisition of cFC for subsequent learning (no extinction, no erasure).

2.6.2 Brain network required during consolidation of cFC for subsequent flexible learning

In the second part of my analysis of the network related to ACC and implicated in subsequent flexible learning, I have concentrated on interfering with the activity of the structures and their connections during consolidation of cFC memory. Like for the first step of the analysis, silencing was induced by using the same combination of two viral injections into the selected areas (Fig 2.15). For all the areas and their connections silencing involved Sternson inhibitor, except for DMS where I have instead used DREADD coupled with Gi.

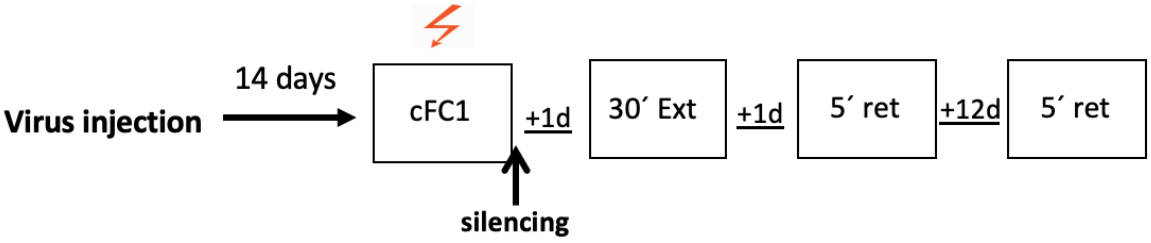


Fig 2.15 Schematic of the experimental protocol for the analysis of brain networks required during consolidation of cFC for subsequent flexible learning.

I have started with silencing ACC and the input it receives from RSC (Fig 2.16, Fig 2.17). ACC is required during consolidation of cFC to enable subsequent alternative learning (Fig 2.16). Silencing RSC->ACC projecting neurons during memory consolidation led to subsequent erasure (Fig 2.17).

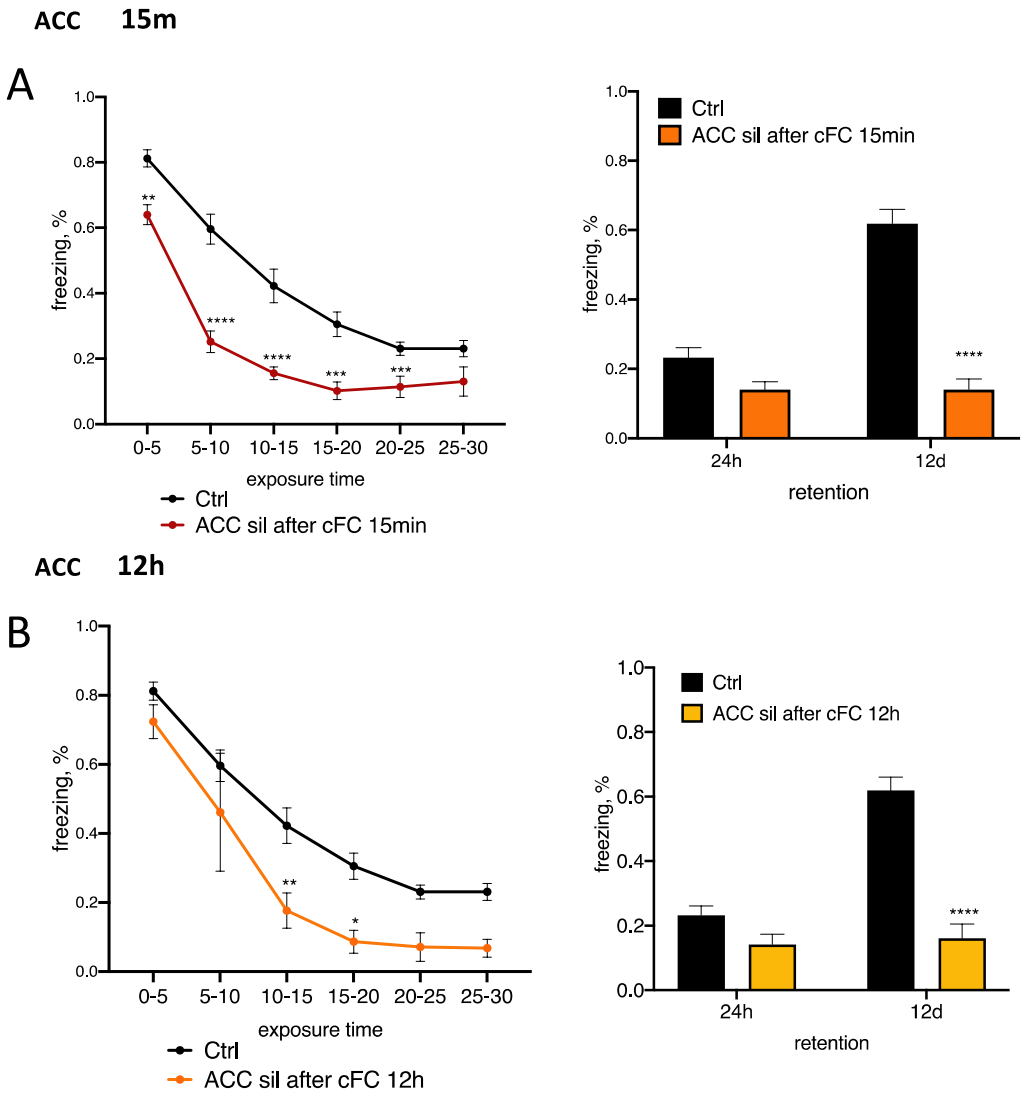


Fig 2.16 Effects of silencing ACC during consolidation of cFC.
Silencing ACC during consolidation of cFC (15 minutes (A) or 12 hours (B) after acquisition) prevents alternative learning 24h later. N = 3-8 each.
*Statistical analysis – two-way ANOVA followed by multiple comparisons test *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001*

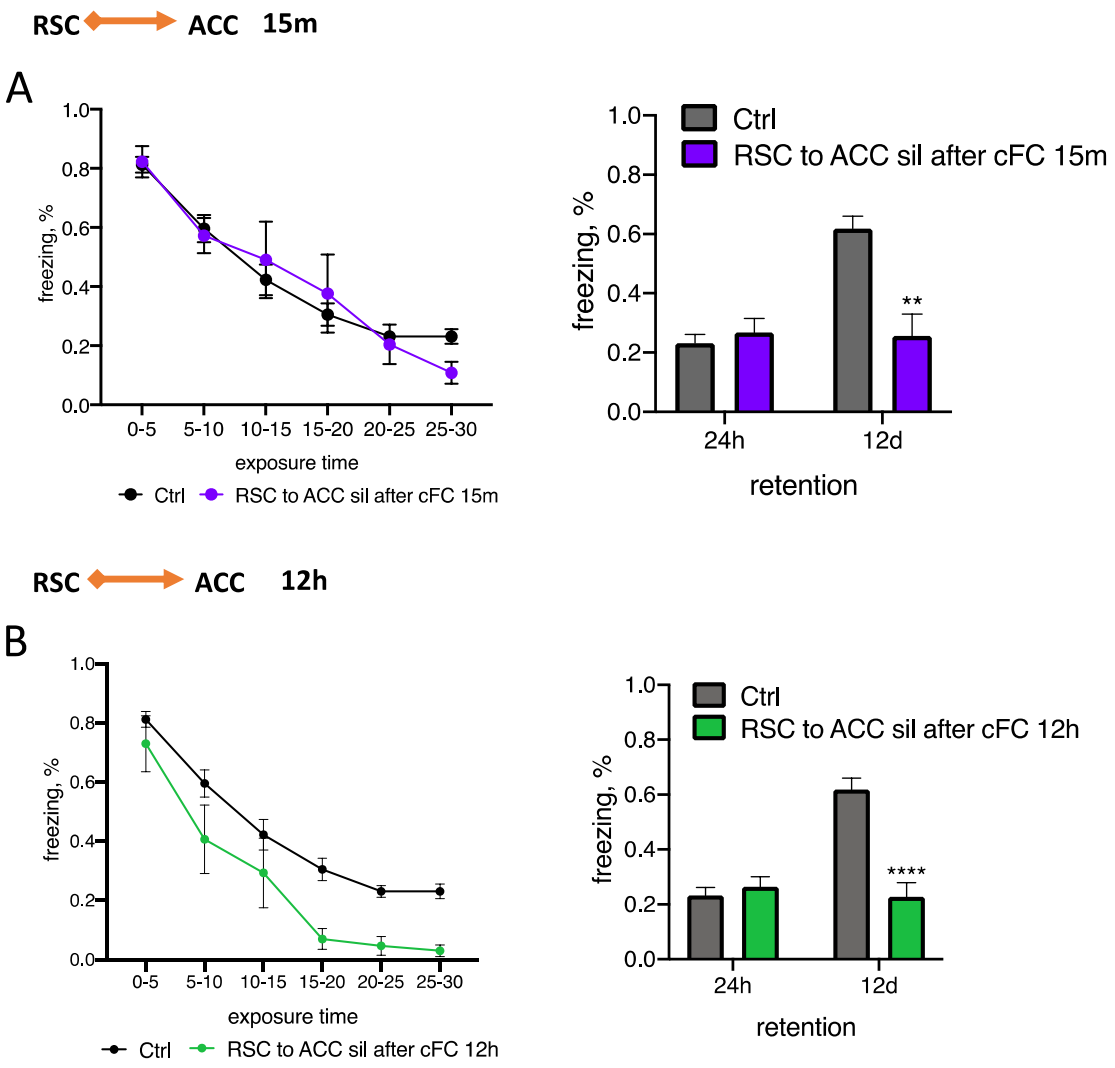


Fig 2.17 Effects of silencing the input to ACC from RSC during consolidation of cFC. Silencing of RSC to ACC input during cFC consolidation 15 minutes (A) or 12 hours (B) after acquisition prevents alternative learning 24h later. N = 3-5 each.
*Statistical analysis – two-way ANOVA followed by multiple comparisons test *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001*

By contrast, silencing ACC->RSC projection neurons during consolidation of cFC suppressed subsequent learning (no extinction, no erasure; Fig. 2.18); at the same time, silencing RSC at consolidation of cFC led to abolishment of subsequent learning (Melissa Serrano', unpublished results). Likewise, silencing DMS during consolidation of cFC suppressed subsequent learning (Fig. 2.19A). Silencing ACC->DMS, ACC->PF or RSC->PF projection neurons during consolidation of cFC led to subsequent erasure (Figs. 2.19B, 2.20A, 2.20B), whereas silencing RSC->DMS projection neurons did not perturb subsequent extinction learning (Fig. 2.19C).

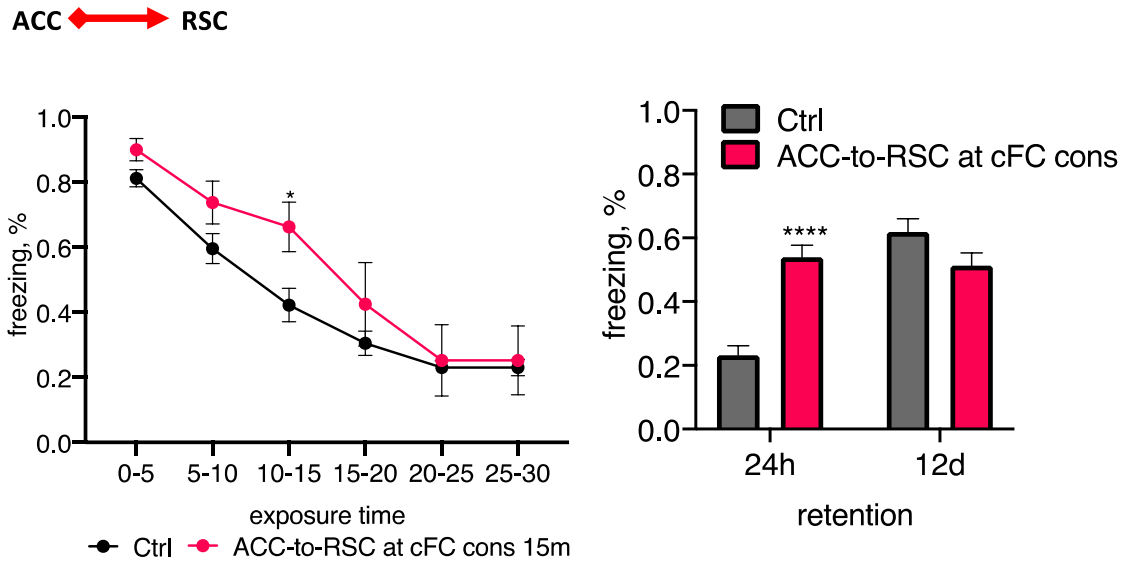


Fig 2.18 Effects of silencing the input to RSC from ACC during consolidation of cFC. Silencing of ACC to RSC input during cFC consolidation (15 minutes after acquisition) prevents alternative learning 24h later. N = 6-8 each.
*Statistical analysis – two-way ANOVA followed by multiple comparisons test *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001*

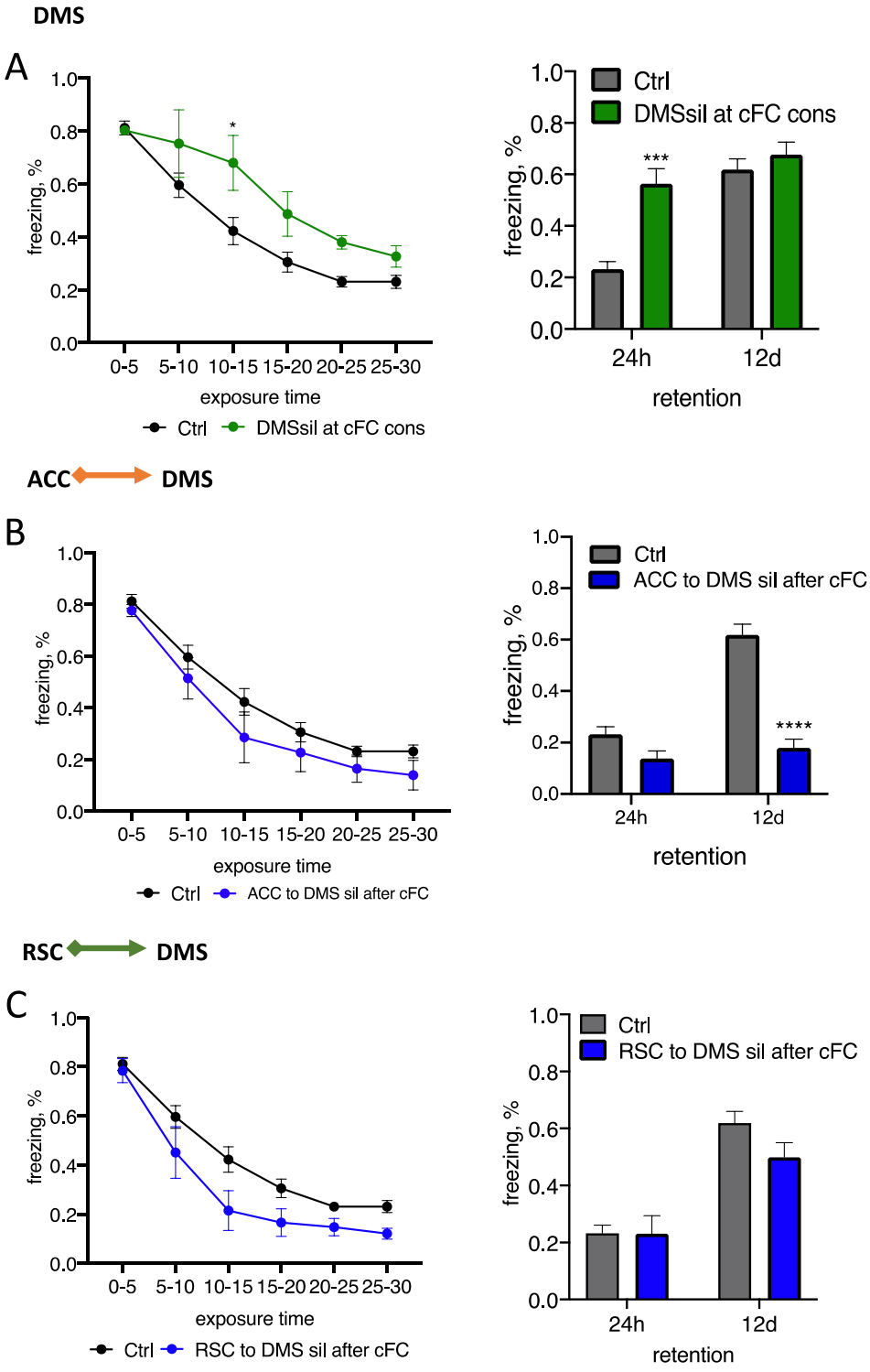


Fig 2.19 Effects of silencing DMS or the input it receives from ACC or RSC during consolidation of cFC.
(A) Activity in DMS is required at consolidation of cFC for subsequent learning. N = 3-5 each. (B) Connectivity from ACC to DMS is required during consolidation of cFC to enable subsequent alternative learning. N = 5-8 each. (C) Connectivity from RSC to DMS is not required during consolidation of cFC for subsequent learning. N = 5-6 each.
*Statistical analysis – two-way ANOVA followed by multiple comparisons test *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001*

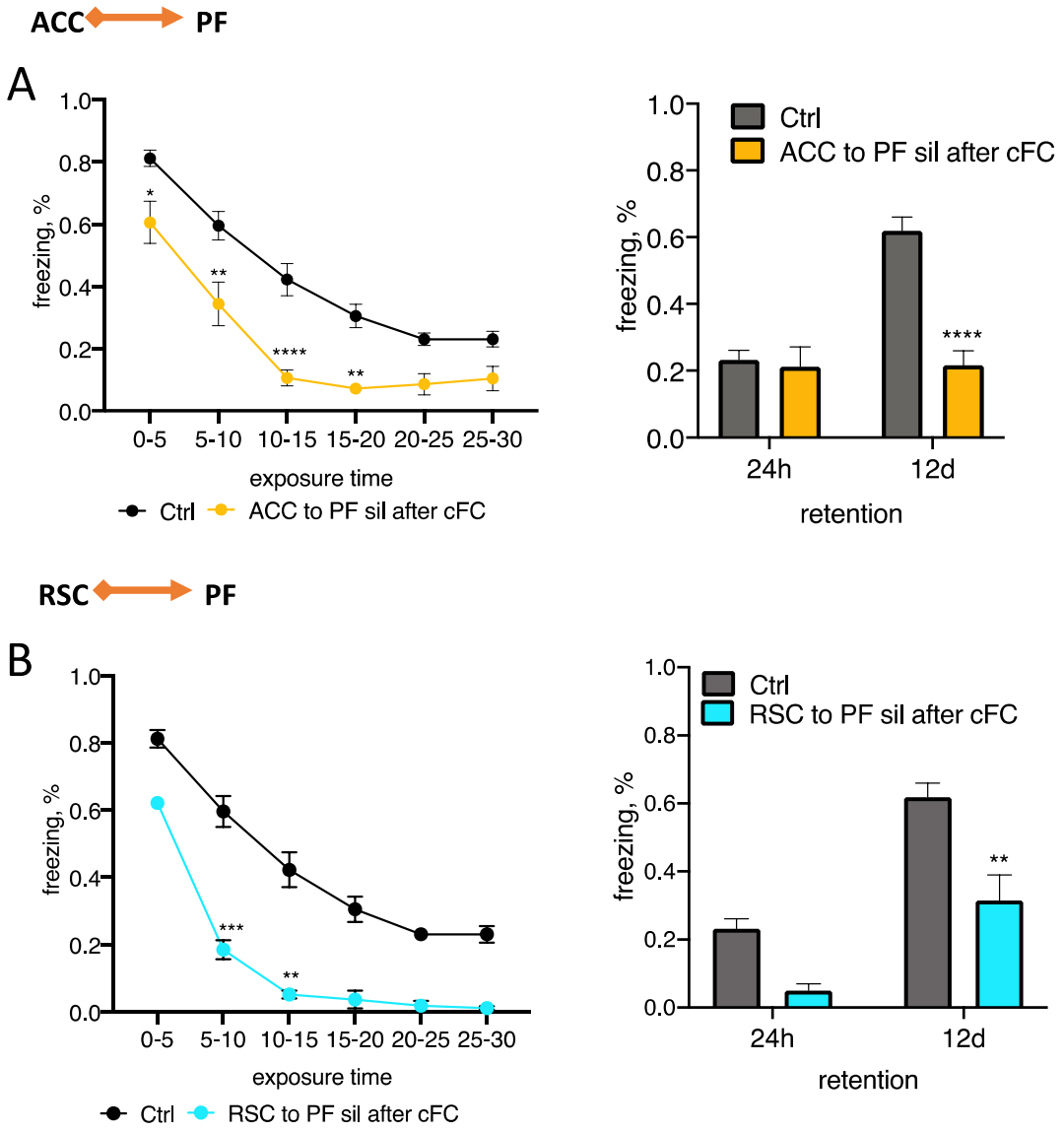


Fig 2.20 Effects of silencing connectivity from ACC to PF, or from RSC to PF during consolidation of cFC.
Connectivity from ACC to PF (A) or RSC to PF (B) is required during consolidation of cFC to enable subsequent alternative learning. N = 5 each (A), 2-5 each (B).
*Statistical analysis – two-way ANOVA followed by multiple comparisons test *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001*

These results demonstrate, that DMS is required during consolidation of cFC to allow subsequent learning. However, the input DMS receives from ACC is not required for subsequent learning, and instead is needed to learn alternative learning, whereas silencing projection neurons from RSC to DMS during cFC consolidation did not affect extinction learning.

2.7 Brain networks involved in distinct forms of subsequent learning

To further specify the role of an ACC-related brain network in flexible learning, I next silenced these areas and projection neurons during subsequent learning (i.e. duirng the extinction protocol). This approach also allowed me to contrast mechanisms required for alternative as opposed to subsequent relearning. I first investigated silencing during alternative learning (Fig 2.21). Sternson Inhibitor was used to silence RSC, ACC and their connections, whereas DREADD coupled with Gi was used for silencing dHP and DMS; ligand was injected 15-20 minutes (for Sternson) or 25-30 minutes (for DREADD) before the beginning of extinction.

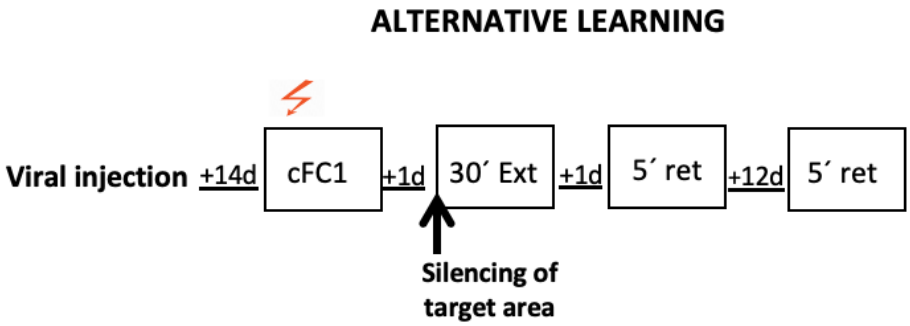


Fig2.21 Schematic of the experiments addressing alternative learning

As was shown by experiments performed in our lab by Melissa Serrano (manuscript in preparation), RSC and its projections to PF and DMS are required during the extinction protocol for alternative learning. I have then asked if RSC depends on input from ACC to perform this function. Silencing ACC->RSC projection neurons did not affect extinction learning (Fig. 2.22A). Likewise, silencing ACC during the extinction protocol did not affect extinction learning (Fig. 2.22B).

By contrast, silencing dHP (Fig. 2.23A) or DMS (Fig. 2.23B) during the extinction protocol prevented extinction learning.

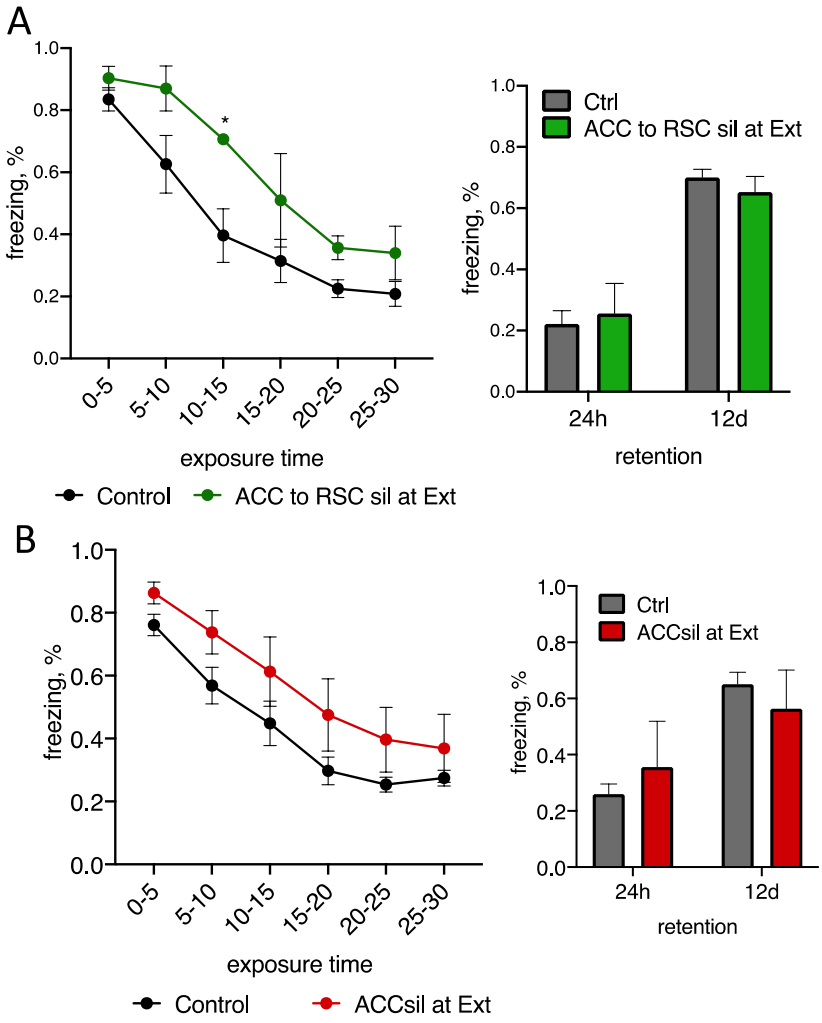


Fig 2.22 No involvement of ACC and its projection to RSC during alternative learning. (A) ACC to RSC connectivity silencing does not affect extinction learning. N = 3-5 each. (B) Silencing ACC during extinction does not affect extinction learning. N = 3-5 each. *Statistical analysis – two-way ANOVA followed by multiple comparisons test *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001*

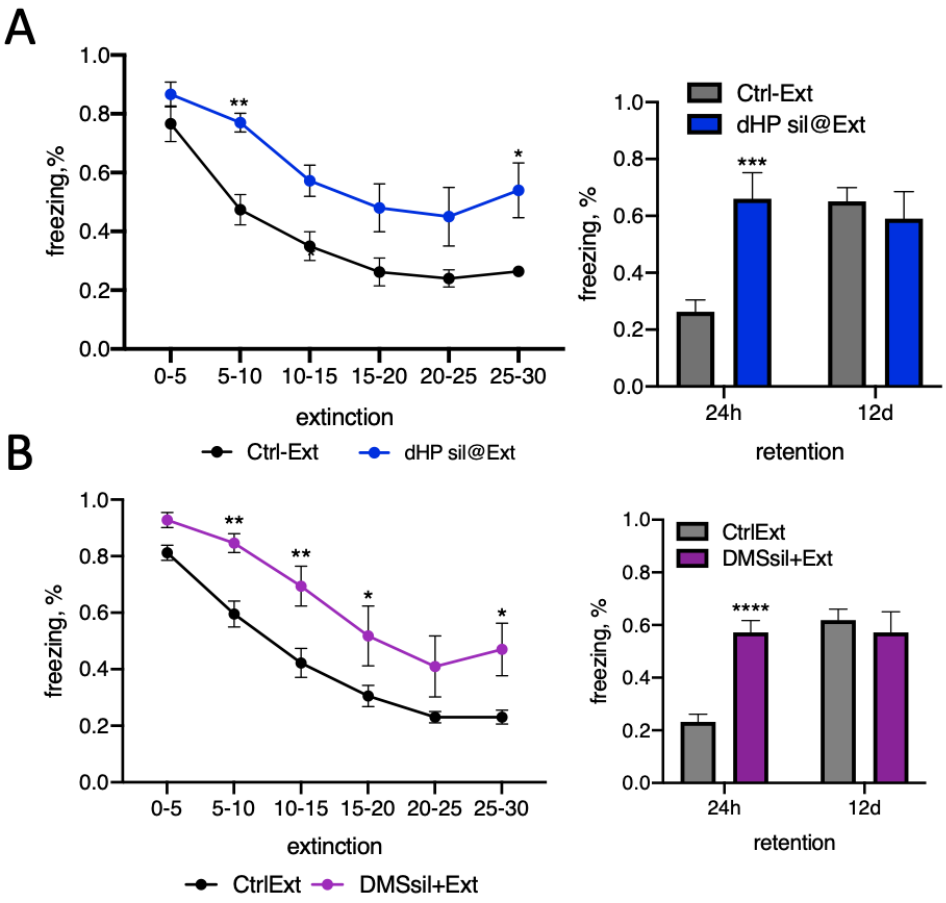


Fig 2.23 Requirement for activity in dHP and DMS for extinction learning
Silencing dHP (A) or DMS (B) during extinction prevents extinction learning. N = 5 each.
*Statistical analysis – two-way ANOVA followed by multiple comparisons test *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001*
Silencing done using DREADD Gi

Next, I addressed requirements for erasure learning. To investigate ACC-related brain networks underlying relearning (erasure of cFC), I have compared two different relearning protocols. The first one is the two-stage silencing protocol schematically represented in Fig 2.24. In this approach I silenced ACC during consolidation of cFC with the GABA-A agonist muscimol, and then examined the impact of area silencing during the subsequent extinction/erasure protocol.

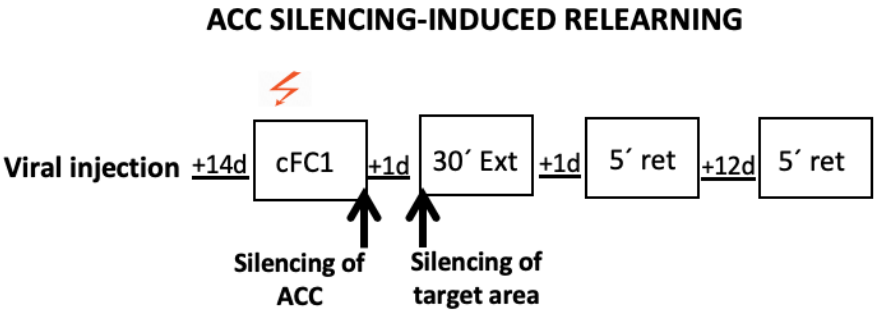


Fig2.24 Schematic of the experiments addressing subsequent learning in ACC silencing-induced relearning protocol

Silencing RSC during the extinction/relearning protocol prevented relearning (Fig. 2.25A). Likewise, silencing ACC->RSC connectivity during the extinction/relearning protocol prevented relearning (Fig. 2.25B). Silencing ACC prevented relearning (Fig. 2.26A), but silencing RSC->ACC connectivity did not affect relearning (Fig. 2.26B). Finally, silencing dH or DMS during the extinction/relearning protocol did not affect relearning (Fig. 2.27A, B).

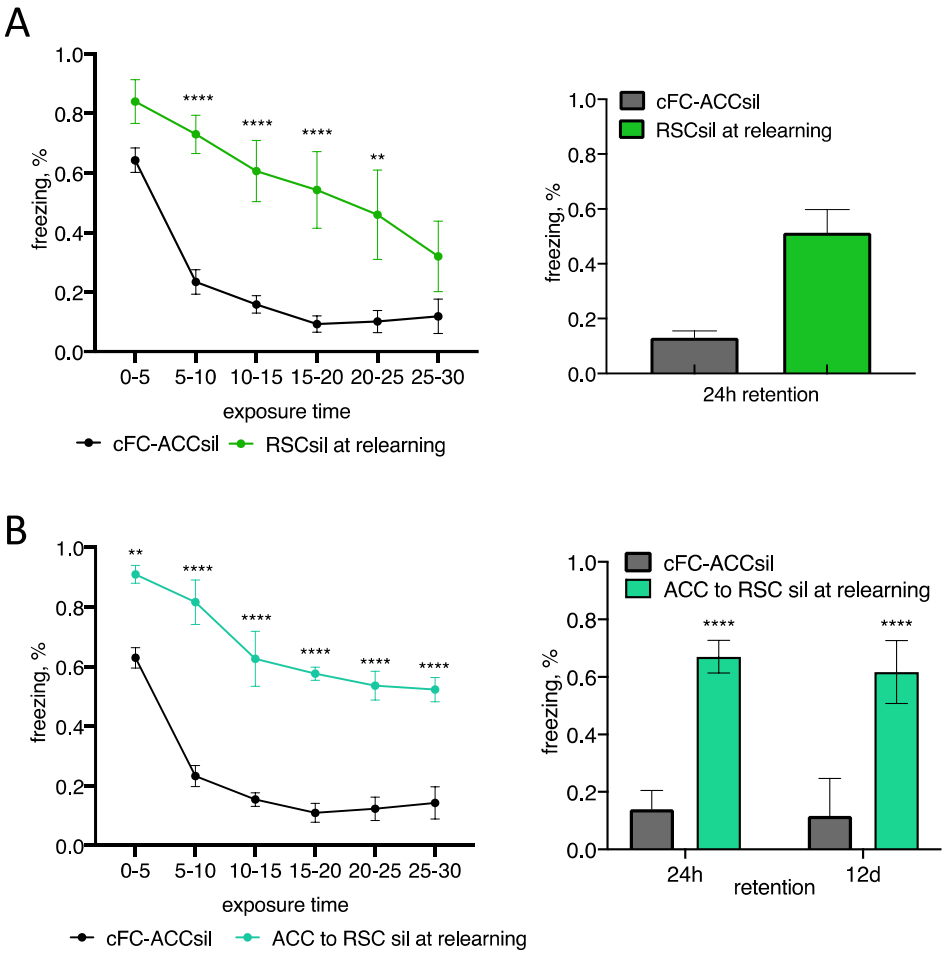


Fig 2.25 Requirement for RSC (A) and the input it receives from ACC (B) in relearning.
N = 2-5 each (A) and N = 3-5 each (B).
*Statistical analysis – two-way ANOVA followed by multiple comparisons test *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001*

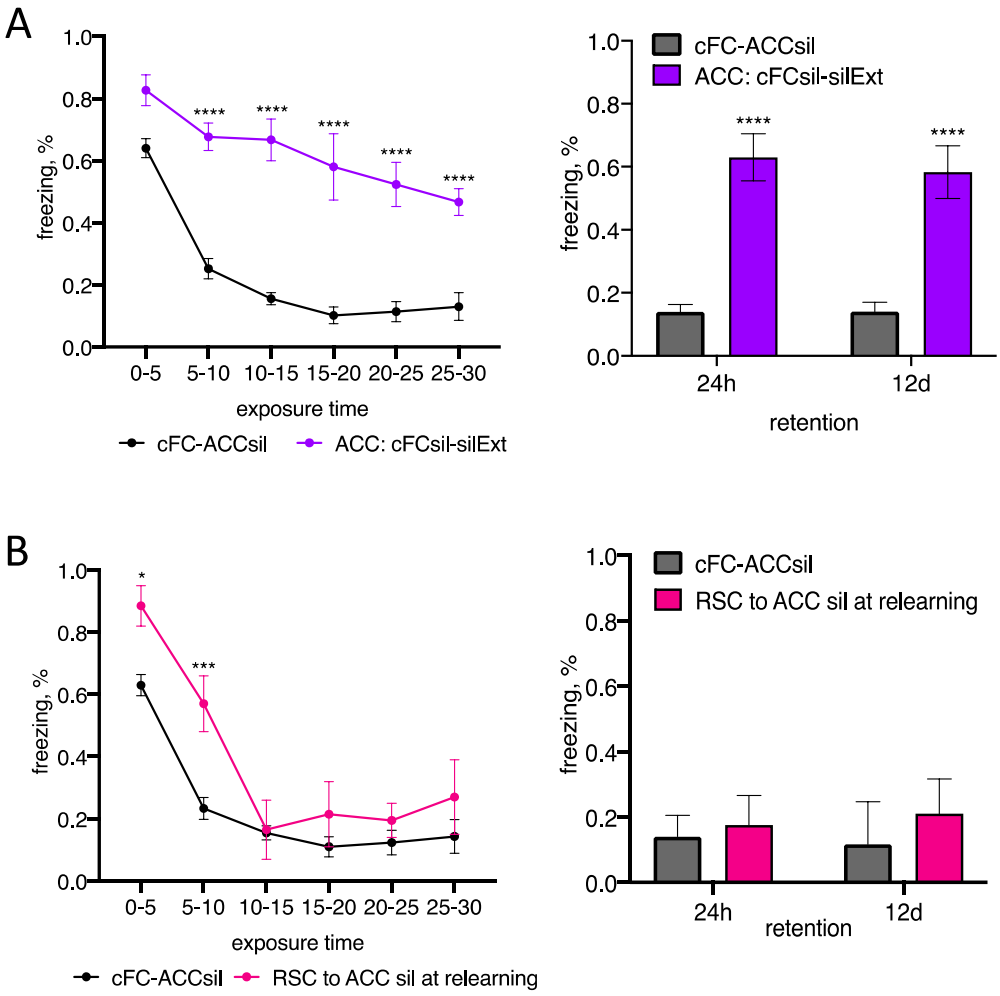


Fig 2.26 Requirement for activity in ACC (A) and in RSC-to-ACC connectivity (B) for relearning.
N = 3-5 each.
*Statistical analysis – two-way ANOVA followed by multiple comparisons test *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001*

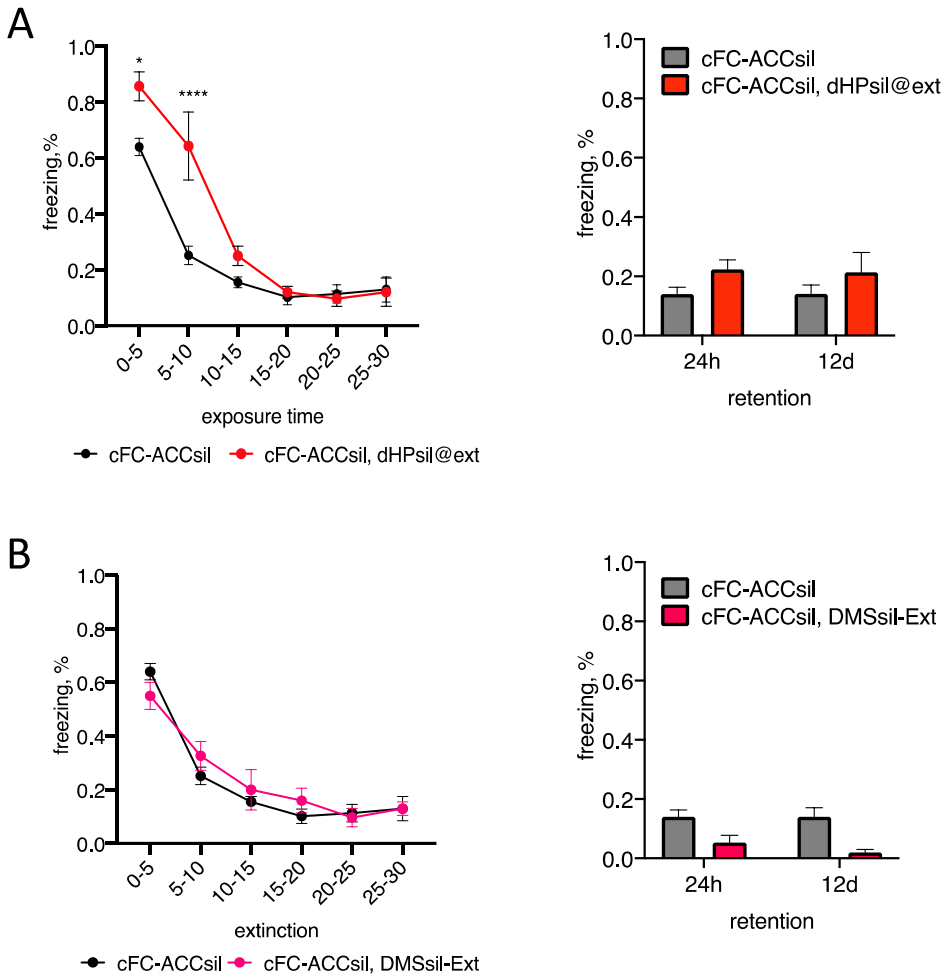


Fig 2.27 No requirement for activity in dHp (A) or DMS (B) for relearning.
N = 3-5 each.
*Statistical analysis – two-way ANOVA followed by multiple comparisons test *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001*

In the next step I have addressed requirements for behavioral erasure learning. Protocol for behavioral erasure is comprised of 5-minutes recall and 25 minutes of extinction, performed within the plasticity (reconsolidation) window initiated by recall (Kindt et al., 2009). This transient state coincides with the 1st window of c-Fos expression and lasts up to 5h, during which the initial memory can be updated with the new information. Unlike alternative learning, in which the initial memory remains unchanged, extinction given within the plasticity window instead erases the fear memory.

To probe the role of activity in respective brain areas during behavioral erasure, I have done silencing in the 2nd phase of the protocol – during 25-minute contextual exposure

(Fig2.28). The resulting memory was assessed by the freezing levels immediately, at 24h retention and at 12 days recovery.

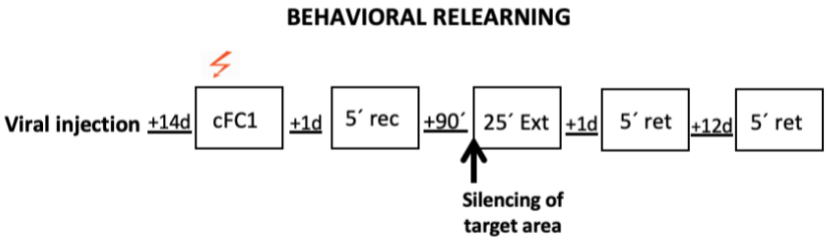


Fig2.28 Schematic of the experiments addressing subsequent learning in behavioural erasure protocol

Silencing DMS during the 2nd phase of the behavioral erasure did not impact relearning (Fig 2.29). Likewise, silencing ACC as well had no effect on the result of relearning, although it led to partial increase in immediate freezing levels (Fig 2.30). On the contrary, silencing RSC or dHP during 2nd phase of the protocol completely prevented relearning. The roles of RSC and dHP in behavioral erasure were established (using the same protocol) by Melissa Serrano and Maria Lahr respectively (unpublished data, not shown). The summary of experimental results is represented in the scheme (Fig2.36).

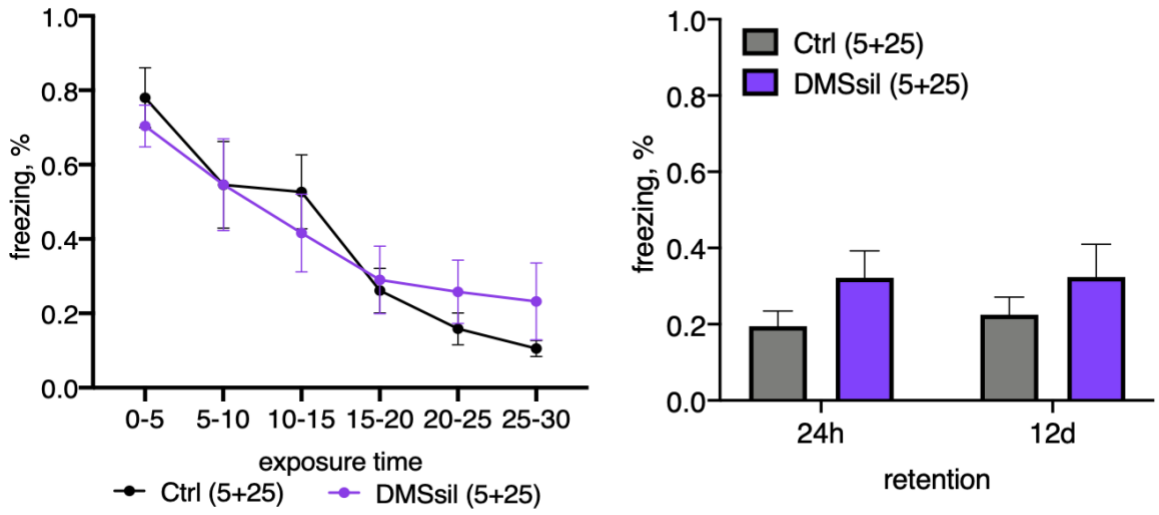


Fig 2.29 No requirement for DMS at behavioral relearning.
N = 5 each.
*Statistical analysis – two-way ANOVA followed by multiple comparisons test *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001*

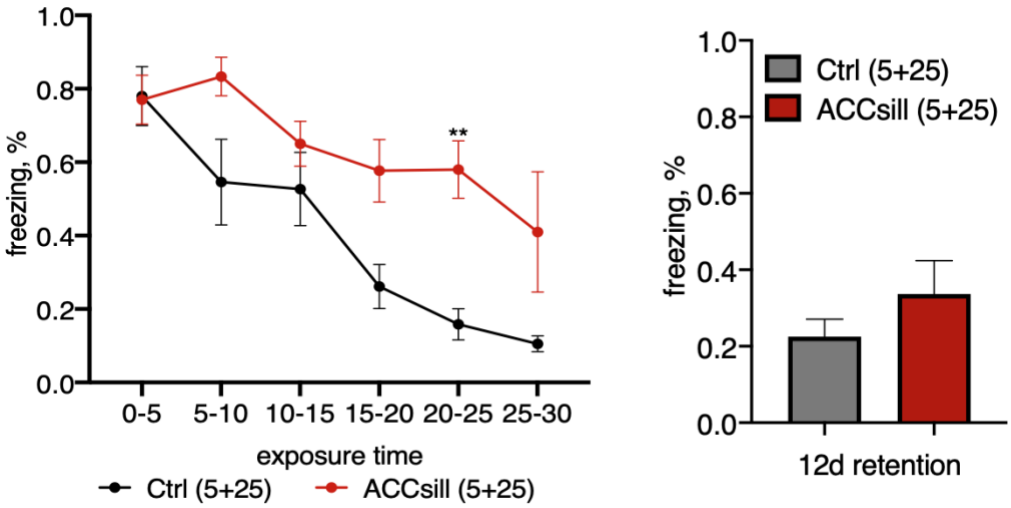


Fig 2.30 No requirement for ACC at behavioral relearning.
N = 3-5 each.
*Statistical analysis – two-way ANOVA (left) and t-test (right) *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001*

2.8 Experimental conditions for reversal of re-learning

Although relearning is characterized by at least partial rewriting of the original fear memory **(**Suppl 2**)** and absence of spontaneous fear recovery, it remained unclear if the initial fear memory is being irreversibly altered already during the extinction protocol or whether there might be a time window when fear can still be reinstated by the delivery of a footshock..

To reinstate fear in the previously conditioned mice, I have used a paradigm in which one footshock is delivered at the 25th minute of the contextual exposure. This footshock serves as the contradictory evidence to the alternative (safety) value acquired during extinction, and reinstated the meaning of the context back to fear. this led to behavioural return of freezing and to suppression of extinction learning (2.31 A).

Notably, delivery of one footshock also fully restored freezing and fear memory in mice in which relearning was induced by preceding ACC silencing at cFC consolidation (Fig. 2.31 B, C). This result is particularly noteworthy in view of the fact that for relearning to occur, mice only had to be exposed to conditioning context in the absence of footshocks during 10 min (Suppl. 2). This result suggests that the footshock delivered

at the 25th minute interferes with the outcome of learning as a whole for both alternative learning and relearning.

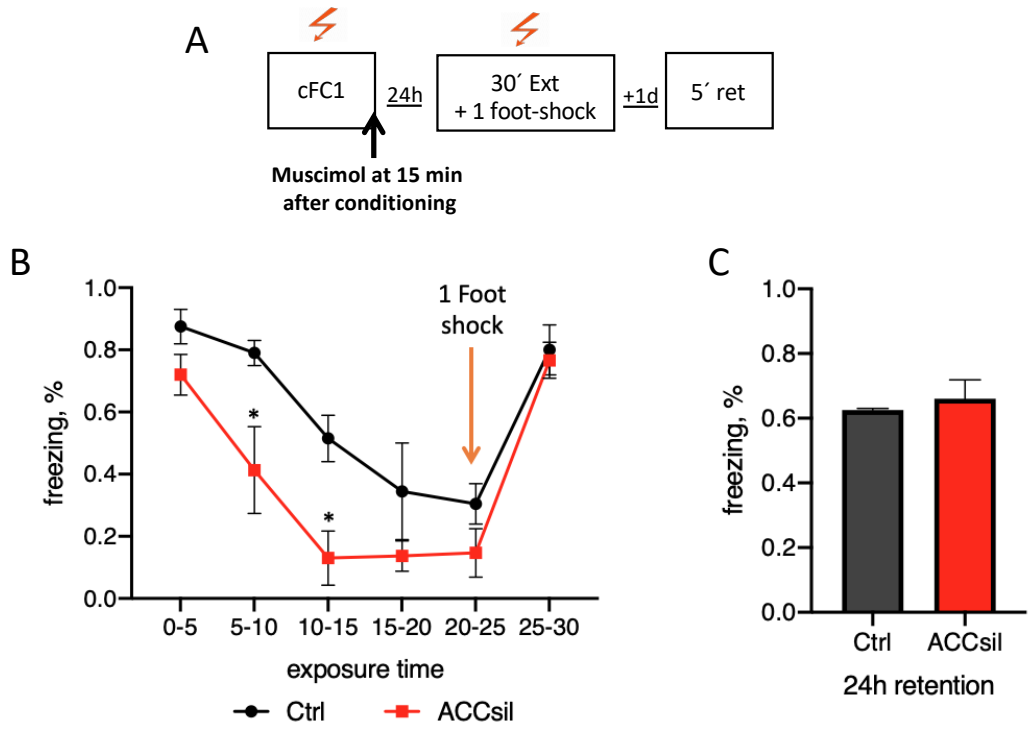


Fig 2.31 Single footshock during context exposure reinstates fear during extinction or erasure.
(A) Schematic of the experiment. One footshock during extinction or erasure learning is sufficient to restore immediate (B) and retention-time (C) freezing. N = 3 each.
*Statistical analysis – two-way ANOVA followed by multiple comparisons test *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001*

I then determined whether relearning might become irreversible after completion of the memory consolidation process. To this end, I have applied a reinstatement protocol, which consists of delivering one footshock at different time intervals post extinction or erasure (Fig 2.32, A).

I subdivided animals in two groups – one that underwent extinction learning, and the other underwent relearning due to silencing of ACC at consolidation of cFC. Each group was further subdivided in three different cohorts of mice, in which one footshock was delivered 3h, 7.5h and 24h after the extinction protocol (Fig 2.32, A).

One footshock delivered at 3h was ineffective in reinstating fear after extinction or erasure (Fig 2.32, B1-2). By contrast, and remarkably, a footshock delivered at 7,5 h caused reinstatement of fear in both extinction and erasure groups of mice (Fig 2.32, C1-2). Finally, a footshock delivered in training context 24h after subsequent learning led to reinstatement of fear memory after extinction learning, but not after erasure (Fig 2.32, D1-2). This finding suggested that once erasure learning is consolidated at the 12-hour time window, it becomes resistant to reinstatement of fear.

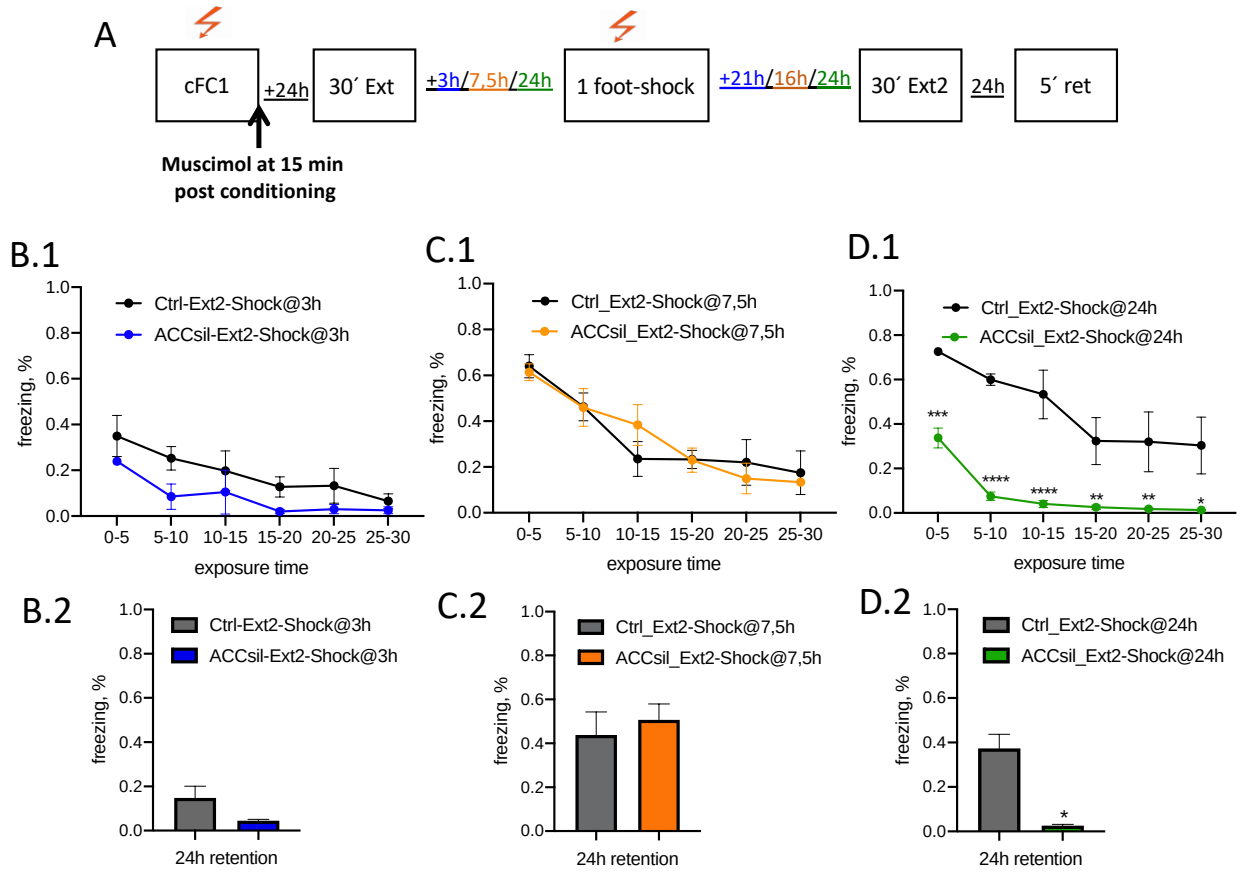


Fig 2.32 Time-dependent sensitivity to the reinstatement of fear memory by a single footshock post extinction or erasure.
(A)Schematic of the experiment.
(B1, B2) A single footshock delivered in conditioning context 3h after extinction or erasure learning does not reinstate the fear memory.
(C1, C2) A single footshock delivered in conditioning context 7.5h after extinction or erasure learning reinstates fear memory.
(D1, D2) A single footshock delivered in conditioning context 24h after extinction or erasure learning reinstates fear memory upon extinction, but not upon erasure learning.
N = 3-4 each.
*Statistical analysis – two-way ANOVA followed by multiple comparisons test *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001*

3. Discussion and Outlook

3.1 Summary of main findings

I have investigated the role of ACC in flexible learning. For this purpose, I interfered with activity in ACC during acquisition and consolidation of the original memory, and analysed the consequences for subsequent learning. Notably, while these interventions had profound and specific consequences on subsequent learning, interfering with activity in ACC during subsequent alternative learning itself did not affect those learning processes. These findings point to an important distinction between ACC and other prefrontal (prelimbic and infralimbic cortices), and cingular (RSC) areas, which were also required during alternative learning. This suggests that distinct brain networks are required during different stages of flexible learning.

Taking into consideration dense reciprocal connections of the ACC with numerous areas of the extra-hippocampal circuit, it was important to include in the study of ACC function its input and output targets that have been implicated in flexible learning. The study of the broader network for flexible learning provided important insights on how, on one hand, the function of flexible memory formation is supported by a network of structures implicated at acquisition and consolidation, and, on the other hand, how the same structures are involved during subsequent learning. The main findings of my study include the observations that a network of ACC-related brain areas is involved in setting up conditions for subsequent flexible learning, and that the relative roles of these structures are opposite at acquisition and consolidation of the original memory. A further important finding is that once the initial memory is acquired, subsequent learning depends on a different network of structures, albeit some of them are involved in both stages of learning (initial and subsequent). Moreover, different types of subsequent learning, namely alternative learning and relearning, depend as well on a different set of structures. My findings concerning the time-course of relearning memory consolidation highlight distinct roles of a 5h time window, as opposed to the 12h time window for consolidating relearning. Finally, my results reveal how the

incorporation of subsequent evidence related to the same context depends on activity in ACC.

3.2 Role of ACC in the formation of flexible memories

The results presented in my thesis demonstrate a previously undiscovered role of the ACC in flexible memory formation.

The deficits caused by ACC silencing at consolidation or acquisition of the initial memory lead to abolishment of subsequent flexible learning. Thus, silencing ACC at consolidation of cFC or of the IDS phase of ASST prevents subsequent alternative learning, and dramatically facilitates switch during reversal phases of behaviour, whereas silencing at acquisition of cFC prevents retention of subsequent learning without affecting immediate behaviour. At the same time, I have found that rule application, generalisation and expression, which are based on the initial memory trace, are not affected by ACC silencing. Similarly, a published study found that expression of recent fear memory itself does not depend on ACC (Frankland, 2004).

A deficit in subsequent learning caused by ACC silencing at acquisition of the initial memory is evident not only at reversal learning, but also in subsequent learning involving reinforcement of the previous related memory trace. Thus, a second session of cFC in the same setting fails to reinforce the initial cFC when the latter was acquired while ACC was silenced. Inability to integrate subsequent learning in turn leads to deficits in behavioural adaptation. In particular, inability to integrate repeated reinforcement leads to decreased adherence to current behavioural strategy, whereas inability to integrate the evidence to the contrary prevents the switch to an alternative one.

Published studies have described the role of ACC as being crucial for integration of reinforcement history to guide voluntary behaviour (Holroyd and Coles, 2008; Kennerley et al., 2006; Wittmann et al, 2016). However, these studies were limited to investigating the role of ACC activity within one learning session, and did not consider possible contributions of ACC to flexible learning involving multiple learning sessions

spanning over multiple days. Adding to those previous studies, my data reveal how activity in ACC is important to form a memory which serves as a basis for subsequent learning sessions.

3.3 ACC connectome

Studying the role of ACC within a broader brain network context is essential in order to better understand its role in flexible learning. New genetic tools allowed us to not only trace, but also manipulate selected circuits within this network.

Especially interesting in the context of this study were ACC connections with distinct memory systems, including the extrahippocampal system, and two specialized routes (White et al., 2002*)* – one constituted by DMS and the other by the amygdala. The elements of the first two were addressed in the current work, whereas the one comprising amygdala circuits could be an interesting alternative direction to study.

Unlike some other current studies (Bian et al. 2019; Rajasethupathy et al., 2015), I have not observed a direct connection between ACC and hippocampus in any of the tracing experiments. However, ACC has 2nd order connections with the hippocampal system, mainly via RSC and ATNs (AM).

The numerous connections of ACC strengthen its position as a major hub interconnecting several brain systems crucial for learning. This, however, makes dissection of the particular contribution by ACC a non-trivial task, since the deficits observed upon ACC silencing can be expected to affect a large number of systems, and can be partially compensated by the other numerous routes implicated in memory formation.

The approach used in this work includes separate analysis of the brain areas and their connections during different phases of the same flexible learning task. Accordingly,

the resulting picture reflects the cooperative role of the broader network in flexible learning, as opposed to individual contributions considered in isolation.

3.4 Establishment of brain networks for subsequent learning

I summarize here my findings addressing the role of an ACC-related brain network in flexible learning (Fig 2.33, Fig 2.34). Generally, perturbation had three possible outcomes: no effect, suppression of any subsequent learning (i.e. subsequent evidence to the contrary did not readily lead to a modification of behaviour), or suppression of alternative learning (i.e. subsequent evidence to the contrary led to re-learning instead of secondary learning (e.g. extinction)). Remarkably, silencing of any of the tested areas (ACC, RSC and DMS) or of the connectivity between these brain areas exhibited opposite consequences depending on whether silencing was carried out during acquisition or consolidation of the initial rule.

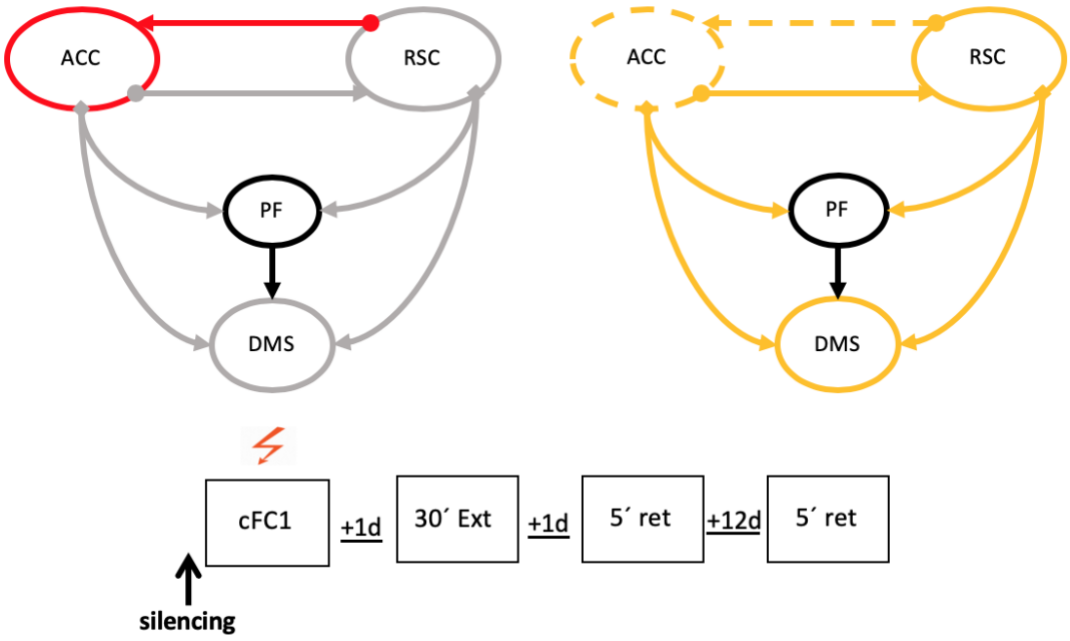


Fig 2.33 Brain networks for flexible learning involved during acquisition of cFC.
Brain networks required at acquisition of cFC to enable subsequent (left) learning or alternative (right) learning.
In the schematics, black colour indicates connections and brain areas, which were not tested; red indicates connections and brain areas whose silencing abolishes subsequent learning (and possibly alternative learning); orange (on the right; masked by grey on the left) indicate connections and brain areas whose silencing abolishes alternative learning.

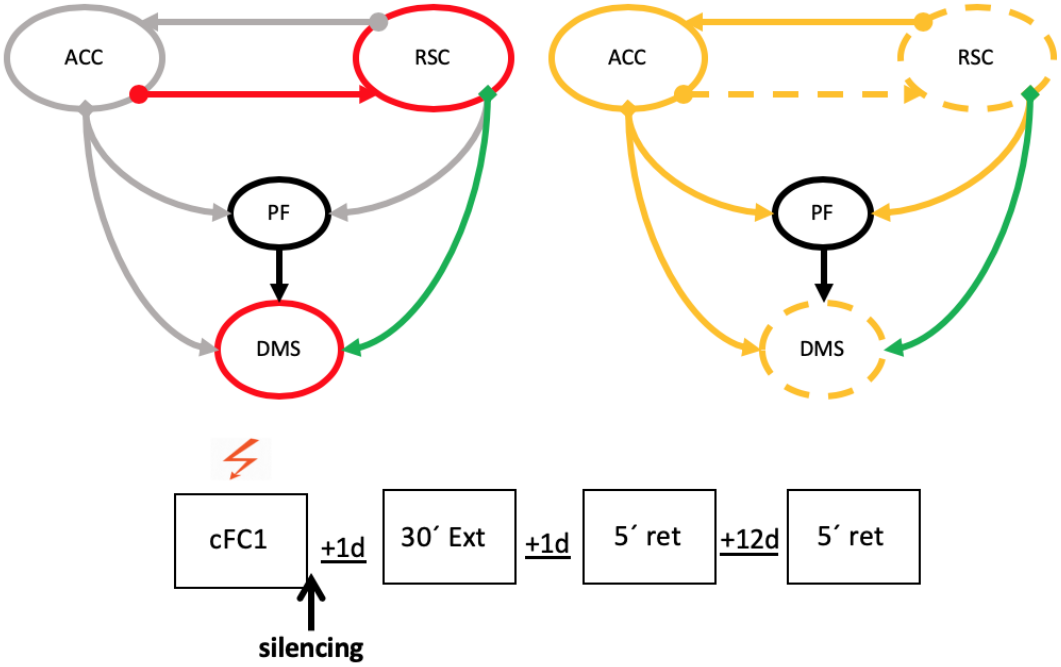


Fig 2.34 Brain networks for flexible learning involved during consolidation of cFC.
Brain networks required at consolidation of cFC to enable subsequent (left) learning or alternative (right) learning.
In the schematics, black colour indicates connections and brain areas, which were not tested; red indicates connections and brain areas whose silencing abolishes subsequent learning (and possibly alternative learning); orange (on the right; masked by grey on the left) indicate connections and brain areas whose silencing abolishes alternative learning; green indicates connections whose activity is not required during subsequent (extinction) learning.

Especially interesting in the light of this finding are the relative roles of ACC and RSC. During acquisition and consolidation of the initial memory the roles of these two cortical areas consistently alternated and remained opposite. Moreover, ACC function depended on inputs from RSC, and RSC function on inputs from ACC. This result strengthens the notion of ACC and RSC as components of a brain network, working in a sequential manner, to produce a memory trace which can be subsequently modified by new learning. This data complements the general view on ACC and RSC roles in flexible learning (Pearson et al., 2011, Kolling et al., 2016), but from a new perspective – namely, their roles in flexible memory acquisition and consolidation rather than their activity during single learning sessions. Furthermore, these data challenge the notion that a particular form of flexible learning might require activity in either ACC or RSC, highlighting instead the major role of their reciprocal interactions across learning stages. My results provide a framework to understand the remarkable similarities in functions attributed to ACC and RSC with respect to behavioural

flexibility and integration of reward history (Pearson et al., 2011, Wittmann et al, 2016; Kolling et al., 2016)

One possibility, which could not be addressed by this experimental protocol, is that activity in the brain network, which is involved in setting-up conditions for subsequent learning, might coincide with the activity in the same network, which is required for setting-up alternative learning. Since alternative learning cannot be executed without conditions for subsequent learning being set-up, overlapping activity remains a plausible alternative explanation for the underlying mechanism.

Analysis of subsequent learning revealed that the brain network required for extinction learning differs from the one required during acquisition and consolidation of the initial memory (Fig 2.35).

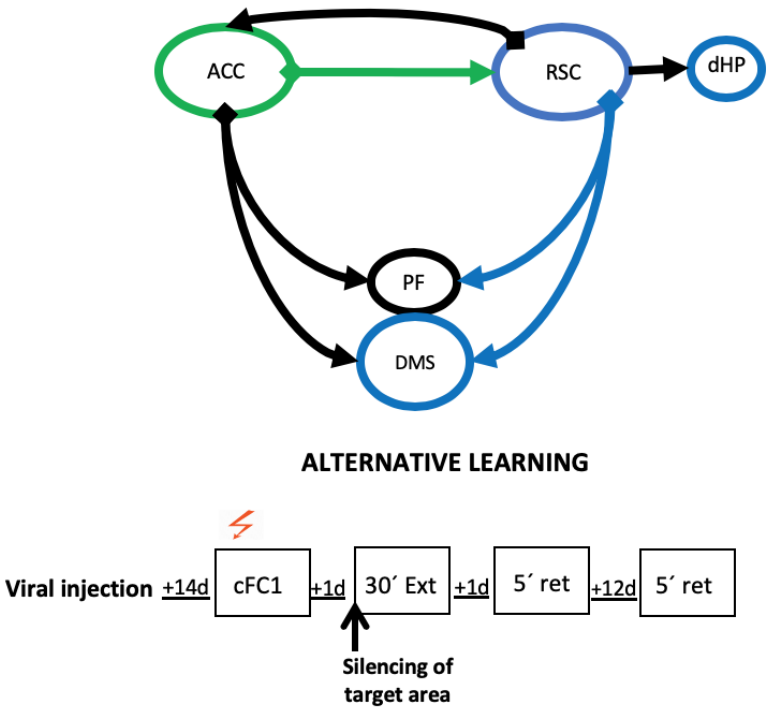


Fig 2.35 Brain networks for alternative learning.
In the schematics, black colour indicates connections and brain areas, which were not tested; blue indicates connections and brain areas whose silencing abolishes alternative learning; green indicates connections whose activity is not required during alternative learning.

Thus, activity in DMS, RSC and their connections was essential during subsequent learning and during formation of the initial memory, whereas activity in ACC and its projection to RSC was not required during subsequent learning. This finding is unexpected, considering the existing claims, according to which ACC is required during instrumental learning to adjust behavior (Holroyd and Coles 2008; Kennerley et al., 2006; Wittmann et al., 2016). However, it might point to more specific functions of ACC during particular learning tasks, such as higher demand for attention or cognitive control during execution of the task, rather than involvement of ACC in subsequent learning per se (Weissman 2004; Bush et al. 1999; Kolling et al., 2016).

A second important finding of my study was that interventions at subsequent learning never resulted in erasure of the fear memory. The possible outcomes of interference with the ACC-related brain network at extinction included either the absence of extinction, or no effect. This could be explained by assuming that the formation of the initial memory includes a process of schema or template formation in an ACC-dependent manner (Wang et al., 2012; Ghosh and Gilboa, 2014). The schema is then further used to integrate future learning episodes, including alternative learning. In this framework, the initial template is not being modified during extinction learning, but instead it allows to add new associations to the schema. Alternative learning, at the same time, relies on a separate set of requirements. This set of requirements is executed by a distinct brain network, consisting of DMS, RSC and dHP (among other possible areas). For instance, a requirement for mismatch detection depends on RSC (Melissa Serrano, unpublished results).

Further insights concerning the mechanism for subsequent learning were obtained by comparison of its two different forms – namely relearning (either involving a behavioral protocol, or caused by ACC silencing at cFC consolidation) and alternative learning (extinction) (Fig 2.35, 2.36, 2.37).

Remarkably, these forms of learning each rely on a different network of brain areas. The shared requirement for all, however, is activity in RSC. This fact strengthens the

basic role of RSC, possibly as a mismatch detecting structure, to initiate the switching process, guided by the rest of the brain network.

At the basis of the behavioural erasure protocol (Fig. 2.36) lies a reconsolidation process of the original contextual fear memory upon 5 min recall. In this way, the process involves recruitment and activation of the intact initial memory, followed by learning that modifies its meaning (Suppl. 4). This difference in learning involves, at the same time, a different set of brain areas, which partially overlaps with extinction. Thus, it requires activity in RSC and dHP, but, unlike extinction, DMS is not involved in relearning.

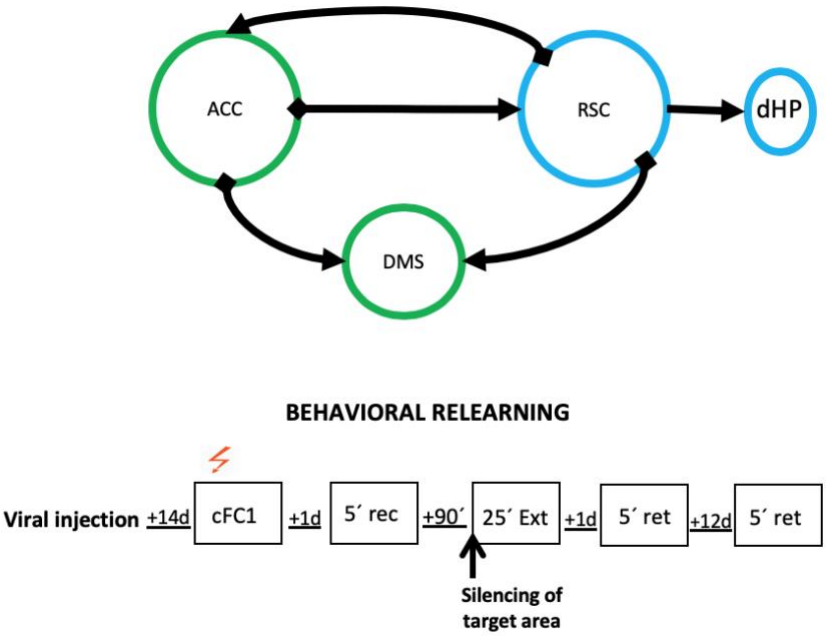


Fig 2.36. Brain networks for behavioural relearning.
In the schematics, black colour indicates connections and brain areas, which were not tested; light blue indicates connections and brain areas whose silencing abolishes relearning; green indicates structures whose activity is not required during behavioural relearning.

Relearning caused by prior ACC silencing at cFC consolidation (ACC-silencing-based erasure) lacks the intact initial memory and in this way is different from the prior two cases (Fig. 2.37). Moreover, modification of this changed memory now depends on ACC and ACC->RSC connectivity (unidirectionally). A possible explanation of this result is that the memory trace which was consolidated without the frame for an

alternative learning, now depends on ACC for its modification. I provide additional evidence for this idea in the supplementary part of my thesis (Suppl. 8). I show that after the memory was consolidated as relearned (by behavioral erasure), its expression depends on ACC. It seems plausible that memory which was initially consolidated as primed for relearning also depends on ACC.

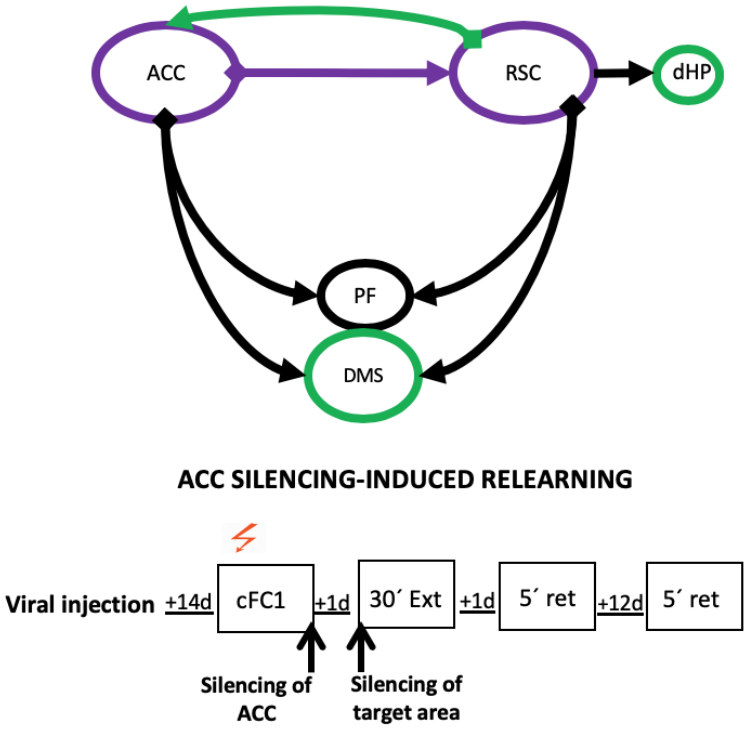


Fig 2.37 Brain networks for ACC silencing-induced relearning.
In the schematics, black colour indicates connections and brain areas, which were not tested; violet indicates connections and brain areas whose silencing abolishes relearning; green indicates connections whose silencing does not lead to observable learning deficits.

The actual process of subsequent learning (including alternative learning and relearning) exhibited a complex pattern of dependences on activity in ACC-related flexible learning systems and their connectivities.

RSC stands out as having a critical role in all forms of subsequent learning, possibly due to its implication in mismatch detection as a basis for new learning. dHP is required during learning involving modification of the intact initial memory, both in relearning and in extinction, possibly because of its role in memory. Activity in DMS, on the other hand, is specifically required during alternative learning, but not during relearning,

possibly because it serves as a mechanism to drive gradual evidence-based new learning. Lastly, ACC is required during subsequent learning when the initial memory was consolidated as primed for erasure due to interference with ACC activity during cFC consolidation.

Studies of system consolidation have provided evidence that memories initially dependent on the hippocampus for their retrieval, increasingly rely instead on cortex for their retrieval (Kitamura et al., 2017). How and whether memories primed for relearning also undergo time-dependent changes has not been investigated. It is possible, that upon erasure the initial memories are not rewritten everywhere in the brain, but are instead only modified in critical local circuits such as the hippocampus. My most recent experiments on post-erasure cortical memory ensembles provide evidence in favor of this hypothesis (Suppl. 8). This in turn could explain why silencing-based erasure required ACC, but not dHP activity.

3.5 Reversal of re-learning

In an additional part of my thesis I have addressed the mechanism of relearning in greater detail, monitoring the time at which relearning becomes apparently irreversible. My results suggest time-sensitivity for reversal of relearning, and revealed distinct roles of a 5h time window for consolidation and memory binding, as opposed to a 12h time window for consolidating relearning.

Relearning can be reversed during the relearning session itself by immediate contradiction to the evidence accumulated online. This is followed by the 5-hour window of consolidation, during which both extinction and erasure are resistant to reinstatement of fear through delivery of a foot shock. Sensitivity to reinstatement of fear after erasure opened after the closure of the 5-hour window and closed after the 12-hour consolidation window.

These findings demonstrate that the definite rewriting of the cFC memory is a process which follows the course of long-term memory consolidation. This process includes reassignment of the new meaning to the previous memory trace in HP (Suppl. 3).

This result is reminiscent of how memories are combined before and after the 12h time window for memory consolidation. Combination after the 12-hour time window might involve mechanisms involved in distinguishing the two events as separate learning episodes. In this case, the second learning event would be compared to the previously consolidated experience as an independent new association. In the case of alternative learning, one footshock is sufficient to immediately actualise the fear memory, whereas upon relearning there might no longer be an alternative memory to reinstate. By contrast, if the second learning event is delivered before the 12-hour window for memory consolidation, but after the closure of the 5-hour window of consolidation, this might lead to revaluation of the two learning processes (relearning and subsequent fear association in the same setting) during the 12-hour consolidation. Similarly, one footshock delivered at the end of a 25 min of contextual exposure without foot shocks, might lead to re-evaluation of the whole preceding learning session.

3.6 Conclusions and outlook

The results of my thesis work provide a novel understanding of the role of ACC in flexible learning. Previous studies had provided a variety of separate theories, each sharing the notion that ACC is important for flexible adjustment of behaviour. However, those studies did not provide a coherent model of how ACC and its related network might contribute to flexible learning. Furthermore, while it is widely accepted that ACC is required to evaluate reward history within one learning session, the role of ACC in subsequent learning had not been addressed.

My findings highlight how activity in ACC during the acquisition and consolidation of initial memory traces has critical and specific roles for subsequent adaptive learning. Two kinds of learning deficits were analysed separately, demonstrating different requirements for subsequent and alternative learning. Furthermore, subsequent learning itself exhibited distinctive requirements depending on whether it involved alternative learning or relearning.

So far, requirements for flexible learning have rarely been addressed at the system-wide level. Most published studies instead focused on a single brain structure or on the connections between two structures. In addition, previous studies did not address the involvement of an ACC-related brain network during the different stages of the learning and subsequent learning process. The approach, which I have used in my work takes into account different kinds and stages of flexible learning and demonstrates how the brain network of closely connected areas supports this function.

The specific circuit and network mechanisms underlying these phenomena remain to be determined. Further studies addressing the logic of these systems interactions might provide novel insights into mechanisms of flexible learning. Considering the numerous connections between the ACC and extrahippocampal area, it seems plausible that investigating the role of ACC connections with particular

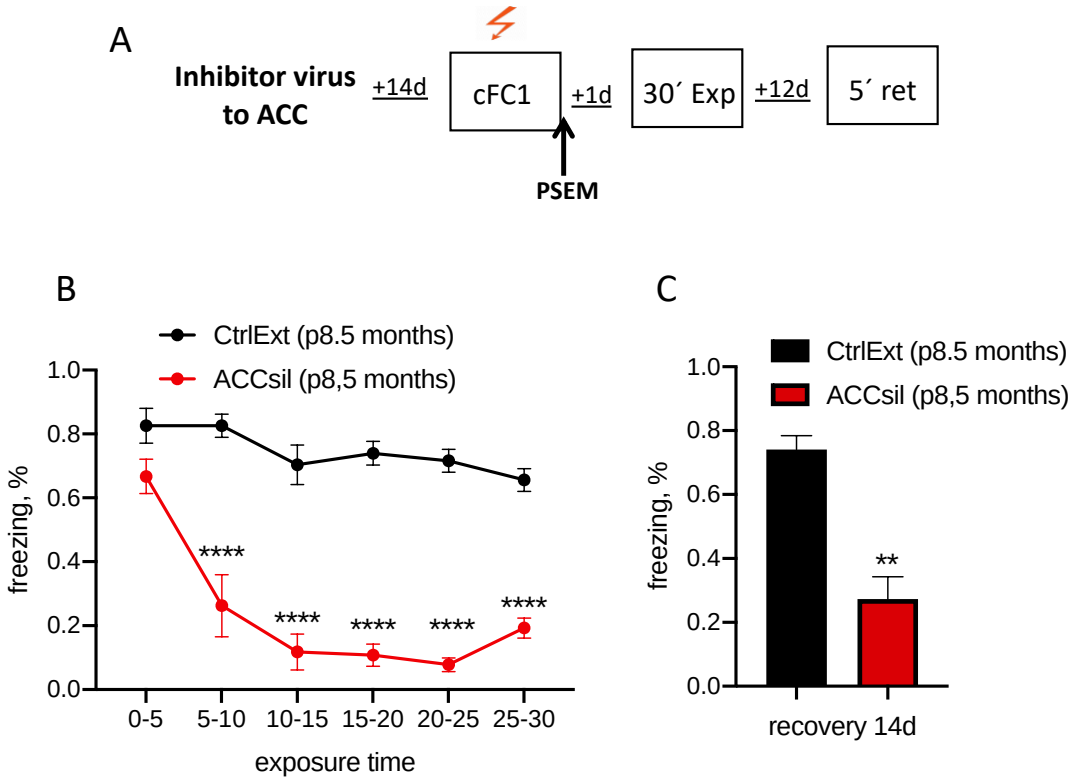
extrahippocampal structures, e.g. anterior thalamic nuclei, perirhinal cortex and postsubiculum, might be particularly informative to understand flexible learning.

I compare the role of two consolidation windows during the course of alterative learning and relearning memory formation. It remains yet to be studied, which mechanism prevents the reinstatement by one footshock during the onset of the 5-hour window of consolidation. For instance, an interesting question could be, how a footshock delivered at 5-7 hours post extinction/erasure, is being consolidated – whether it starts another 5-hour cFos window and thus interferes with the 12-hour window or it is a separate learning event with its own time course of consolidation.

Overall, in my thesis I demonstrate how flexible learning is supported by a network of brain structures, with respect to their interchanging roles and specialised functions during acquisition and consolidation of adjustable memory formation. This knowledge was further deepened by determining specific requirements for activity of the respective structures during different forms of subsequent learning. This data lays the groundwork for subsequent studies of the flexible learning function and principles of its organisation in the brain.

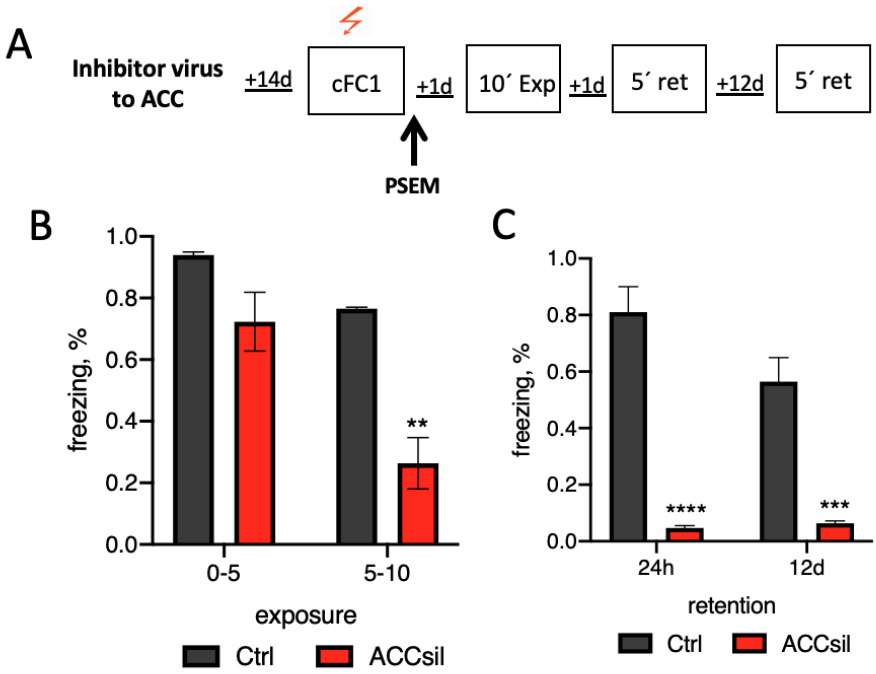
Appendix

In old mice (8-9 months) silencing ACC at consolidation of cFC dramatically facilitates freezing reduction during subsequent extinction and leads to erasure of fear memory. The erasure occurrence and facilitated decrease of freezing do not dependent on the age of the mice, unlike normal extinction learning.



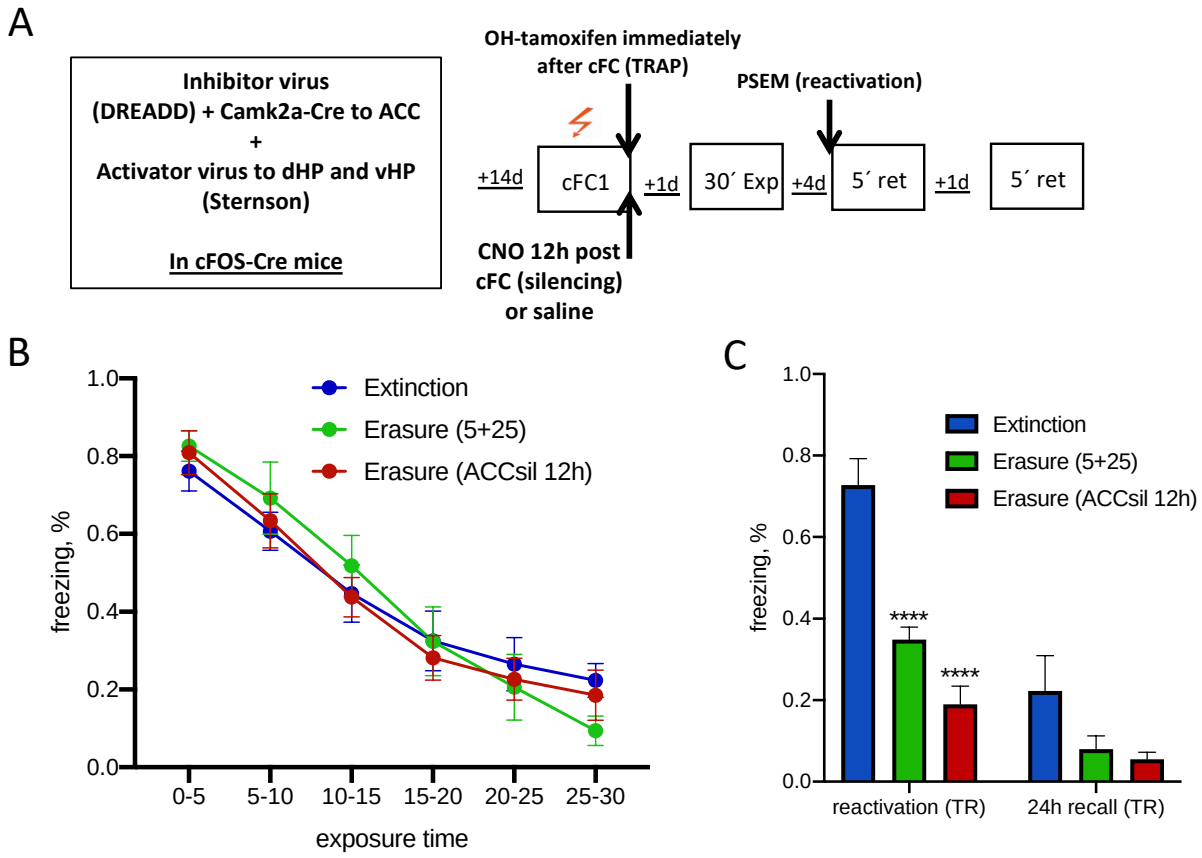
Suppl.1 Effects of ACC silencing in the group of old mice (8.5 months).
(A) Schematic of experiment – silencing is done after cFC acquisition and extinction learning follows 24h later. (B) Extinction speed in experimental (red) and control (black) groups of old mice. (C) Recovery of cFC 14 days later in the same two groups of mice.
N=4-5 each.
*Statistical analysis – two-way ANOVA followed by multiple comparisons test *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001*

Upon ACC-silencing at acquisition of cFC, subsequent contextual exposure lasting 10-minutes is sufficient for dramatic reduction of freezing and results in an erasure of fear memory, even though the 10-minute exposure is not sufficient to produce extinction in control group.



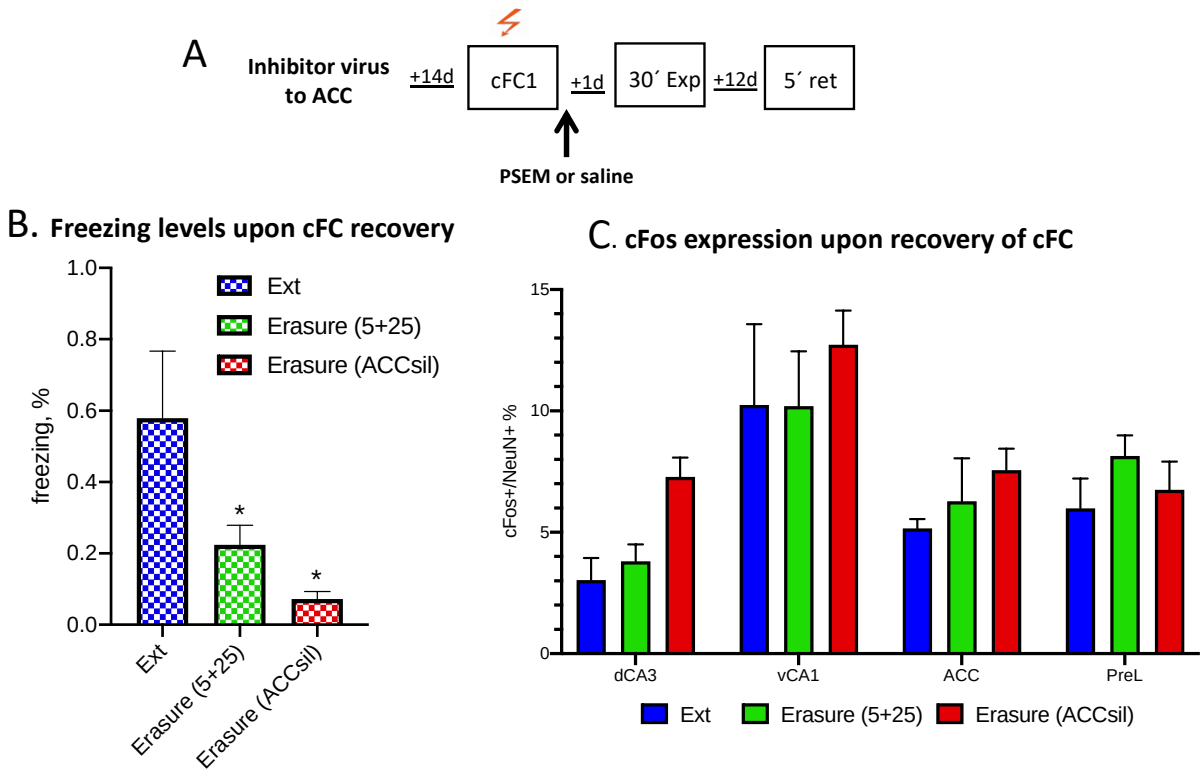
Suppl.2 Erasure caused by 10-minute exposure to the training context.
(A). Schematic of experiment – silencing is done within 20min post cFC, 10-minute exposure follows 24h later. (B) Rapid decrease in freezing level in treated compared to control group at the end of 10-minute exposure. (C) Retention of 10-minute exposure memory shows high freezing in control group both 24h and 12d post exposure and complete absence of freezing in the treated group.
N=3 each.
*Statistical analysis – two-way ANOVA followed by multiple comparisons test *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001*

Behavioral and ACC-silencing induced erasure prevent normal fear induction in the training context upon reactivation of fear-memory engram in hippocampus. On the contrary, after extinction activation of hippocampal fear-engram in training context leads to substantial freezing levels. Additionally, reactivation does not lead to fear reinstatement, as is shown by recall 24h later in the same context.



Suppl.3 Reactivation of original cFC ensemble post erasure and extinction.
(A) Schematic of experiment. (B) Comparison of extinction learning and relearning (behavioral and by ACC silencing at 12h post cFC). (C) Activation cFC ensemble in HP is capable of eliciting high freezing in training context after extinction, but not after relearning; this does not lead in turn to reinstatement 24h post reactivation.
N=3-5 each.
*Statistical analysis – two-way ANOVA followed by multiple comparisons test *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001*

In three different groups of animals (extinction, behavioural erasure and ACC-silencing based erasure) 12-days retention test in training context leads to cFos induction pattern which is identical in all groups across several brain areas involved in learning and memory, regardless of spontaneous fear memory recovery (post extinction) or absence of such (post erasure).



Suppl. 4 Freezing levels and cFos expression upon retention test at 12 days post extinction or erasure.

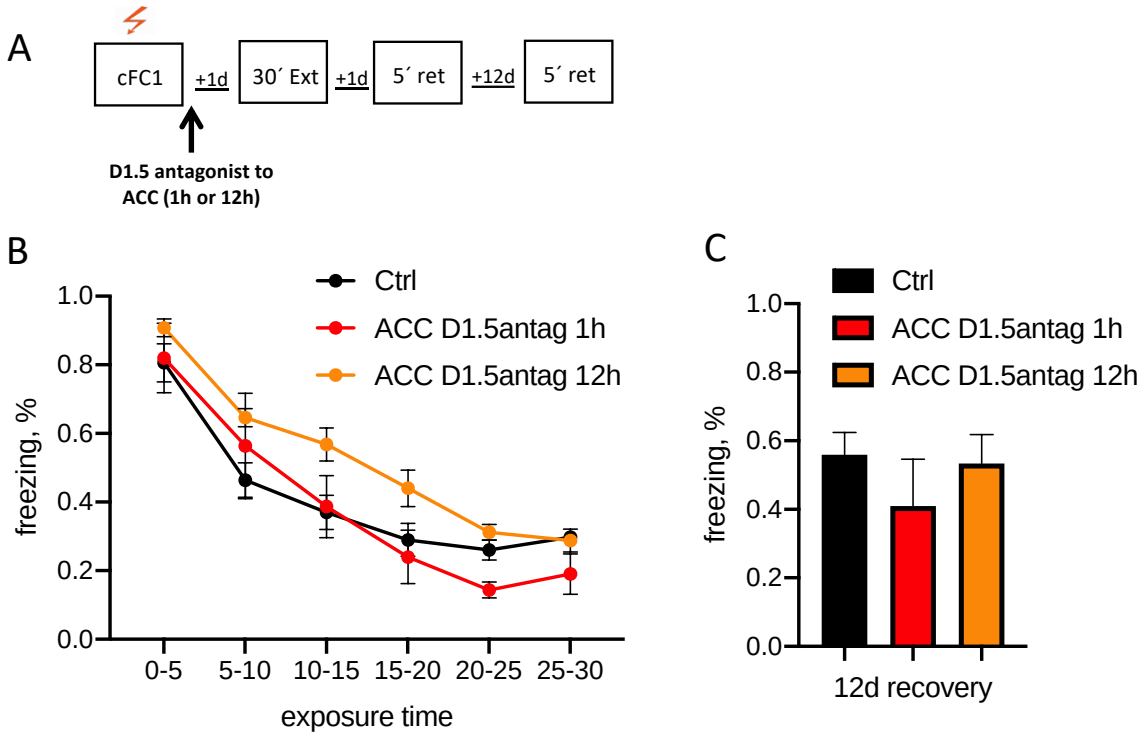
(A) Schematic of experiment. (B) Freezing levels at retention after extinction show substantial amount of freezing, whereas, behavioural erasure and erasure, caused by ACC silencing at cFC consolidation, show no fear memory recovery. (C) cFos expression levels 90 minutes after 12days reintroduction in training context in corresponding animals´ groups shows no significant differences between control and both erasure groups

N = 3-5 each.

Immunohistochemistry is done using Millipore antibodies for cFos

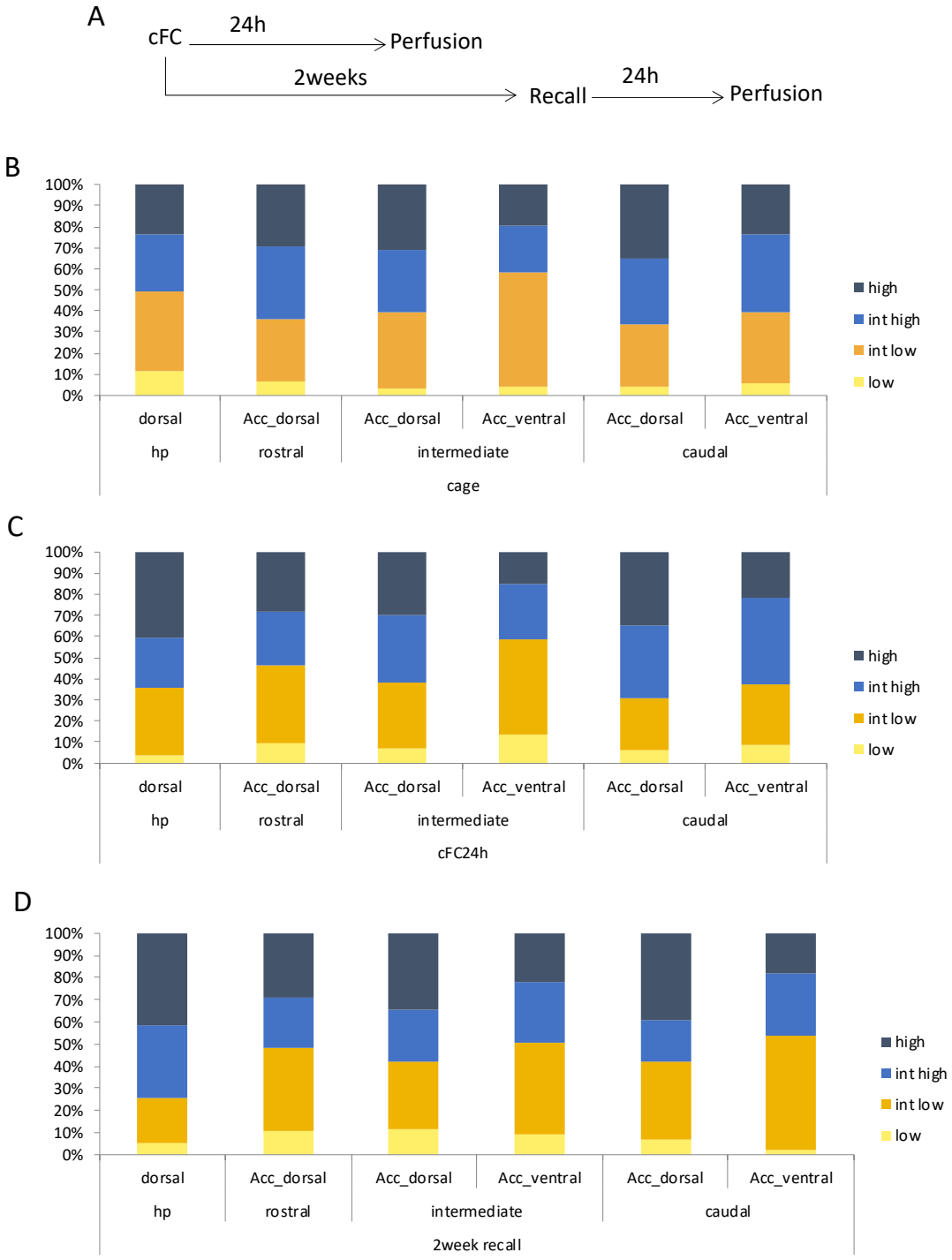
*Statistical analysis – t-test (erasure groups compared to extinction) *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001*

Blocking dopamine signal in D1.5 receptors in ACC during 1st or 2nd window of consolidation of cFC does not affect subsequent extinction learning.



Suppl. 5 Dopamine injection to the ACC during consolidation of cFC at 1h or 12h post cFC acquisition.
(A) Schematic of experiment. (B-C) Immediate and retention-time freezing are unaffected by D1.5 antagonist injection performed during consolidation of the preceding cFC.
N = 3-5 each.
*Statistical analysis – two-way ANOVA (B) and t-test (C); experimental groups compared to control *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001*

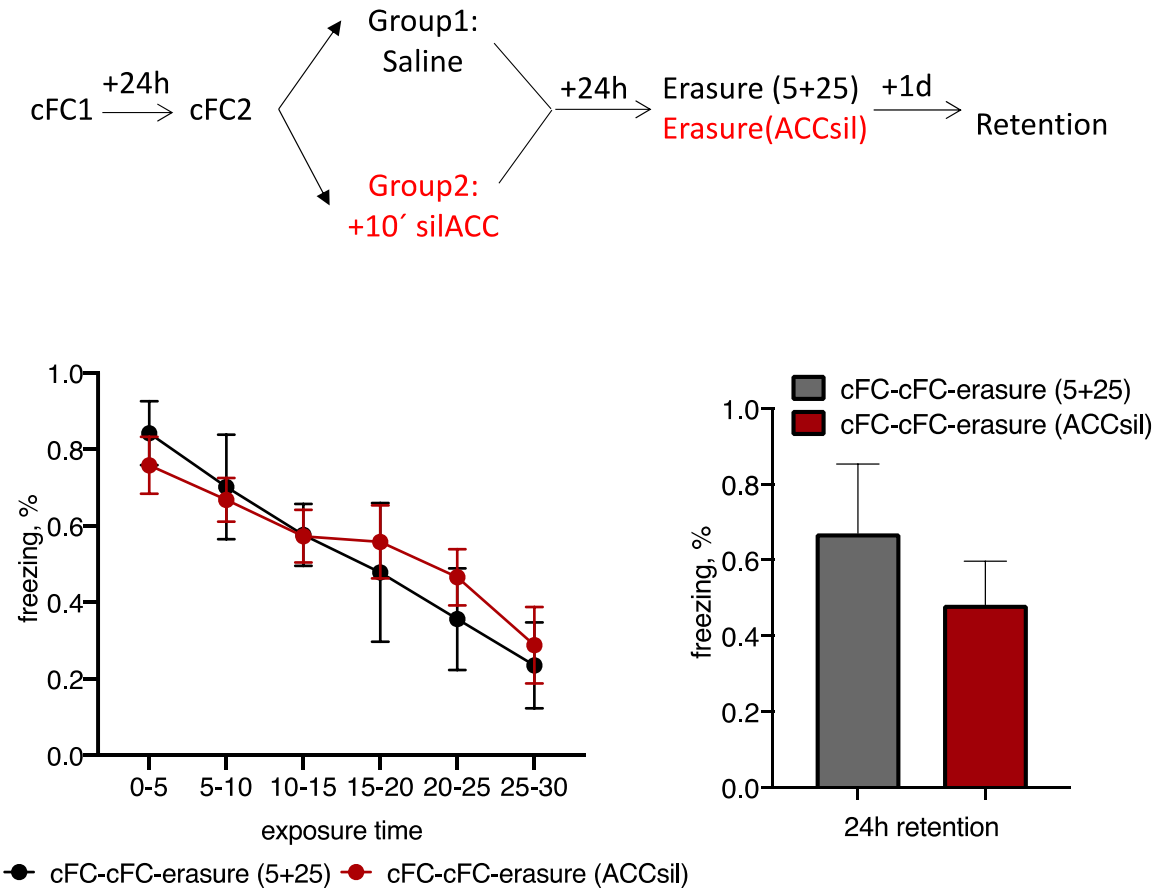
No high PV induction was observed in ACC area 24h post cFC or 24h post remote (14days) recall of cFC (samples were taken from respective dACC or vACC regions ranging from +1.8 to +0.4 AP from Bregma).



Suppl. 6 PV levels in dorsal hippocampus and several subareas of the ACC 24h post cFC acquisition or recall

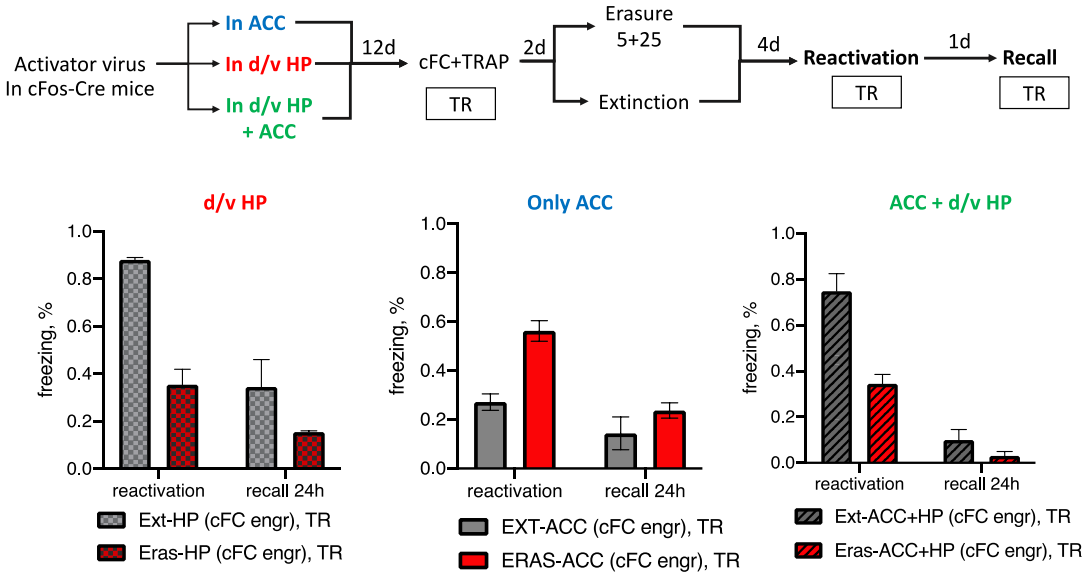
(A) Schematic of the experiment. (B) PV levels in the cage control animals. (C) PV levels 24h after cFC. (D) PV levels 24h after remote recall of cFC (2 weeks).
Colors on the scheme correspond to the proportion of cells with particular PV intensity: dark blue – high, light blue – intermediate high, orange – intermediate low, yellow – low.
N = 4-6 each.

Repeated cFC results in reinforced fear memory which cannot be subsequently changed neither in behavioral erasure protocol, nor in ACC-silencing based erasure (silencing is done at consolidation of 2nd cFC).



Suppl.7 Compound cFC-cFC memory cannot be erased.
Repeated cFC memory cannot be erased by utilizing 5+25 behavioral erasure protocol or by ACC silencing at 2nd cFC consolidation.
N = 3-4 each.

Expression of cFC memory in training context depends on cFC memory ensemble in hippocampus post extinction and on cFC memory ensemble in ACC – post erasure. Coactivation of cFC memory ensemble in hippocampus together with the one in ACC post erasure block ACC-dependent freezing, whereas coactivation of ACC-fear ensemble and HP-fear ensemble in training context post extinction results in high freezing.



Suppl. 8 Dependency of cFC memory expression on ACC and d/vHP post extinction and erasure.
Left: cFC engram in HP is able to elicit freezing in training context (TR) after extinction, but not after erasure
Center: cFC engram in the ACC is able to elicit freezing in training context after erasure, but not after extinction
Right: Activation of HP together with ACC prevents ACC-dependent fear memory expression after erasure; at the same time, activation of HP together with ACC elicits HP-dependent freezing after extinction.
N = 3 each.

Materials and methods

Mice

Camk2a-Cre mice (B6.Cg-Tg(Camk2a-cre)3Szi/J) were from Jackson laboratories, Fos-CreER (B6.129(Cg)-Fostm1.1 (Cre/ERT2)Luo/J) mice were a kind gift from S. Arber (Friedrich Miescher Institut) and C57Bl6/J mice were from Janvier.

Before the onset of behavioural experiments, mice were single caged for 1-3 days; no water and food restrictions applied, unless otherwise stated. All animal procedures were approved and performed in accordance with the Veterinary Department of the Kanton Basel-Stadt.

Behavioural procedures

All behavioural experiments were carried out with mice aged from p75 to p110 (age at the onset of the experiment).

Contextual fear conditioning

Contextual fear conditioning experiments were performed as described in Donato et al 2013. The training phase consists of free exploration of the lit test chamber for 3 minutes, which is followed by 5 footshock separated by 30 seconds each (duration of the footschock is 1 second, power is 0.8mA). Contextual fear memory is estimated on the next day by placing the mouse in the same chamber for 5 minutes and measuring the total duration of freezing (defined as the suppression of all visible movement except that required for respiration), starting from the end of the 1st minute and until the end of the test.

Extinction of the fear memory was done by placing the mouse for 30 minutes into the test chamber without shocks 24h post acquisition of cFC. The freezing was counted separately during 5-minute intervals of the test (except the first 5-minute interval of the test, in which similarly to recall the 1st minute was excluded from analysis). Retention of extinction was tested 24h post extinction learning in the same context (total freezing during 5-minute test, excluding 1st minute).

Behavioural erasure was done 24h after cFC acquisition by combining 5-minute recall in the training context with 25-minute exposure to the same context, which followed

90-minutes post the initial recall. The freezing was estimated the same way as described above.

Spontaneous fear recovery after extinction or erasure was tested by putting the mice into the training context 12 days after erasure/extinction and measuring the total freezing during 5-minute test.

Attentional set shifting task

In ASST experiments, mice were trained to dig in transparent plastic bowls (internal diameter 40mm, depth 40mm) filled with wood shaves to retrieve a hidden food reward consisting of one-third of a Honey Loop (Kellogg, Manchester, UK). The test apparatus consists in a rectangular non-transparent Plexiglas box divided by two sliding doors into separated compartment with baited bowls, comprising one-third of its length, and starting chamber.

The bowls are placed in two sub-compartments divided by a wall. Access to the chambers is regulated by the experimenter and is closed at the start and at the end of every learning session. Once the animal makes a choice by digging in one of the bowls, the access to the other one is closed (except for the first 4 trials at every new learning day, when the mouse is allowed to explore the 2nd chamber regardless of the initial choice).

The training consists of 3 days of habituation (free exploration of the chamber and the bowls, which contain food pellets, but no odour) and subsequent learning phases, each performed on a separate day with a gap of 24h hours. Habituation stage is completed once the mouse eats a food pellet twice from every bowl. During training phases, mice are required to reach the learning criterion of 8 correct choices out of 10 in the rolling window. The test is completed either when the learning criterion is reached, or once the mouse exceeds 50 trials without learning the task.

Training phases are comprised of several substages, performed on separate days: simple discrimination includes two different odors, one of which is baited; complex discrimination apart from odors has added textures (which are irrelevant to the reward); interdimensional shift is done with a new pair of odors (relevant dimension) and textures (irrelevant dimension) to test the rule generalisation; interdimensional shift reversal changes (reverses) the salience of the two odors and is aimed to

estimate flexibility of the alternative learning; interdimensional shift 2 is identical to interdimensional shift and restores the original salience of the initial stimuli.

To increase the food search motivation and facilitate learning of the task, mice are kept under food restriction 24h prior to the test. The animals are fed only once a day, upon the end of the learning phase - they are given 1 full food granule (from the usual food supply), mashed with water.

Immunohistochemistry and image processing

Antibodies were used as follows: monoclonal rabbit anti-cFos (Millipore) 1:5000; mouse anti-NeuN (Millipore, MAB377) 1:1000; α-Bungarotoxin, Alexa 488 Conjugate, to detect the expression of rAAV9-CAGflox-PSAM(L41FY116F)5HT3-WPRE (Molecular Probes, Life Technologies, B-13422) 1:500. Secondary antibodies were Alexa Fluor 488 (Molecular Probes; A150077), or 647 (Molecular Probes; A150131, A150107); 1:500. At the end of the experiment some mice will receive an intra-peritoneal injection of a Ketamine/Xylazine cocktail (100mg/kg and 10mg/kg), followed by transcardial perfusion with, first PBS and then 4% Paraformaldehyde (PFA) in PBS (pH7.4); brains were collected, kept overnight in 4% PFA at 4°C, and subsequently 40 um coronal sections were prepared using vibratome.

The standard procedure for immunostainings was as follows: sections were blocked for 1-2 hours at room temperature in 10% BSA in PBS-T (0.3% Triton X-100 in PBS). Incubation in primary antibody was done overnight in the antibody solution containing 3% BSA and 0.3% PBS-T. After three washing steps in PBS-T, sections were incubated in secondary antibody solution (also in 3% BSA and 0.3% PBS-T) at room temperature for 2 hours. After another three washing steps (one with PBS-T and the following with PBS), sections were mounted in Prolong Gold antifade reagent (Molecular probes) and kept at room temperature to ensure correct polymerisation of the Prolong until imaging (not less than 3 days). Between long-separated imaging sessions the specimen were kept at 4°C and broight to room temperature at least 24h before imaging.

Images for the analyses were acquired at 40x (objective EC Plan-Neofluar 40x/1,30 Oil DIC M27) using a spinning-disk confocal microscope (Axio Imager M2 upright microscope + Yokogawa CSU W1 Duel camera T2 spinning disk confocal scanning

unit) equipped with Visiview 4.4.0.18 software. All the samples belonging to one experimental set were processed in parallel and using the same imaging settings.

Image analysis was performed using the Imaris 9.0 software; all c-Fos and/or NeuN immunopositive cells were quantified using an automatic spot-detection and cFos induction was quantified as a fraction of cFos positive cells over the total neuronal population expressing NeuN.

Stereotaxic surgery:

All surgeries were conducted under aseptic conditions using a Stereotaxic alignment system (David Kopf instruments). For experiments involving viral or ligand injections into the brain, mice were anaesthetized with 2-3% isoflurane using air (O2) as a carrier gas, using an OXYMAT3, which yields 95% oxygen concentration. Mice were kept under anaethesia during the surgery/injection in the stereotaxic frame with isoflurane 1-2% using air (O2) as a carrier gas and body temperature was maintained stable with a heating pad. Half an hour before initiation of surgery, Buprenorphine (Temgesic) (0.1 mg/kg) was applied subcutaneously as pre-emptive analgesia. After induction of anesthesia and fixation in the stereotactic frame, mice were injected subcutaneously with a 1:1 mixture of Lidocain: 10mg/kg and Ropivacain: 3mg/kg (Naropin, Astra Zeneca) in the area of the surgery to reduce post-operative pain. In addition, after surgical procedure, Buprenorphine (0.1 mg/kg) was again applied subcutaneously 1-2x on the day of surgery (with a break not exceeding 4-6h). Meloxicam (Metacam, 5 mg/kg), was given at the day of surgery, when the animals are awake to assure analgesia overnight, and again on the next two days at an interval of 24 h.

Topical drug and virus injections were carried out using glass pipettes (tip diameter 10–20µm) connected to a picospritzer (Parker Hannifin Corporation).The glass pipette was inserted at the desired coordinate and a maximum of 250nl of the virus solution was slowly injected over a period of 5 minutes. After the end of the injection the pipette was left in its place for a further 10-15 minutes to allow for diffusion of the virus and avoid backflow and then slowly withdrawn. Mice were left to recover for 10-14 days and to let the virus fully express in the neuronal cells. Post-surgical recovery was monitored daily until the start of behavioural protocols. All injections were paired with saline injected control animals to account for any effect due to tissue damage or surgical procedure.

The coordinates used were as follows.

ACC: AP +0.75mm, +0,35mm; ML ± 0.35mm; DV – 1.4mm, 1.5mm

RSC: AP -1.2mm,-2.2mm,-3mm; ML: ± 0.35mm; DV -0.55mm

DMS: AP +0.8mm, +0.4mm; ML: ± 1.65mm DV: - 2.35mm

PF: AP -2.3mm; ML: ± 0.7mm; DV: - 3.2mm

dHP: AP – 1.7mm; ML ± 1.6mm; DV -1.65mm, -1.9mm

vHP: AP – 3mm; ML ± 3.25mm; DV -3.0mm, -3.7mm

Pharmacology and pharmacogenetics in vivo

Drugs were used as follows: SCH23390, Tocris, 1.5mM in saline (D1-D5 dopamine receptor antagonist).

For acute silencing of ACC and RSC, floxed PSAM-dependent inhibitor channel (rAAV9-CBA-flox-PSAM(Leu141Phe,Tyr116Phe)GlyR-WPRE in combination with Cre-delivering rAAV8-CamKII-mCherry-Cre (UNC GTC Vector Core) was delivered bilaterally in the respective areas of wild type mice. To allow for transgene expression, mice were kept under home cage conditions for 10-14d before any behavioural experiment. The PSAM agonist, PSEM308 was injected i.p. (5mg/kg) 15-20 minutes before behavioural testing to activate the PSAM channels (for silencing at acquisition) or immediately after completion of the learning session (for silencing at consolidation) (Magnus et al., 2011).

For silencing of DMS, vHP and dHP CNO-activated inhibitor channel FLEXed DREADD-carrying AAV8 (B. Roth, UNC Vector Core; silencing, rAAV8-AAV-hSyn-DIO-hM4D(Gi)-mCherry) in combination with Cre-delivering rAAV8-CamKII-mCherry-Cre was delivered bilaterally in the respective areas of wild type mice. Clozapine-N-oxide (CNO; 5mg/kg, i.p, Tocris; Sternson and Roth, 2014) was injected 25-30 minutes before the onset of the experiment (for interference with acquisition) or immediately after completion of the learning (for interference with consolidation).

Silencing of circuits specifically involving projections from one area to the next was achieved by combining injections of two viruses according to the following protocol: retroAAV carrying the genetic sequence for Cre-recombinase (pAAV-pCAG-iCRE-2A-

H2BGFP-WPRE) and AAV carrying the genetic sequence for PSAM-dependent inhibitor channel (rAAV9-CBA-flox-PSAM(Leu141Phe,Tyr116Phe)GlyR-WPRE) were injected into the area receiving projections and into the area which sends those projections respectively.

Genetic targeting of active populations

FosCreER mice were used for selective manipulation of memory ensembles by using the TRAP (targeted recombination in active populations) method. To label neurons, 4-Hydroxytamoxifen (50 mg/kg in sunflower oil, Sigma Adrich) was injected i.p. immediately after the completion of behavioural session. Mice were kept under control conditions for 5 days to allow for the expression of the construct. Subsequently, the selected memory ensemble was inhibited or reactivated by respective viral constructs (excitation was done by rAAV9-CAG-flox-PSAM(Leu41Phe,Tyr116Phe)5HT3-WPRE and inhibition by rAAV9-CBA-flox-PSAM(Leu141Phe,Tyr116Phe)GlyR-WPRE) .

Statistical analysis

All statistical analyses were based on two-tailed comparisons and were done using GraphPad Prism (GraphPad software. Inc.). Results are presented as mean ± SEM. Number of animals to be used for a standard behavioural analysis was determined based on our preliminary behavioural experiments. Data distributions were assumed to be normal but this was not formally tested. Male mice of closely comparable age were assigned randomly to experimental groups. Intensity analysis and freezing data were done either by using automated workflow in Imaris 9.0 or, when manual analysis was required, additionally verified by a co-worker blind to experimental conditions.

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