Behavioural and Neural Analyses of Higher-Order Fear Conditioning

Dilara E. Gostolüpçe

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By: Dilara E. Gostolüpçe

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Signed by the final examining committee:

Chair
Dr. Norman Segalowitz

External Examiner
Dr. Kate Wassum

Examiner
Dr. Andrew Chapman

Examiner
Dr. Matthew Gardner

Examiner
Dr. Christopher Steele

Thesis Supervisor (s)
Dr. Mihaela Iordanova

Approved by
Dr. Andrew Chapman, Graduate Program Director

March 21, 2024
Date of Defence

Dr. Pascale Sicotte Dean,

Abstract

Behavioural and Neural Analyses of Higher-Order Fear Conditioning

Dilara E. Gostolupce, Ph.D.

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Memories about aversive events that elicit fear can imbue fear to other stimuli. This is studied using Pavlovian higher-order fear conditioning. A stimulus that is directly paired with an aversive outcome (i.e., Pavlovian first-order fear conditioning) can support learning about another stimulus (i.e., Pavlovian higher-order fear conditioning). That is, by virtue of its links with a first-order stimulus (i.e., S1), a higher-order stimulus (i.e., S2) controls behaviour by eliciting conditioned defensive responses. This occurs in two ways: either S2 is paired with S1 before the latter is paired with an outcome (i.e., foot shock) as exemplified in sensory preconditioning (SPC), or after S1 is paired with an outcome as exemplified in second-order conditioning (SOC). Reduction in fear to S1 by presenting it in the absence of the shock transfers to sensory preconditioned but not to second-order conditioned fear, showing that two types of fear are supported by distinct behavioural (and neural) structures.

Extensive work investigated the neural substrates underlying first-order fear conditioning, while our understanding of the neural structures mediating higher-order fear conditioning is still relatively limited. The present thesis investigated the role of some of the neural substrates that support sensory preconditioning and second-order conditioning. In Chapter 4 we confirmed that SPC but not SOC required the integrity of first-order fear and provided neural evidence for this dissociation using a chemogenetic approach to delete first-order fear memory, which disrupted SPC but not SOC. In Chapter 5 we investigated the role of lOFC in regulating the expression of both types of fear and showed that lOFC inactivation prior to test disrupted SPC but enhanced SOC. In Chapter 6 we identified the neuronal ensembles that are activated by SPC and SOC in the BLA, a region critical for the expression of fear to SPC and SOC, and showed that a subset of these ensembles showed projections to the lOFC. These projections, when silenced disrupted both types of fear. Lastly, we silenced lOFC input to the BLA and showed that this pathway is crucial for SPC but not SOC. Our findings delineate neurobiological structures differentially supporting SPC and SOC types of fear and characterize the role of lOFC in the fear circuit.

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Contribution of Authors

Chapter 1: Sections 1.1 and 1.3 of this chapter are individually published as parts of review (Gostolupce et al., 2021) and mini-review (Gostolupce et al., 2022) articles, respectively and the relevant sections received minor edits to improve coherence and the flow of the thesis.

Chapter 2: Introduction written by Dilara E. Gostolüpçe and revised by Dr. Mihaela Iordanova.

Chapter 3: General methods written by Dilara E. Gostolüpçe and revised by Dr. Mihaela Iordanova.

Chapter 4: Dilara E. Gostolüpçe conducted all the experiments, analyzed the data and prepared the figures under the guidance and supervision of Dr. Mihaela Iordanova.

Chapter 5: Dilara E. Gostolüpçe conducted Experiments 5.1, 5.2, 5.4a. Dr. Matthew Williams-Spooner conducted Experiment 5.3 and Dr. Czarina Evangelista conducted Experiment 5.4b. Dilara E. Gostolüpçe analyzed the data and prepared the figures under the guidance and supervision of Dr. Mihaela Iordanova.

Chapter 6: Dr. Czarina Evangelista contributed to viral infusion surgeries for Experiment 6.2b. Dilara E. Gostolüpçe conducted all the experiments, analyzed the data and prepared the figures under the guidance and supervision of Dr. Mihaela Iordanova.

Chapter 7: General discussion written by Dilara E. Gostolüpçe and revised by Dr. Mihaela Iordanova.

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Chapter 1. Higher-Order Conditioning

Pavlovian Conditioning

Adaptive behaviour is controlled by environmental stimuli. These stimuli can acquire value by forming associations with motivationally significant outcomes. The simplest form of such associative learning is known as Pavlovian or classical first-order conditioning (Pavlov, 1927). For example, if you get bit by a dog, you become fearful of dogs. Here, the dog stimulus which was originally devoid of any biological significance is learned to predict the painful bite (the unconditioned stimulus, [US]) and becomes a first-order conditioned stimulus (CS or S1). Subsequent to this experience, the sight of a dog is sufficient to evoke responses that would naturally occur towards the painful bite (unconditioned responding, [UR]). These responses include running away, changing directions, sweating, and freezing (i.e., conditioned responses, [CR]). In the lab, this type of learning is studied using animal models and conditioned stimuli such as tone and USs such as footshock. Pairings between a tone and a footshock makes the former a reliable predictor of the latter. This predictive relationship between the tone and the shock is evidenced by tone-evoked conditioned responses that reveal the expectation of shock delivery. Depending on the type of the US, be it aversive or appetitive, such conditioned responses include species-specific defensive responses (e.g., freezing) or approaching to where the reward is delivered.

Higher-order Conditioning

Pavlovian conditioning is not restricted to such direct pairings between S1 and a US. Another neutral stimulus (denoted as S2) can be conditioned *indirectly* as a result of its pairings with a first-order stimulus (i.e., S1). As a result of such S2-S1 pairings, S2 comes to elicit conditioned responses. This type of learning is known as higher-order conditioning (Pavlov, 1927), and it has two examples: sensory preconditioning (Brogden, 1939) and second-order conditioning (Pavlov, 1927). The former is established when S2→S1 pairings occur *before* S1→US learning, whereas the latter occurs when S2→S1 pairings occur *after* S1→US learning (Figure 1.1). In both examples, S2 elicits conditioned responding despite never having been directly paired with the US (Holland, 1980; Gewirtz & Davis, 2000; Wessa & Flor, 2007; Parkes & Westbrook, 2010).

Auditory (e.g., tone, clicker, noise) or visual (e.g., light, color, shape) stimuli are used to serve as S2 and S1 in a within- or cross-modality manner.

Sensory preconditioning. There are two training phases that establish sensory preconditioning (i.e., SPC, Figure 1.1b). Phase 1 consists of sensory training between two neutral stimuli (i.e., S2 and S1). Phase 2 consists of first-order conditioning of one of the stimuli (i.e., S1) with the US. On test, presentations of sensory preconditioned S2 (i.e., SPC-S2) on its own elicit conditioned appetitive or aversive responses depending on the nature of the US. We can illustrate sensory preconditioning using the dog example. In Phase 1, you may encounter dogs in parks, forming a link between dogs (i.e., S1) and parks (i.e., SPC-S2). In Phase 2, getting bit by a dog renders it a learned danger signal (i.e., first-order conditioned stimulus). Subsequently, passing by a park will likely trigger conditioned fear responses as you anticipate seeing dogs due to your prior experience. In this way, fear of the bite transfers to the park. In the lab, we can study sensory preconditioning by pairing SPC-S2 and S1 in Phase 1, followed by conditioning S1 with a US. On test, SPC-S2 is able to elicit conditioned responding as a result of its association with S1 although it was never directly paired with the US.

Second-order conditioning. Similar to sensory preconditioning, second-order conditioning (i.e., SOC) is also established in two phases of identical training sessions (Figure 1.1c). However, the order in which these phases occur is reversed. That is, in Phase 1, S1 is conditioned with the US. In Phase 2, second-order S2 (i.e., SOC-S2) is conditioned with S1. On test, presentations of S2 elicit conditioned responding. We can illustrate how an S2 elicits conditioned responding using the dog example. In Phase 1, you get bit by a dog. In Phase 2, you see the dog at the park, which likely will evoke fear. Subsequently passing by the park triggers fear responses as the dog-evoked fear in Phase 2 was linked with the park. Similar to sensory preconditioning, the fear of the bite transfers to the park. In the lab, second-order conditioning is achieved by conditioning an S1 with the US prior to pairing S1 with S2. On test, presentations of S2 alone result in conditioned responding by virtue of its associations with S1 despite never having been directly paired with the US.

Controls. Demonstration of successful sensory preconditioning and second-order conditioning require comparisons with a control group which receives same number of stimuli presentations but does not develop higher-order conditioning. Successful higher-order conditioning requires two training phases, training between S2 and S1 and a training between S1 and the US. Therefore, it is

possible to control for higher-order conditioning in two ways: either by unpaired presentations of S2 and S1 during sensory training or by unpaired presentations of S1 and the US during fear conditioning. In this way, stimuli are presented equal number of times as experienced in sensory preconditioning and second-order conditioning but in an unpaired manner. Therefore, a control S2 does not elicit conditioned responding to the level of sensory preconditioned or second-order conditioned S2 when tested.

The strength of sensory preconditioning and second-order conditioning is influenced by design parameters and the procedures used to obtain higher-order conditioning. Following sections describe these parameters and procedures. The thesis mainly covers literature in the aversive domain, however, where informative, evidence from appetitive designs is also considered.

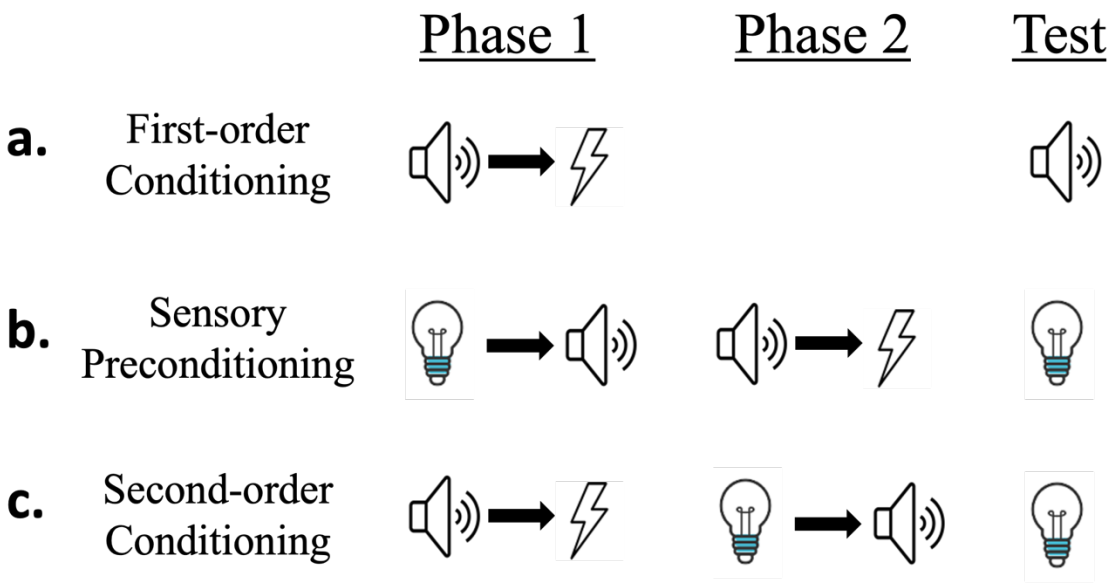

Figure 1.1. Behavioural designs (Gostolupce et al., 2021). (A) In first-order conditioning, a tone is paired with the foot shock. (B) In sensory preconditioning, pairings between light and tone occur prior to first-order conditioning. (C) In second-order conditioning pairings between light and tone occur after first-order conditioning.

Parameters used to obtain higher-order conditioning

There are a number of factors that influence the content of learning in sensory preconditioning and second-order conditioning. These are detailed in Gostolupce and Lay (2022) and the relevant factors are included below with minor edits.

Stimulus type. Various stimuli have been used in higher-order conditioning experiments including colour (Rashotte et al., 1977), shape (Rescorla, 1980a), odour (Holland, 1983), flavour (Holland, 1981, 1983) auditory cues such as tone (Rizley & Rescorla, 1972), white noise (Holland & Ross, 1983), clicker (Ward-Robinson & Hall, 1998) and visual cues such as key light (Rashotte et al., 1977), flashing light (Parkes & Westbrook, 2010; Wong et al., 2019) and context (Archer & Sjödén, 1982a; Iordanova et al., 2009). The types of USs used in higher-order designs are similar to those used in first-order conditioning studies including foot shocks, rewards such as food to hungry rats, lithium chloride (LiCl) – induced illness (e.g., Archer & Sjödén, 1982; Rizley & Rescorla, 1972; Holland & Rescorla, 1975; Ward-Robinson & Hall, 1998). Other aspects of stimulus type such as the intensity of the US with which S1 is paired, and the physical similarity between S2 and S1 (Garcia & Koelling, 1966; R. Rescorla, 1980a; R. A. Rescorla & Furrow, 1977) influence the strength of higher-order conditioning.

Stimulus similarity. An important contributing factor to learning in higher-order conditioning is stimulus similarity. Specifically, when similar stimuli are used in the roles of S2 and S1, higher-order conditioning is facilitated compared to using dissimilar stimuli. Rescorla and Furrow (1977) showed that second-order conditioning proceeded more rapidly when S1 and S2 belonged to the same, compared to a different, class of stimuli (e.g., colour: blue or green; orientation: horizontal or vertical lines). These effects were not due to stimulus generalization or pseudo-conditioning, that is, responding that accrues to an originally neutral stimulus due to physical similarity to a conditioned stimulus or following a train of unconditioned responding, respectively (Wickens & Wickens, 1942; R. A. Rescorla & Furrow, 1977; Martin & Pear, 1999). Cue similarity also facilitates second-order conditioning when the cues form a part-whole relationship. For example, in a pigeon autoshaping design, Rescorla (1980a) used achromatic shapes (triangle or square) as S2 and red shapes (triangle or square) as S1. Congruency in the shape, that is, when the achromatic shape was the same as the coloured shape, resulted in better second-order conditioning. Similar effects were reported in sensory preconditioning (Holland & Ross, 1983) and in appetitive second-

order conditioning (Holland, 1977) using same cue modality or spatial similarity (Rescorla & Cunningham, 1979).

Trial number. The number of trials used to establish higher-order conditioning depends on various factors including the nature of the design (e.g., fear, reward, taste aversion), cue modality, stimulus arrangement, the model organism (e.g, rat, pigeon, rabbit) and the response measure (e.g., magazine approach, freezing, conditioned suppression). Higher-order fear conditioning progresses fairly rapidly: four trials of serial S2→S1 pairings is sufficient to obtain second-order learning (Rizley & Rescorla, 1972; Parkes & Westbrook, 2010; Lay et al., 2018) and sensory preconditioning can be achieved in eight serial S2→S1 trials (Rizley & Rescorla, 1972; Parkes & Westbrook, 2011; Wong et al., 2019). Higher-order conditioning designs involving rewards require more extensive S2→S1 training. In particular, second-order conditioning is successful using 100 trials across 10 days in pigeons (Rashotte et al., 1977), or 40 trials across four days in rats (Holland & Rescorla, 1975a) whereas sensory preconditioning has been obtained with 200 trials across 10 days in pigeons (Reid, 1952), but with as few as 12 trials across two days in rats (Jones et al., 2012; Sadacca et al., 2018).

The large number of trials often required for appetitive second-order conditioning can have unintended effects. As the number of S2→S1 trials increase in second-order conditioning, responding to S2 decreases, which is in contrast with the increase in responding to S1 across S1→US pairings. When S2→S1 pairings are alternated with continued S1→US pairings, the S2 can become a signal for the *absence* of the US (i.e., a conditioned inhibitor, Herendeen & Anderson, 1968; Holland & Rescorla, 1975b; Yin et al., 1994). That is, conditioned inhibition to S2 accrues, competing with its ability to exhibit second-order conditioning (Gewirtz & Davis, 2000; Parkes & Westbrook, 2010). In a lick suppression study in rats, 20 simultaneous S2→S1 pairings favored conditioned inhibition over second-order conditioning and a hundred such trials rendered S2 a conditioned inhibitor regardless of whether S2 and S1 were paired simultaneously or serially (Stout et al., 2004). The transition of S2 from a second-order excitor to a conditioned inhibitor was quicker when S2 and S1 were presented in compound (Stout et al., 2004). To limit the development of conditioned inhibition in second-order conditioning, fewer S2→S1 pairing should be employed. This is possible in conditioned taste aversion. Indeed, a single pairing between a gustatory S2 and a contextual S1 was sufficient to obtained sensory preconditioning and

second-order conditioning provided the US used to conditioned S1 was very salient (i.e., LiCl; Archer & Sjödén, 1982). These data, among others, reveal the importance of the strength of S1→US association on higher-order conditioning (N. Bond & Harland, 1975; N. W. Bond & Di Giusto, 1976).

Procedures that support higher-order conditioning

The crucial learning phase in higher-order conditioning is the sensory training between S2 and S1. Procedurally, these stimuli can be paired in several distinct ways. Here we describe these procedures and how they influence the strength of S2-elicited conditioned responses.

Sensory preconditioning. In the classical design, pairings between S2 and S1 proceed serially in a forward conditioning arrangement where S2 is immediately followed by S1 presentation (i.e., S2→S1). This forward serial arrangement optimizes learning about the predictive relationship between the cues. That is, upon learning, S2 leads to the expectation of S1 delivery. In a sensory preconditioning preparation, serial S2→S1 pairings resulted in faster acquisition relative to simultaneous arrangement on a savings test where S2 was reinforced directly with the US (Silver & Meyer, 1954). This suggests stronger sensory preconditioning in the serial arrangement relative to simultaneous pairings. However, Rescorla argued that such a comparison between serial and simultaneous pairings is inherently biased. That is, in the serial arrangement stimuli are better processed or attended to given their isolated presentation, whereas in the simultaneous case, processing of stimulus elements may be more difficult as they are experienced in compound. Moreover, a decrement in response generalization from training phase to test phase can result from training two cues but testing to only one. Therefore, serial arrangement would be biased to elicit more responding relative to simultaneous arrangement. To circumvent these problems, Rescorla used a three-stimulus arrangement that included simultaneous and serial components (i.e., LightTone→Noise). Light and tone were in simultaneous arrangement and noise was serially paired with LightTone compound. Light served as S2 while either the tone or the noise served as S1 by pairing it with the US. When S1 was presented simultaneously with S2 (i.e., when the tone was paired with the US) sensory preconditioning was superior relative to the case when S1 was presented serially (i.e., when the noise was paired with the US Rescorla, 1980; Holland & Ross,

1983). This type of an arrangement, also known as compound training, facilitates associating the sensory features of the two stimuli (Rescorla, 1982).

It is also possible to obtain higher-order conditioning when S2 and S1 are arranged in reverse order during training, such that S1 is followed by S2 (i.e. S1→S2; Ward-Robinson & Hall, 1998). However, the strength of sensory preconditioning can be weaker in a backward design relative to forward. Wynne and Brogden (1962) showed that increasing the duration of S1 prior to S2 presentations, therefore backwardly conditioning S1 and S2 resulted in lower levels of sensory preconditioning in cats. As S1 precedence increased, the level of S2 elicited responding decreased and approached the level of the controls. Reversing the stimulus order during first-order conditioning (i.e., US→S1), however, resulted in robust sensory preconditioning in a lick suppression preparation with water-deprived rats (Barnet et al., 1997). Backward sensory training and backward first-order conditioning allow for an overlap between the associatively retrieved S2 to form links with the US, a phenomenon known as mediated conditioning (Holland, 1981). In both scenarios, associatively retrieved S2 temporally coincides with the US during Phase 2. Importantly, the level of conditioned responding that indicates the strength of associations between the US and the S1-mediated representation of S2 is lower relative to the association a physically present S2 forms directly with the US.

Interestingly, a trace between S1 and the US enhances sensory preconditioning. Lin and Honey (2011) showed that S2 elicited greater responding when there was a 40 sec trace between S1 and the US relative to when there was a 10sec trace despite reduction in S1-elicited responding on test in the former.

Second-order conditioning. In second-order conditioning, serial and simultaneous arrangements of S2 and S1 yield comparable levels of conditioned responding on test (Rescorla, 1982). Rescorla (1982) showed that both serial and simultaneous arrangements result in similar levels of conditioned responding in second-order conditioning. Similarly, backward or forward arrangements of S2 and S1 during sensory training yielded comparable levels of second-order conditioning in a conditioned flavour aversion preparation (Mowrer et al., 1988). Moreover, backward pairings between S1 and US (i.e., US→S1) also resulted in robust second-order conditioning, in a lick suppression preparation with water-deprived rats (Barnet et al., 1997).

In a classical second-order conditioning design sensory training is comprised of serial S2 and S1 pairings in the absence of the US. However, it is possible to obtain second-order

conditioning when S2→S1 presentations are reinforced (S2→S1→US, Seger & Scheuer, 1977; Williams-Spooner et al., 2019). Such reinforced presentations result in robust responding to S2 as a result of direct and indirect (i.e., via S1) pairings between S2 and the US (Holland, 1980). To show this, Holland (1980) tested S2 under food satiation to reveal the presence of direct S2→US association. Devaluation of the US resulted in lower levels of responding to S2 on test relative to the group that did not receive US devaluation, indicating the presence of direct S2→US association.

There have been reports showing enhanced second-order conditioning when the trace between S1 and US increased and sensory training proceeded in forward arrangement (Kehoe et al., 1981; Lin & Honey, 2011). The opposite was true when the trace between forwardly arranged S1 and US increased but sensory training occurred in backward arrangement (Cole et al., 1995b).

The content of learning in higher-order conditioning

Despite the procedural simplicity of Pavlovian conditioning, the underlying associative architecture that supports learning and the expression of conditioned responding is far from simple (e.g., (Pearce & Bouton, 2001; Pearce, 2002; Hall, 2002; Harris, 2006, but see also Pearce, 1994). The most rudimentary account focuses on the role of binary links between the events (stimuli or responses) that are present during the learning experience. Two types of binary associations have been proposed to underlie associative learning, namely an association between two stimuli (stimulus-stimulus [S-S]) or an association between a stimulus and a response (stimulus-response [S-R]). In the case of S-S associations, the stimuli can be (neutral) sensory events (e.g., S2→S1) or of biological significance (S1→US). Importantly, an S-S association implies that the presentation of one of these stimuli (e.g., S1) is able to activate a representation of its associate (i.e., US, see Fig 2a) leading to behavioural responding. In the case of S-R associations, the presentation of a stimulus directly enables behavioural responding, bypassing the activation of its associate (Fig 2c). The nature of the associations can be behaviourally examined using revaluation and extinction methods. These methods are discussed below and the role of binary associations in sensory preconditioning and second-order conditioning are considered.

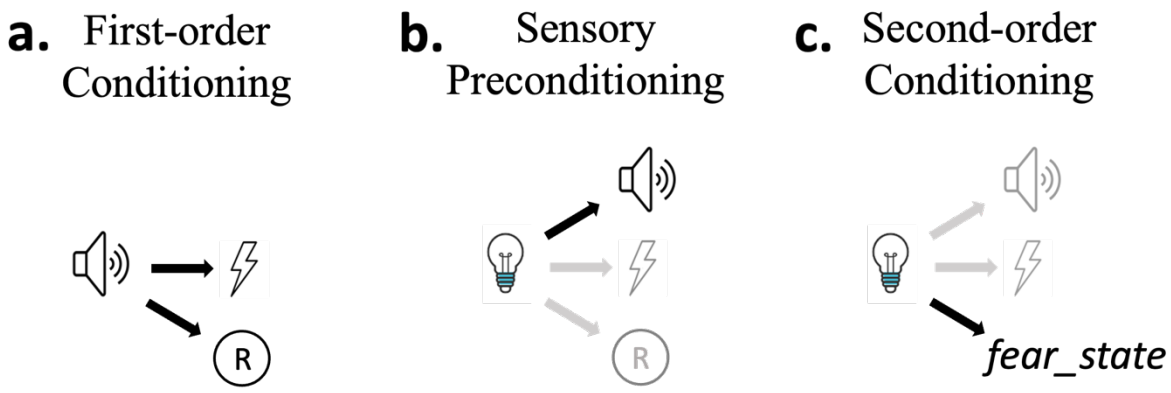

Figure 1.2. Nature of associations formed during first-order and higher-order learning. Black lines refer to dominant associations, while grey lines represent potential associations that may be formed during learning but have been ruled out as the primary mediator of conditioned responding. The tone represents the first-order CS, the light represents the higher-order CS, the shock represents the US, and R refers to the behavioural response. Pairings of the tone and shock during first-order conditioning leads to the formation of a stimulus-stimulus (S-S) association as well as a stimulus-response association (S-R). The nature of association that underlies sensory preconditioning is said to be S-S in nature between the higher-order light cue and the first-order tone cue (but see text), whereas associations between the higher-order light and the central motivational state (S-*fear_state)* are critical for the conditioned responding in second-order conditioning.

First-order conditioning. Considerable effort has been made to understand the associations that underlie first-order conditioning. To determine whether first-order conditioning depends on S-S associations for behavioural expression, the outcome is de/re-valued. How this is done depends on the experimental design. For instance, an aversive US can be devalued through habituation, that is, through repeated presentations of the US which reduces the unconditioned response to the US. Rats conditioned to fear an S1 through pairings with a loud startle-eliciting noise show a reduction in conditioned responding if startle to the noise was habituated prior to test (Rescorla, 1973a). Similarly, in appetitive studies where the US is a food reward, devaluation occurs by pairing the food with illness (e.g., lithium chloride) or inducing sensory specific satiety through pre-feeding. If a cue enables conditioned responding such as magazine approach as a result of activating a representation of the food US, then following devaluation of the US, conditioned responding to the cue is reduced. Prior studies have shown that devaluation of the food US either by satiation

with ad libitum access to food (Holland & Rescorla, 1975b; Holland, 1981) or by pairing food with high-speed rotation to induce illness (Holland & Rescorla, 1975b) reduced responding to the first-order cue, suggesting that first-order conditioning is mediated by an internal representation of the US. In addition to the formation of an S-S association in first-order conditioning, Holland (1990) demonstrated that a stimulus-response (S-R) association can also be learned during pairings of an S1 and a US. This was evident when comparing responding to the S1 to that during the pre-S1 period. This comparison showed that despite the reduction in conditioned responding following US devaluation, conditioned responding was greater to the S1 compared to the pre-S1 interval. Taken together, these results suggest that responding to a first-order conditioned cue depends on both S-S and S-R associations.

Higher-order conditioning. Sensory preconditioning and second-order conditioning are strikingly similar. Both consist of sensory training and first-order conditioning, and in some cases this training is parametrically identical (Parkes & Westbrook, 2010; Holmes et al., 2013, 2014). The key difference is the order of the training phases. This order, however, is important as it determines the nature of the associations learned. There are typically three types of associations that underlie responding to the higher-order S2: a relationship about the two stimuli, S2 and S1 (an S-S association; Rizley & Rescorla, 1972, an association between S2 and the conditioned response evoked by S1 (an S-R association; Hull, 1943) or, an association between S2 and an internal representation of the US mediated by S1 (a stimulus-US association; Fig 1.2; Konorski, 1948).

Sensory preconditioning. The nature of the sensory preconditioning design necessitates the formation of an association between the sensory properties of the two cues (i.e., S-S). Pairings between S2 and S1 occur prior to the introduction of the US, therefore the cues have neutral valence and do not signal any rewarding or aversive events, nor do they elicit US-based behavioural responses. Therefore, for sensory preconditioning to be evidenced, and irrespective of any other associations that may develop later, an association between S2 and S1 must be acquired during the sensory learning phase. Empirical evidence supports this proposal. Repeated presentations of S1 alone (i.e., extinction) subsequent to sensory preconditioning and first-order conditioning reduced conditioned responding not only to S1 but also to S2 compared to animals that did not receive S1 extinction (e.g., Rizley & Rescorla, 1972; Archer & Sjödén, 1982b). Therefore, these data demonstrate that responding to the sensory preconditioned cue (S2) is dependent upon its association with S1 and the S1→US relationship. That is, if S1 no longer

reliably signals the occurrence of the US as a result of S1 extinction, then S2 is also no longer able to elicit conditioned responding on test. An alternative account holds that S2 may undergo extinction in its own right via *mediated* associations while S1 is presented nonreinforced (Holland, 1983; Holland & Sherwood, 2008). In either case, the S2→S1 association is fundamental to sensory preconditioning.

Second-order conditioning. In contrast to sensory preconditioning, the sensory phase in second-order conditioning occurs following first-order conditioning. The implication of this is that when S2 is paired with S1, S1 is able to activate a representation of the associated US, that is, it carries motivational valence and is also able to elicit conditioned responding. Therefore, the opportunity for learning during the sensory phase in second-order conditioning extends beyond S2→S1 associations. Specifically, S2 is able to enter into its own direct link with the associatively activated US, the motivational state elicited during this phase or with the conditioned response.

Stimulus-Stimulus associations (S-S). Rizley and Rescorla (1972) ruled out the possibility of an S2→S1 association governing second-order conditioning using the same extinction method as the one described above. They found that rats that received S1 extinction training after Phase 2 but before test exhibited similar levels of fear to S2 as non-extinguished rats, indicating that conditioned responding to S2 is independent of its link with S1 and the current fear-eliciting properties of the latter stimulus. This finding was replicated using reward as a US. Holland and Rescorla (1975a) showed that extinction of an appetitive first-order cue also had no disruptive effect on second-order responding. Conditioned responding to S2 could be a result of mediated conditioning, that is, a link between S2 and the associatively-activated representation of the US (S2→mediated_US). For instance, during second-order conditioning, presentations of S1 activates the representation of the US such that S2 could become directly associated with that S1-activated US representation (e.g., Konorski, 1948). However, responding to S2 is also unaffected by changes in the value of the US. For example, Rescorla (1973b) paired S1 with a fear-eliciting startle stimulus and then paired S2 with S1. These pairings were followed by habituation of the response to the startle-eliciting stimulus and subsequent tests of S1 and S2. Rescorla reported that habituation of the startle-eliciting stimulus reduced fear responses to S1 but left intact such responses to S2. Holland and Rescorla (1975b) demonstrated similar findings when the value of a food US was reduced after second-order conditioning: pairing the food US with an illness-inducing treatment significantly reduced general activity to the first-order cue, but left second-order

responding intact. These findings were further supported by Holland (1981). Ad libitum access to the food US after second-order conditioning reduced all aspects of responding evoked by the first-order cue (i.e., general activity, magazine entries, head-jerking, and startle), but still had little to no effect on behaviours elicited by the second-order cue. Together, the data rule out the possibility that an S2-US association forms the basis of second-order conditioning.

Stimulus-Response associations (S-R). Provided that neither the extinction of S1 or devaluation of the US following second-order conditioning affected second-order conditioned responding on test, Rescorla (1973b) suggested that perhaps second-order conditioning is based on an association between the second-order S2 and the response elicited by S1 (a stimulus-response [S-R] association). However, Holland (1977) ruled out this possibility when he found that two different first-order cues (i.e., a visual and an auditory S1) that predicted the delivery of a food US supported similar patterns of conditioned behaviours to a common second-order cue, despite producing different conditioned responses themselves. Instead, the most widely accepted view suggests that second-order conditioning involves a direct association between S2 and the central motivational state elicited by S1 (Rescorla, 1973; Holland, 1977; Gewirtz & Davis, 2000; Winterbauer & Balleine, 2005). Notably, this conclusion regarding the nature of second-order conditioning may be specific to cases where S2 and S1 are taken from different modalities, e.g., an auditory S2 and a visual S1 or vice versa. When S2 and S1 are from the same modality, two auditory or two visual cues, responding to S2 is contingent on the current value of S1, as test responding to an auditory (or visual) S2 was reduced when the test was preceded by extinction of an auditory (or visual) S1. Such results imply that two cues from the same modality biases the subjects to encode an association between S2 and S1 (Rescorla, 1979, 1982).

Overall, the nature of associations formed during higher-order conditioning parallels those formed during first-order conditioning. However, behavioural evidence indicates that the learning underlying sensory preconditioning and second-order conditioning are distinct despite being parametrically similar. Further similarities and differences will be revealed when comparing the neural substrates and mechanisms that underlie these forms of learning.

Chapter 2. Neural Substrates in Higher-Order Conditioned Fear

Pavlovian conditioning has proved to be a valuable tool to investigate the neural substrates underlying learned behaviours. Yet, despite the extensive research focused on understanding the cellular and molecular basis of first-order learning in the last three decades (Johansen et al., 2011), our understanding of the neural mechanisms underpinning the higher-order phenomena are still relatively limited (but see Holmes et al., 2022). Work in the aversive domain involve structures such as the basolateral amygdala (BLA, Parkes et al., 2010), perirhinal cortex (PRh, Holmes et al., 2013; 2014), retrosplenial cortex (RSP, Robinson et al., 2011) and dorsal hippocampus (DH, Huff and Rudy, 2004) while research in the appetitive domain showed a role for orbitofrontal cortex (lOFC, Jones et al., 2012), nucleus accumbens (NAc, Setlow et al., 2002b; McDannald et al., 2013) and dopaminergic activity in the ventral tegmental area (VTA, Nader & LeDoux, 1999b; Maes et al., 2020). Common and different regions crucial in guiding behaviour in SPC and SOC are highlighted with a focus in the aversive domain but where informative, evidence from the appetitive literature is also considered.

Basolateral Amygdala

The amygdala is situated in the rostral portion of the temporal lobe and is comprised of several interconnected subnuclei. The main regions include the central (CN), the basal (BA) and the lateral (LA) nuclei (Sah et al., 2003). Amygdala receives inputs from all sensory modalities: olfactory (Scalia & Winans, 1975), auditory (Shi & Cassell, 1997), visual (Shi & Davis, 2001), somatosensory and gustatory (C. J. Shi & Cassell, 1998). It also receives inputs from frontal cortices (Mcdonald et al., 1996), perirhinal cortex (Shi & Cassell, 1999) and the hippocampus (McDonald, 1998). Moreover, the amygdala sends projections to many regions including the perirhinal cortex (Pitkanen, 2000), orbitofrontal cortex (Shi & Cassell, 1998), prefrontal cortex (Mcdonald et al., 1996), hippocampus (Petrovich et al., 2001) and the nucleus accumbens (NAc, McDonald, 1991) all of which are critical in mediating higher-order conditioning.

A wealth of data from lesion, pharmacological and neurophysiological studies demonstrate the amygdala as the critical site for conditioned fear (Davis, 1997; Fendt and Fanselow, 1999; Lavond et al., 1993; LeDoux, 1996) and reward (Corbit & Balleine, 2005; Cole, Powell, & Petrovich, 2013; Ramirez & Savage, 2007; Tye & Janak, 2007; Everitt, Cardinal, Parkinson, & Robbins, 2003;

Ambroggi, Ishikawa, Fields, & Nicola, 2008). Here we discuss the evidence for associative learning in the amygdala underlying Pavlovian first-order conditioning and draw parallels to higher-order conditioning.

Associative learning within the basolateral amygdala. Sensory inputs about the conditioned and unconditioned stimuli converge in the lateral amygdala. Such a convergence sets the basis of associative Hebbian plasticity (Hebb, 1949). That is, a strong depolarization initiated by the US leads to the strengthening of synapses that are active during the same time frame, those initiated by the CS (LeDoux, 2000; Paré, 2002; Sah & Westbrook, 2008). The strengthening of associations are mediated by *N*-methyl D-aspartate (NMDA, Malenka & Nicoll, 1999) receptors. Subsequent to learning (i.e., convergence of CS and US inputs), presentation of the CS by itself is sufficient to evoke neuronal activity in the BLA (LeDoux, 2000; Maren & Quirk, 2004). Lesions of the amygdala have been shown to impair the acquisition and expression of conditioned fear responses in animal models (Bagshaw and Benzies, 1968; Blanchard & Blanchard, 1972; Kapp et al., 1979; LeDoux, Cicchetti, Xagoraris, & Romanski, 1990). Specifically, reversible pharmacological inactivation of this region with the $GABA_A$ agonist, muscimol (Müller et al., 1997), or neurotoxic lesions of the BLA (Zimmerman et al., 2007) in rats disrupt acquisition and expression of conditioned fear, respectively.

Second-order conditioning. Basolateral amygdala is a crucial region also for second-order conditioning. Parkes and Westbrook (2010) showed that targeting the BLA via intracranial infusions of muscimol, systemic injections of NMDA receptor antagonist MK-801 or NMDA receptor NR2B subunit inactivation via intracranial ifenprofil infusions prior to sensory training disrupted expression of fear to SOC-S2 on test. These mechanisms are also known to underlie fear to S1 (Gewirtz & Davis, 1997; Rodrigues et al., 2001). Although first- and second-order Pavlovian conditioning require similar mechanisms in the BLA, the neural bases of two types of learning are not identical (Lay et al., 2018; Leidl et al., 2018). For example, Lay and colleagues (2018) showed that activation of PKA/PKC and ERK/MAPK signaling pathways in the BLA are not necessary following second-order fear conditioning although they are known to be crucial immediately after first-order fear conditioning (PKA: Goosens et al., 2000; ERK/MAPK: Schafe et al., 2000). The absence of effects as a result of PKA and ERK/MAPK inactivation were not due to the inefficiency of the drugs. Subsequent to test, training SOC-S2 in a first-order protocol disrupted expression of first-order fear when these pathways were inactivated relative to when they were intact. Moreover,

blockade of *de novo* protein synthesis following sensory training had no effect on fear to SOC-S2 on test, a process that disrupts expression of fear to S1. These results show that while first-order and second-order learning require activity in the BLA, they likely rely on distinct processes.

Leidl and colleagues (2018) investigated whether *de novo* protein synthesis requirement in first- but not in second-order conditioning was due to the presence of shock in the former and absence in the latter. That is, the shock reinforcer drives learning in S1→shock but not in S2→S1 episode. It could then be argued that direct pairings with the reinforcer requires newly synthesized proteins while indirect pairings, as exemplified in second-order conditioning does not. To show this, they used a reinforced second-order conditioning protocol where S2→S1 pairings in Phase 2 were followed by shock delivery (i.e., S2→S1→US) providing an opportunity for direct pairings between S2 and the shock. However, blockade of protein synthesis via cycloheximide infusions following Phase 2 showed equivalent levels of fear to S2 as the vehicle infused controls despite the opportunity of forming direct S2→US pairings. This shows that fear to SOC-S2 does not require newly synthesized proteins (even under conditions of shock delivery). However, it is known that protein synthesis is necessary subsequent to new learning (Davis & Squire, 1984; Hernandez & Abel, 2008). Therefore, one possibility is that the proteins synthesized as a result of S1→shock learning are used in Phase 2. To investigate this possibility, the use of such proteins is eliminated either by omitting Phase 1 training or by omitting S1 presentation during Phase 2 (i.e., S2→ … →US). When Phase 1 of SOC is omitted blockade of protein synthesis via cycloheximide infusions resulted in disruption of fear to reinforced SOC-S2 relative to rats that received vehicle infusions, demonstrating the requirement for newly synthesized proteins. Similarly, the omission of a fear conditioned S1 in Phase 2 also disrupted fear to S2 in the group that received cycloheximide relative to the group that received vehicle. These results indicate that when the proteins synthesized as a result of S1→shock episode are not available during Phase 2 of reinforced SOC, new proteins need to be produced in the BLA.

Williams-Spooner and colleagues (2019) expanded on these findings by delineating the conditions under which newly synthesized proteins are required in Phase 2 in an aversive reinforced SOC protocol. They showed that only when S1 is fear conditioned in Phase 1 and responding to S1 is intact prior to Phase 2, fear to reinforced SOC-S2 does not require newly synthesized proteins. In contrast, when S1 and shock are unpaired in Phase 1, or fear to S1 is reduced via extinction following Phase 1 but prior to Phase 2, or when S1 is paired with a food US

in Phase 1 and Phase 2 is comprised of S2→S1→shock trials, fear to reinforced SOC-S2 is disrupted by cycloheximide infusions following Phase 2 relative to vehicle controls. Taken together, these findings highlight common and distinct mechanisms in the BLA underlying first- and second-order fear and provide insight into the content of learning. As discussed in Chapter 1, first-order conditioning is based on S-S associations while second-order conditioning is governed by S-fear_state associations. More specifically, SOC-S2 becomes linked with the fear state that is elicited by S1. The data summarized above provide neurobiological evidence in line with the behavioural basis of second-order fear. That is, manipulations that interfere with the ability of S1 to elicit fear prior to but not after sensory training, disrupt second-order conditioning.

Most of the work on the function of the BLA focuses on fear. However, evidence in the appetitive domain shows parallels to the work in aversive literature (Everitt et al., 2003; Seitz et al., 2021; Wassum & Izquierdo, 2015). Important work from Peter Holland's lab provide insight into the role of BLA in appetitive Pavlovian conditioning. They showed that BLA lesions interfered with the ability of an appetitive S1 to serve as a reinforcer for an SOC-S2. On test, BLA-lesioned rats showed lower levels of conditioned responding to SOC-S2 relative to control rats (Hatfield et al., 1996). Further, Setlow and colleagues (2002a) showed that if lesions to the BLA occur subsequent to Phase 1 but prior to Phase 2 the expression of second-order responses is not impaired. That is, if a first-order stimulus is trained prior to BLA damage it can condition an SOC-S2. However, an S1 trained after the BLA lesions did not support second-order conditioning despite being able to elicit first-order conditioned behaviours (Setlow et al., 2002a). These results demonstrate that responding to appetitive SOC-S2 is dependent on the ability of S1 to serve as a conditioned reinforcer which is dependent on an intact BLA. Another study by the same group examined whether S1 can support second-order conditioning to S2 after its motivational value is extinguished (Lindgren et al., 2003). Subsequent to Phase 1, rats received either BLA or sham lesions and underwent S1-extinction prior to Phase 2. They found that extinguishing responding to S1 impaired its ability to support second-order conditioning in shams but not in BLA lesioned rats. These results indicate that lesions to the BLA rendered acquired motivational value of S1 extinction-resistant. Moreover, Holland (2016) showed that a first-order stimulus trained prior to BLA damage results in more vigorous responding to a second-order S2 relative to sham lesioned rats. These results show that a functional BLA is required for the acquisition but not expression of

the motivational value of a first-order stimulus and this property of S1 is crucial to obtain robust second-order conditioning.

Sensory preconditioning. Basolateral amygdala is important for the expression of fear to SPC-S2 (Holmes et al., 2013). More specifically, Holmes and colleagues (2013) showed that inactivation or NMDA receptor antagonism in the BLA via muscimol or ifenprofil infusions prior to test, respectively, disrupted fear to SPC-S2 relative to vehicle infused controls. However, unlike SOC, the BLA is not involved in sensory training in SPC. Identical muscimol or ifenprofil infusions into the BLA prior to sensory training had no effect on fear to SPC-S2. These effects were reversed when the sensory training took place in a dangerous context. That is, when the shock US was paired with the context prior to sensory training, NMDA receptor blockade in the BLA impaired fear to SPC-S2 on test. The same group also showed that BLA activity is required when the context→US pairings occur following sensory training (Qureshi et al., 2023). Infusions of NMDA receptor antagonist DAPV into the BLA prior to sensory training impaired fear expression to SPC-S2 when context→shock_US is experienced 5 minutes following sensory training in the same context. Identical NMDA receptor inactivation in the BLA had no effect on fear to SPC-S2 in the animals that were exposed to the context in the absence of the shock. That is, NMDA receptor activity in the BLA was required following sensory training only when danger ensued. These results suggest that the BLA forms a representation of SPC-S2 during sensory training (Holmes et al., 2021; Qureshi et al., 2023). If danger is experienced either before or immediately after sensory training the S2→S1 representation resides in the BLA, otherwise this representation in the BLA decays and the S2→S1 representation elsewhere (e.g., Perirhinal Cortex, PRh) drives sensory preconditioning.

Perirhinal cortex

The perirhinal cortex (PRh) is comprised of two strips of cortex, area 35 and area 36, which are situated adjacently along the rhinal sulcus. These areas receive cortical inputs from the auditory and visual cortices while reciprocating with the cingulate, infralimbic, prelimbic and the orbitofrontal cortices (Deacon et al., 1983; Sesack et al., 1989; Hwang et al., 2018), regions involved in guiding emotional behaviours and decision making (Öngür & Price, 2000). The PRh also has reciprocal projections with subcortical regions. For example, half of the subcortical

projections received in areas 35 and 36 come from the basolateral amygdala (BLA; McDonald & Jackson, 1987; Pikkarainen & Pitkänen, 2001). These connections are important in imbuing complex polymodal representations formed in the PRh (Suzuki et al., 1993) with affective value provided by the BLA (Yaniv et al., 2000; Yaniv & Richter-Levin, 2000). The role of PRh in forming sensory representations is evidenced in sensory preconditioning (Iordanova et al., 2009). For example, pharmacological inactivation of the PRh prior to Phase 1 sensory training via $GABA_A$ agonist muscimol or blockade of PRh neural activity via NMDA receptor antagonist ifenprofil disrupted fear to SPC-S2 on test relative to vehicle infused controls (Holmes et al., 2013; Qureshi et al., 2023). This demonstrates that learning the relationship between two neutral stimuli in sensory preconditioning involves the PRh (Holmes et al., 2013).

Interestingly, the PRh is also implicated in Phase 2 of SPC although S2 is not presented in this phase. A study by Wong and colleagues (2019) demonstrated that silencing the PRh via sodium channel blocker bupivacaine infusions either before or after Phase 2 impaired fear to S2 on test while having no effect on fear to S1. This shows that fear to SPC-S2 requires neural activity in the PRh during S1-shock episode. Moreover, protein synthesis in the PRh was also required following Phase 2 to obtain SPC. Infusions of protein synthesis inhibitor cycloheximide immediately after S1-shock episode disrupted fear to SPC-S2 on test relative to vehicle infused controls. These results provide evidence that SPC-S2 is linked with fear during Phase 2, in line with the argument that mediation drives SPC effect (see Chapter 1). This linking requires neural activity and protein synthesis in the PRh. Notably, silencing the PRh via bupivacaine infusions prior to expression of fear to SPC-S2 also disrupted performance relative to vehicle infused controls. This suggests that either the expression of mediated associations or the retrieval of chained associations are at play during SPC test, and it requires neural activity in the PRh.

Other brain areas

Orbitofrontal cortex. In addition to the PRh and the BLA, research in the appetitive domain implicates the lateral orbitofrontal cortex (lOFC) in SPC. A study by Sadacca and colleagues (2018) recorded lOFC neurons across training and at test in an appetitive SPC protocol. They trained two cue pairs during sensory training (i.e., A→B and C→D). In Phase 2, B was reinforced with food US and D was non-reinforced. On test, behavioural responding to A would exceed that

of C as A's associate was paired with food and C's associate was not. This evidences SPC. Using in vivo electrophysiology, they found that different neurons in the lOFC show activity to different pairs of neutral stimuli. That is, sensory training between distinct neutral cue pairs (i.e., A→B and C→D) recruited distinct neuronal populations in the lOFC for the A→B pair and the C→D pair. Moreover, neurons that showed high firing rate to a single cue (i.e., A or C) were more likely to respond to its pair (i.e., B or D). Subsequently, in Phase 2, a greater fraction of neurons showed increased firing to B as a result of its pairings with food US exceeding the fraction that increased firing to D. Furthermore, the population of neurons that responded to B was distinct from that of D. Finally, on test, the neurons that responded to A were much more likely to respond to its associate B relative to D. These results provide evidence that lOFC neurons track progression of SPC and can distinguish SPC-S2 from a control-S2. Importantly, Hart and colleagues (2020) showed that optogenetic inhibition of lOFC prior to Phase 1 impaired appetitive SPC providing causal evidence for lOFC in S2→S1 learning. The same group also showed that in an appetitive SPC task neural activity in the lOFC was necessary at test as silencing this region via muscimol infusions disrupted SPC effect (Jones et al., 2012).

The role of lOFC in higher-order fear learning remains unexplored. However, studies that examined its role in an aversive setting support its function in tracking and updating the associations between stimuli. One study found that the lOFC shows significantly more activity when there is an unexpected shock on test compared to when the shock is expected (Furlong et al., 2010). In this study, neural activity was measured by quantifying *c-fos*, a biological marker for cue evoked neuronal activity. They found that the unexpected shock resulted in more robust *c-fos* expression in the lOFC along with infralimbic, prelimbic, cingulate and insular cortices relative to the group that expected the shock. These results implicate a role for the lOFC in the aversive Pavlovian conditioning. Several other studies showed that fear regulation depends on intact lOFC (Ray et al., 2018; Lay et al., 2020). For example, Ray and colleagues (2018) showed that in a fear discrimination task comprised of a danger, an uncertainty and a safety cue, lOFC lesions resulted in generalization of fear to all cues when the shock probability increased only for the uncertainty cue. In contrast, sham rats that did not receive lOFC lesions increased fear only to uncertainty cue when shock probability increased while fear towards danger and safety cues remained unchanged as shock probabilities did not change for these cues. These findings indicate that lOFC is necessary for appropriate regulation of fear. Similarly, Lay and colleagues (2018) showed that lOFC is

required for learning from aversive overexpectation, a task that critically requires lOFC in an appetitive setting (Takahashi et al., 2009, 2013). In Phase 1 of overexpectation two stimuli are individually reinforced by the same US. Subsequently in Phase 2, these stimuli are presented together and reinforced by an identical US. Strikingly, responding to stimuli is reduced despite continued delivery of the same US. Importantly, the compounded stimuli signal twice the amount of US while only a single US is delivered. This results in reduction of fear to each individual stimulus. However, when lOFC is pharmacologically inactivated via muscimol/baclofen mixture during Phase 2, rats failed to learn from overexpectation as evidenced by high levels of fear to either of the stimuli relative to vehicle infused controls. These data provide evidence for the role of lOFC in fear regulation and suggest that lOFC is important for keeping track of multiple, dynamic cue-outcome relationships and adjusting behaviour accordingly.

Nucleus Accumbens and dopaminergic signaling in the Ventral Tegmental Area. The nucleus accumbens (NAc) is part of the ventral striatum and is involved in the processing of learned motivational significance of stimuli. It receives inputs from the BLA (Wright et al., 1996), prefrontal cortex (Brog et al., 1993) the ventral tegmental area (VTA; Zahm & Brog, 1992), and the hippocampus while sending projections to the VTA and the ventral pallidum (Zahm, 1999). Given its anatomical arrangement, NAc is proposed to be a region where limbic and motor information is integrated to drive goal-directed behaviours (Mogenson, 1984; Pennartz et al., 1994). Such behaviours along with Pavlovian behaviours are regulated by the dopaminergic neurons in the VTA (Grace et al., 2007; Darvas et al., 2014). Located in the midbrain, the VTA sends dopaminergic inputs via two main projections: mesocortical (prefrontal cortex; Weele et al., 2019) and mesolimbic (NAc and amygdala; Blaess et al., 2020) in regulating behaviour. Here we review the evidence for the role of NAc and VTA dopaminergic signalling in sensory preconditioning and second-order conditioning.

Similar to the effects obtained via BLA lesions, damage to the NAc prior to Phase 1 of an appetitive SOC task impaired SOC in the lesioned rats relative to sham (McDannald et al., 2013). However, BLA damage had no effect on conditioned approach responses to S1. This shows that damage to the NAc interfered with the motivational value of S1 and rendered it a weak conditioned reinforcer for SOC-S2. Moreover, disconnecting BLA and NAc via contralateral lesions impaired SOC relative to shams and ipsilaterally lesioned rats (Setlow et al., 2002b). These results suggest that BLA and NAc are part of a system that mediate learned motivational value. However, the

function of NAc does not seem to be restricted to value. In a sensory preconditioning task, NAc neurons showed greater firing rates to S2 and S1 during Phase 1 in the Paired groups relative to the Unpaired controls (Cerri et al., 2014). Moreover, this difference was only observed in the rats that learned from SPC relative to the rats that did not. That is, the Poor Learners that did not show the effect of SPC on test showed firing rates at the level of the Unpaired during sensory training.

The involvement of NAc during Phase 1 of SPC may be modulated by dopaminergic input. Young and colleagues (1998) showed that dopamine levels were higher during sensory training for the stimuli that were paired relative to the levels observed in the Unpaired controls. Another study showed that optogenetically silencing VTA dopamine neurons during sensory training disrupted SPC indicating that dopamine signalling in the VTA is necessary for appetitive SPC (Sharpe et al., 2017). Dopaminergic signaling in the VTA is also necessary to obtain appetitive SOC (Maes et al., 2020). Maes and colleagues (2020) found that optogenetically silencing VTA dopamine neurons during S1 delivery in Phase 2 impaired SOC in halorhodopsin-transfected animals relative to eYFP controls.

Dopamine signaling is mostly studied in reward learning but it is also involved in fear. For example, in an SOC protocol where S2 and S1 were both auditory, systemic injections of quinpirole, a D2 dopamine receptor agonist prior to sensory training impaired SOC (Nader & LeDoux, 1999a). The impairment was caused by disrupting the motivational value elicited by S1 as rats that received quinpirole showed lower levels of fear to S1 relative to vehicle injected controls. In a separate study using the same-modality SOC protocol, the authors showed that antagonising BLA D1 dopamine receptors or agonising VTA D2/D3 dopamine receptors prior to Phase 2 impaired SOC on test relative to vehicle infused controls (Nader & LeDoux, 1999b). Similar to Nader and LeDoux (1999a) this impairment was due to a disruption in S1's ability to elicit conditioned fear during Phase 2 to reinforce SOC-S2.

Blockade of D1 or D2 receptors via systemic SCH39166 or eticlopride injections, respectively, prior to sensory training both disrupted appetitive SPC (Roughley et al., 2021). These results are in line with Sharpe and colleagues (2017). In contrast, agonising D2 receptors via systemic quinpirole injections prior to sensory training had no effect on aversive SPC (Nader & LeDoux, 1999a). The lack of effect in Nader and LeDoux (1999a) in contrast to Sharpe et al., (2017) and Roughley et al., (2021) could be due to methodological differences such that the former *activated* D2-type in an aversive protocol, while the latter two reports *inactivated* D2- as well as

D1-type receptors in appetitive protocols. Perhaps, activation of D2 receptors does not interfere with the pairing of SPC-S2 and S1 during sensory training while inactivating D2 and/or D1 receptors does. Currently, exact mechanism underlying dopaminergic regulation of fear and reward is not clearly understood (Seitz et al., 2021; Holmes et al., 2022). However, these data along with *in vivo* electrophysiological recordings that show cue-evoked dopaminergic neural activity in the absence of any rewarding events (Sadacca et al., 2016) clearly demonstrate the role of dopamine during sensory training.

Retrosplenial cortex. The retrosplenial (RSC) is a long cortical structure that extends around the midline along the anteroposterior axis in the rat brain (Todd & Bucci, 2015). It is extensively connected with the thalamic regions (van Groen & Wyss, 1992), parahippocampal areas as well as the hippocampus (Burwell & Amaral, 1998; Sugar et al., 2011). Given its connectivity pattern RSC is involved in processing hippocampal-dependent contextual information, integration of sensory stimuli and is a critical component of '*where/when*' pathway that integrate spatial and temporal information (Vann et al., 2009; Miller et al., 2014). However, RSC function is not restricted to learning about spatial and contextual stimulus and it extends towards auditory (Gabriel et al., 1983) and visual stimuli as well (Robinson et al., 2014a). For example, permanent electrolytic lesions to the RSC prior to training disrupt appetitive sensory preconditioning (Robinson et al., 2012). Temporary chemogenetic silencing of the RSC using inhibitory DREADDs prior to sensory training also disrupted SPC, demonstrating its role in the formation of S-S associations (Robinson et al., 2014b). These studies used cross-modality stimuli (i.e., auditory and visual) to serve as SPC-S2 and S1. The role of RSC is also shown in sensory preconditioning using cues of the same modality (Fournier et al., 2020). These results indicate that RSC is necessary to link neutral stimuli in appetitive SPC, while its role in aversive SPC is unknown (Holmes et al., 2022).

One study explored the role of RSC in associating S2 and S1 in an aversive second-order conditioning task (Todd et al., 2016). In contrast to the effects obtained in SPC, electrolytic lesions of the RSC had no effect on aversive SOC. The authors argue that this may be due to the S→fear_state associations that govern SOC. Therefore, the role of RSC in Phase 2 of SOC can be investigated in a protocol where SOC-S2 and S1 are presented simultaneously, which give rise to S-S associations in SOC (Rescorla, 1982).

Taken together, evidence shows that the BLA is important for both sensory preconditioning and conditioning while the PRh and RSC are shown to be crucial for the former but not the latter. The lOFC is important in appetitive sensory preconditioning while evidence from the aversive literature indicate a role for this region in keeping track and updating the relationships between stimuli, similar to its function in the appetitive field. Moreover, connections of NAc with the BLA are necessary for second-order conditioning while research that investigate the role of dopamine show that VTA dopaminergic signalling regulates sensory preconditioned and second-order behaviours.

The thesis focuses on the roles of the BLA and the lOFC in regulating the expression of higher-order fear. The former is critical for the expression of both SPC and SOC types of fear. In Chapter 4, we investigate how both types of fear are differentially reliant on first-order fear and its neuronal ensemble in the BLA. The role of lOFC in higher-order fear remains unexplored but data show its involvement in fear regulation (Lay et al., 2020; Ray et al., 2018) and appetitive sensory preconditioning (Hart et al., 2020; Jones et al., 2012; Sadacca et al., 2018). Moreover, lOFC has dense reciprocal connections with both the BLA (Carmichael & Price, 1995a; Mcdonald et al., 1996; Murray & Fellows, 2022) and the PRh (Hwang et al., 2018; Kealy & Commins, 2011), a region important for aversive SPC. Given the role of lOFC in fear and its connections with regions critical for SPC and SOC, in Chapter 5, we investigate lOFC function in the expression of sensory preconditioned and second-order fear. In Chapter 6, we characterize BLA ensembles engaged in the expression of SPC and SOC, and target its projections to the lOFC prior to test. We also investigate lOFC input to the BLA in regulating fear expression in SPC and SOC.

Chapter 3. General Methods

Chapter 3 outlines the general methods used in experimental chapters (Chapters 4, 5, 6).

Subjects

The subjects used were 361 Sprague-Dawley (SD) WT male, 104 SD WT female rats, 81 Fos-LacZ male and 91 Fos-LacZ female rats on SD background. The individual numbers by group and sex as well as weights and breeding source for each experiment are outlined below. The rats were obtained from Envigo (Envigo RMS Ltd. Indianapolis, USA) or bred in-house. The rats were pair-housed, in clear shoebox cages (44.5 cm x 25.8 cm x 21.7 cm) with Sani-Chip (7090A, Envigo) and Corncob (7092, Envigo) bedding mixture, a nylon bone (K3580, Bio-Serv) and a tunnel (K3245, Bio-Serv) toy in a temperature and humidity-controlled environment under reverse light-dark cycle (12:12h light-dark cycle, lights off at 8am). The experimental sessions occurred during the dark cycle between 8am and 3pm. All the rats were handled daily, for five days prior to the start of behavioural experiments. The rats that underwent cannulation or viral infusion surgeries were checked twice daily for 5-7 days post-operatively. Food (5075, Agribrands) and water were available *ad libitum* in the home cages throughout the experiment. All experimental procedures were in accordance with Canadian Council of Animal Care regulations and approved by the Concordia University Animal Care Committee.

Behavioural Apparatus

Stimuli. Visual and auditory cues were used and counterbalanced in the experiments. In the behavioural, pharmacological inactivation and higher-order conditioning cell deletion experiments the visual cue consisted of a 4-Hz flashing light or steady light located on the left-hand side of the right wall 15cm above the floor. The auditory cue was a ~72-dB tone (measured inside the chamber) delivered through a loudspeaker located outside the behavioural chamber. In the pathway specific inactivation studies, the second-order conditioning procedure consisted of a ~75dB white noise or a steady light (counterbalanced) located on the right-hand side of the right wall 15cm above the floor. In higher-order conditioning procedures the directly (S1) and indirectly (S2) conditioned cues were 10s and 30s in duration, respectively. The unconditioned stimulus was a 0.5 s 0.8 mA footshock delivered to the grid floor of each chamber. In the fear discrimination study the cues were a tone and a buzzer (~72dB, counterbalanced) and the unconditioned stimulus was 0.5s 0.5mA footshock. The background noise in the chamber was 48-50-dB. The stimuli were

controlled via a Med-Associates program. The background noise and experimental auditory cues were measured using a digital sound level meter (Tenma, 72-942).

Experimental Chambers. Behavioural procedures were conducted in 8 operant-training chambers, each measuring 31.8 cm in height x 26.7 cm in length x 25.4 cm in width (Med Associates, St. Albans, VT, USA). The modular left and right walls were made of aluminum, and the back wall, front door, and ceiling were made of clear Perspex. Their floors consisted of stainless-steel rods, 4 mm in diameter, spaced 15 mm apart, center to center, with a tray below the floor. The grid floor was connected to a shock generator and delivered continuous scrambled foot-shock. Each chamber was enclosed in a ventilated sound attenuating cabinet. The back wall of each cabinet was equipped with a camera connected to a monitor located in another room of the laboratory where the behaviour of each rat was videotaped and observed by an experimenter. Illumination of each chamber was provided by a near-infrared light source (NIR-200) mounted on the back wall of each cabinet. Stimuli were presented through Med Associates software on a computer located outside the experimental room. The chambers had checkered or striped wallpaper on the door and each wall except for the back wall to allow for video viewing. The back wall of the sound attenuating cabinet was covered with the appropriate checkered or striped pattern. The chambers (walls, ceiling, door, grid floor, and tray) were cleaned with 4% alcohol-based almond-scented solution (Environmental STH) dissolved in water after the removal of each rat. The chamber wallpaper was counterbalanced across groups.

Behavioural Procedures

Context exposure. Rats spent 20 minutes in the behavioural chambers twice daily prior to the first training phase in sensory preconditioning and second-order conditioning studies. The two context exposure sessions were 2,5 hours apart. Two days of context exposure is carried out to eliminate contextual fear prior to the start of the conditioning phases and after first-order conditioning phase.

Sensory training. This session consisted of four presentations of higher-order and first-order cues. The paired groups received successive presentations of audio and visual cues (cues counterbalanced) whereas the unpaired groups were presented with the equal number of higher-order and first-order cues with 2-min intertrial interval between presentations. The first trial in the paired groups started 5 minutes after the rats were placed in chambers, and the intertrial interval was 5 minutes. Rats spent an extra 60 seconds after offset of the last trial.

Fear conditioning. Four trials of S1 and US pairings were carried out to achieve first-order conditioning. The first trial started 5 minutes after rats were placed in behavioural chambers and the intertrial interval was 5 minutes. Rats stayed in the chambers for another 60 seconds after the last trial.

Test(s). Eight trials of the target cue with 2-minute intertrial intervals were presented to assess freezing levels at test. The first trial commenced 2 minutes after the rats were placed in chambers and they remained in the conditioning chambers for another 60 seconds after the last trial.

Fear discrimination training. Over the course of four days, twice daily, rats received two auditory cue presentations per session: one reinforced with foot shock and the other non-reinforced. In each session, the first trial started 10 minutes after the placement in the behavioural chambers and the intertrial interval was 10 minutes. The order of reinforced and non-reinforced trials was pseudorandom (Day 1: ABBA, Day 2: BAAB; Day 3: ABBA Day 4: BAAB, where A is the reinforced cue and B is the non-reinforced cue). The rats remained in the chambers for an extra 10 minutes following the last trial.

Cue exposure. All rats in the fear discrimination study received either two non-reinforced presentations of the target or the non-target cue. Rats were infused with either Daun02 or vehicle solution bilaterally into the BLA 90-120 minutes after the first cue exposure. The first cue exposure occurred 10 minutes after the session started and the intertrial interval was 10 minutes. Rats remained in the chambers for 10 more minutes after the last cue exposure. All rats in the sensory preconditioning and second-order conditioning experiments received two first-order cue exposures with an intertrial interval of 5 minutes. The first trial commenced 5 minutes after the placement in the chambers and rats remained in the boxes for another 5 minutes after the last trial. These intervals are set to match with the training intervals.

Drugs and viral vectors

Pharmacological inactivation. $GABA_A$ agonist, muscimol (M1523, Sigma-Aldrich), and $GABA_B$ agonist, baclofen (B5399, Sigma-Aldrich), were used to pharmacologically to inactivate the lateral OFC (lOFC). A muscimol-baclofen (M/B) cocktail was prepared by dissolving 5 mg of muscimol and 93.65 mg of baclofen in 438 ml of nonpyrogenic saline (0.9% w/v) to obtain a final stock concentration of 0.1 mM muscimol-1 mM baclofen. Nonpyrogenic saline (0.9% w/v) was used as a vehicle solution.

Daun02 inactivation. DNA synthesis inhibitor, Daun02 (A3352, ApexBio), was dissolved in 5% dimethyl sulfoxide (DMSO; Bioshop), 6% Tween 80 (Bioshop), and 89% 0.1M phosphate buffered saline (PBS, pH 7.4) to obtain a final concentration of 4µg/µl. The vehicle solution consisted of 5% DMSO, 6% Tween 80, and 89% 0.1M PBS.

Pathway inactivation using DREADDs. Transfection of the lOFC or the BLA neurons using the inhibitory designer receptors exclusively activated by designer drugs (DREADDs) was achieved by an adeno associated virus (AAV) driving the hm4DG$_i$-mCherry sequence under human synapsin promotor (pAAV2/8-hSyn-HA-hm4DG$_i$-mCherry, titer: 2 x 10^{12} vg/ml; NeuroPhotonics, QC, Canada). The virus lacking the inhibitory hm4DG$_i$ DREADDs gene (pAAV2/8-hSyn-HA-mCherry, titer: 2 x 10^{12} vg/ml; NeuroPhotonics, QC, Canada) was used as the control virus. Drug that is used to activate the DREADDs, Clozapine N-oxide (National Institute for Drug Abuse, Baltimore, MD, USA), was dissolved in a vehicle solution (1% DMSO in 0.9% sterile saline) to obtain a concentration of 1mM.

RNAscope in situ hybridization. Retrograde labelling of the BLA neurons was achieved by microinfusions in the lOFC using mCherry sequence under human synapsin promoter (pAAVrg-hSyn-mCherry; titer: 1 x 10^{13} vg/ml, Addgene, MA, USA).

Surgical procedures

Rats were anaesthetized with 5% isoflurane in oxygen and mounted on a stereotaxic apparatus (David Kopf Instruments) where anaesthesia was maintained through a nose cone for the duration of the surgery. Prior to incision, 5mg/kg carprofen (Rimadyl 50mg/ml, Zoetis, MI) was injected subcutaneously and 2% Lidocaine-HCl (CBmedical) was injected at the incision site as a local anesthetic. Post-operatively rats were handled, weighed, and administered antibiotics orally (TEVA Cephalexin) for 5-7 days.

Cannulation surgeries. Guide cannulae (26 GA) were implanted in the lOFC (Males: AP=3.7, ML= ±2.7, DL=-4.3) or BLA (Males: AP= -2.5, ML= ±4.8, DL=-7.6; Females: AP= -2.4, ML= ±4.6, DL= -7.4). Guide cannulae were fixed using dental cement (Co-oral-ite Dental Mfg. Co., Diamond Springs, CA, USA) and four self-taping jeweler's screws. Dummy cannulae were always kept in the guide except during drug infusions.

Viral infection surgeries. Viral vectors were infused at a volume of 0.8µl (lOFC) or 0.6µl (BLA) in each hemisphere at a rate of 0.1µl/min. Following infusion, the 33GA injectors were left in place

for an extra 10 minutes to ensure diffusion. At the end of the surgery, the incision site was sutured using silk suture thread (Johnson&Johnson Ethicon Silk Suture). Cannulae were implanted 6-7 weeks following the viral infusion surgeries in a manner identical to that described above.

Inactivation procedures

Drugs were infused bilaterally into the target region (lOFC or BLA) by inserting a 33-gauge injector cannula into each guide cannula. The injector cannulae were connected to a 10µL Hamilton syringe attached to an infusion pump (Harvard Apparatus, Holliston, MA, USA). The injector cannula projected an additional 1 mm ventral to the tip of the guide cannula. Infusion of M/B consisted of 0.3 µl delivered to both sides at a rate of 0.1 µl/min prior to test session, Daun02 infusion consisted of 0.5 µl delivered to both sides at a rate of 0.25 µl/min 90-120 minutes following first cue exposure, CNO infusion consisted of 0.3 µl in the lOFC and 0.5 µl in the BLA delivered at a rate of 0.3 and 0.25 µl/min prior to test session, respectively. Drug delivery was monitored with the progression of an air bubble in the infusion tubing. The injector cannula remained in place for an additional 2-3 min after the infusion to allow for drug diffusion before its complete removal. Immediately after the infusion, the injector was replaced with the original dummy cannula. One day before infusions, all rats were familiarized with this procedure by removing the dummy cannula and inserting the injector cannula to minimize stress the following day.

Histology

Rats with DREADDs and the *fos*-LacZ rats received a lethal dose of Euthanyl dissolved in 0.9% saline (1:1) intraperitoneally and perfused transcardially with 0.1M PBS followed by 4% Paraformaldehyde (PFA) in 0.1M PBS (pH 7.4). The *fos*-LacZ transgenic rats were perfused within 90-120 minutes window following the first cue exposure to collect the brains at peak β-galactosidase activity. Following extraction, brains were post-fixed in 4% PFA for 1h and were transferred to 20% sucrose in 0.1M PBS solution overnight. Brains were then stored at -80°C until slicing. Coronal sections were collected onto microscopic slides at 40µm using a Thermo Cryotome FE cryostat. In studies involving *fos-LacZ* transgenics (Chapter 4), additional series of sections were collected in 1.5ml centrifuge tubes (Axygen) containing PB-Azide. These sections were used in X-gal histochemistry to visualize ß-galactosidase activity in determining the effect of Daun02 inactivation.

Rats used in the remainder set of experiments were euthanized using CO_2 (8.6mL/min). Fresh brains were then extracted for cannulated rats, frozen at -80°C, sectioned at 40µm. Sections were mounted directly onto microscopic slides (Fisherbrand) to verify cannulae placements, left to dry at 4°C overnight and stained using Cresyl Violet. Tissues were then dehydrated using graded ethanol (70%, 95% and 99% respectively), cleared with CitriSolv (Decon Labs, Inc.) and coverslipped immediately using DPX mountant (Sigma-Aldrich). These sections were verified for guide cannulae placements under the microscope (Zeiss) using Paxinos and Watson (2007).

X-gal Histochemistry. Series collected in centrifuge tubes were then used in X-gal histochemistry to visualize β-galactosidase expression. The staining procedure started with three 10-minute washes in 0.1M PBS. The sections were then transferred into 1.5ml centrifuge tubes containing X-gal solution (2.4 mM X-gal, 100 mM sodium phosphate, 100 mM sodium chloride, 5 mM EGTA, 2 mM $MgCl_2$, 0.2% Triton X-100, 5 mM K_3FeCN_6, 5 mM K_4FeCN_6). They were incubated at 37°C water bath for 4.5 hours. The tubes were swirled every 20-30 minutes to ensure that all the sections were well covered with X-gal solution. At the end of incubation, one section was mounted on a gel-coated microscopic slide and was checked for the presence of β-galactosidase under the microscope. Once β-galactosidase was verified all the sections were washed in 0.1M PBS for 10 minutes three times and stored in 0.01% sodium azide in 0.1M PBS. Free-floating coronal slices were then mounted onto gel-coated slides using 0.01M PBS. Slides with mounted sections were left to dry overnight, dehydrated with increasing concentrations of ethanol (70%, 95% and 99% respectively), cleared with CitriSolv (Decon Labs, Inc.) and coverslipped using Permount mounting medium (FisherScientific). The coverslipped slides were left to dry for 48 hours at room temperature and β-galactosidase expression was imaged and counted using bright field microscope (Carl Zeiss Microscopy) under 5X magnification.

RNAscope in situ hybridization. Rat brains were flash frozen in isopentane at -50°C and sectioned at 20 µm. The sections are processed according to RNAscope Multiplex Fluorescent kit v2 instructions using c-Fos mRNA probes Rn-Fos-Intron1-C3 (Catalog no. 444861-C3) and Rn-Fos-O1 (Catalog no. 444871; Advanced Cell Diagnostics, Hayward, CA). Images were acquired using Nikon TiE epifluorescent microscope and analyzed in QuPath and ImageJ using guidelines provided by Advanced Cell Diagnostics (ACDbio, CA, USA).

Data analysis

Freezing was used to assess conditioned fear. The criteria for freezing are immobility, heightened back and tail (Blanchard & Blanchard, 1969; Fanselow 1980). Each rat is observed every 2 seconds and scored as freezing or not freezing. The percentage of scores was calculated as the proportion of total freezing for the duration of the cue. In studies where rats received sensory preconditioning prior to second-order conditioning, any animal that showed freezing to the first presentation of the second-order stimulus above 20% was removed from the study. Data were analyzed in RStudio (2022.07.1, PBC) using analysis of variance (ANOVA). Pairwise comparisons were assessed using standardized confidence intervals, Bonferroni adjusted *p*-values and effect sizes reported (η_p^2 for ANOVA and Cohen's *d* for contrasts; see Cohen, 1988). The criterion for rejecting the null hypothesis was set at $\alpha = 0.05$. Violations of sphericity are corrected using Greenhouse-Geisser when $\varepsilon < 0.75$ and Huyn-Feldt when $\varepsilon > 0.75$. Homoscedasticity violations are adjusted by hc3 model of heterescedasticity convenient covariance matrices (hccm, White, 1980).

Chapter 4. Sensory preconditioning and second-order conditioning differentially depend on fear to the first-order conditioned stimulus

As discussed in Chapter 1, fear to a sensory preconditioned stimulus (SPC-S2) and a second order conditioned stimulus (SOC-S2) differentially depend on fear to the first-order conditioned stimulus (S1). Reducing fear to S1 using extinction training (i.e., S1 presentations in the absence of shock) revealed a reduction in fear to SPC-S2 but not SOC-S2 (Rizley & Rescorla, 1972; Gewirtz & Davis, 2000; Parkes & Westbrook, 2011). One of the aims of this chapter was to confirm this behavioural effect while using identical training parameters in SPC and in SOC. That is, I equated for the number of training trials during sensory training and during first-order conditioning, and used identical conditioned and unconditioned stimuli in both tasks.

It is well known that acquisition survives extinction. That is, while fear to S1 is reduced following extinction, the memory that S1 signals shock is not erased (Bouton, 1991; 2002). Therefore, it remains possible that SPC but not SOC is more sensitive to extinction and that SOC may somehow have stronger links with the original fear memory. To determine whether this is the case, it would be beneficial to be able to target the original fear memory. That is, disrupt the

original S1-shock memory directly as opposed to establishing a competing S1→no_shock memory. Therefore, a second aim of this chapter was to disrupt the original fear memory to S1 and examine the effect of this on fear to SPC-S2 and SOC-S2.

To target the memory of fear to S1, we focused on the basolateral amygdala (BLA). We chose the BLA as it has been extensively implicated in the acquisition and expression of first-order conditioned fear (i.e., fear to S1; Davis, 1992; Fanselow, 1994; LeDoux, 2000; Maren et al., 2001). Electrolytic or neurotoxic lesions of the BLA impaired tone-shock learning relative to sham lesions (Bagshaw & Benzies, 1968; Blanchard & Blanchard, 1972; Kapp et al., 1979; LeDoux et al., 1990). Reversible pharmacological blockade of the BLA by $GABA_A$ receptor agonist muscimol infusions prior to S1-shock episode also disrupted first-order fear conditioning (Maren et al., 2001). However, the BLA is also important for fear to a SPC-S2 and SOC-S2 (Parkes & Westbrook, 2010; Holmes et al., 2013). Therefore, we needed a more targeted disruption of the S1-fear memory. To achieve this, we deleted the neuronal ensemble that is activated upon presentation of the fear eliciting S1. We used the Daun02 inactivation method in *fos-LacZ* transgenic rats to target the neuronal ensembles activated by the fear-eliciting S1. This procedure takes advantage of the temporal expression profile of *c-Fos* immediate early gene to induce apoptosis (i.e., cell deletion) in the neurons that are activated as a result of cue exposure. The protein product of the *LacZ* transgene downstream to *c-Fos* promoter allows to delete recently activated neurons in the presence of the Daun02 drug (Figure 4.3a). We targeted the S1-shock memory by deleting BLA neurons activated following S1 presentation and tested for fear to SPC-S2 and SOC-S2.

In this chapter I used extinction to reduce fear to S1 and examine its effect on fear to S2 in SPC and SOC. Next, I used the Daun02 inactivation technique to delete S1-activated neuronal ensembles in the BLA and show that this method results in a reduction of fear to S1. Subsequently, I investigated the dependency of higher-order fear on first-order fear by targeting S1-activated neuronal ensembles in the BLA. We hypothesized that if fear to S2 relies on S2-S1 association in both designs, deletion of S1-activated neural ensembles in the BLA using Daun02 inactivation technique in *fos-LacZ* rats would disrupt both types of fear. However, if fear to S2 differentially depend on the original S1-shock memory such that SOC-S2 is linked with S1-shock more strongly than SPC-S2, deletion of S1-evoked neurons would spare the former and disrupt the latter.

Experiment 4.1: Extinction of a first-order fear conditioned stimulus (S1) reduces fear to sensory preconditioned stimulus (SPC-S2)

In Experiment 4.1, we aimed to investigate the effect of S1-extinction on the expression of fear to a sensory preconditioned stimulus (SPC-S2). To do this, three groups of rats were used: Paired (S2→S1), Unpaired (S2/S1) and Paired-Extinction (S2→S1, S1→no_shock; Figure 4.1a). The Paired and Unpaired groups were included to show that sensory preconditioned fear is contingent on S2-S1 pairings in the former, while the Unpaired group served as a behavioural control and received explicitly unpaired presentations of S2 and S1. Only Paired-Extinction group received S1-extinction by presenting S1 in the absence of the shock following training but prior to test. Comparison between Paired and Unpaired reveal SPC effect. Comparison between Paired and Paired-Extinction reveal the effect of reducing S1-elicited fear on fear to SPC-S2.

Methods

Subjects. Forty (20 Males, 20 Females) experimentally naïve Sprague-Dawley WT rats bred in-house and weighing 220-260g (females) and 330-400g (males) were used. Final group numbers were as follows: Group Paired, n =12 (6 females, 6 males); Group Paired-Extinction, n =16 (8 females, 8 males); Group Unpaired, n =12 (6 females, 6 males).

Behavioural procedures and apparatus. As described in Chapter 3 General Methods (Behavioural Apparatus)

Experimental timeline. Days 1, 2 and 7 consisted of context exposure, Days 3 and 6 consisted of sensory training and fear conditioning, respectively. On Days 4 and 5 rats remained in home cages (no behavioural training). Break days were included to match the passage of time between sensory training and test with that of cell deletion experiments. Days 8 and 9 consisted of test to the sensory preconditioned cue and the first-order cue, respectively. For Paired-Extinction group Day 8 consisted of test to the first-order cue (i.e., extinction training), tests to the sensory preconditioned and first-order cues occurred on Days 9 and 10, respectively.

Results

Phase 1 Sensory Preconditioning. As expected, freezing was low and similar to SPC-S2 among the three groups (Figure 4.1b). A mixed ANOVA for freezing to SPC-S2 revealed no main effect of group ($F_{(2, 37)}$= 2.455, p = 0.10 η_p^2= 0.117), a main effect of trial ($F_{(2.63, 97.15)}$= 4.265, p =0.01, η_p^2= 0.103) and no group x trial interaction ($F_{(5.25, 97.15)}$= 0.853, p =0.52, η_p^2= 0.044). An

identical analysis of freezing to S1 revealed no main effect of group ($F_{(2, 37)}$= 0.449, p = 0.642 η_p^2= 0.024), no main effect of trial ($F_{(2.21, 74.06)}$= 2.131, p =0.12, η_p^2= 0.055) nor a group x trial interaction ($F_{(4.42, 74.06)}$= 0.412, p = 0.818, η_p^2= 0.022).

Phase 2 First-Order Conditioning. Conditioned freezing to S1 increased across trials in all groups (Figure 4.1c, left panel). A mixed ANOVA revealed no main effect of group ($F_{(2, 34)}$ = 0.299, p = 0.743, η_p^2 = 0.01), a main effect of trial ($F_{(3, 102)}$ = 141.438, p < 0.001, η_p^2 = 0.806) and no group x trial interaction ($F_{(6, 102)}$ = 1.108, p = 0.363, η_p^2 = 0.061).

Phase 3 S1 Extinction. Only Paired-Extinction group received non-reinforced presentations of S1, resulting in a reduction in freezing across trials (Figure 4.1c, right panel). A repeated measures ANOVA revealed a significant trial effect as a result of extinction training ($F_{(7, 84)}$ = 6.971, p<0.001, η_p^2= 0.367).

S2 Test. Conditioned freezing to S2 in the Paired group was higher compared to the Unpaired group, providing evidence for the SPC effect. Extinction of S1 prior to S2 test reduced fear to S2 (Figure 4.1d). This was confirmed by a one-way ANOVA ($F_{(2, 37)}$ = 10.360, p < 0.001 η_p^2 = 0.359). Pairwise comparisons revealed a difference between Paired and Unpaired groups ($M_{\text{Paired}} - M_{\text{Unpaired}}$ = 12.15, p<0.001, 95% CI [5.332, 18.956], d= 2.13) and a difference between Paired and Paired-Extinction groups ($M_{\text{Paired}} - M_{\text{Paired-Ext}}$ = 9.83, p = 0.001, 95% CI [3.454, 16.198], d= 1.26). These results demonstrate that fear to SPC-S2 depends on fear to S1.

S1 Test. To confirm that fear conditioning and extinction were successful we tested for fear to S1. Freezing to S1 was higher in the two fear conditioned groups that had not undergone extinction (i.e., Paired and Unpaired) compared to the group that had received S1 extinction (Paired-Extinction). One-way ANOVA revealed main effect of group ($F_{(1, 35)}$ = 4.028, p = 0.027, η_p^2 =0.187; Figure 4.1e).

S1 test occurred 2 days following S1 extinction, which could lead to spontaneous recovery obscuring the extinction effect. Therefore, we examined whether S1 responding reduced faster (extinction savings) in the Paired-Extinction group compared to the Paired and Unpaired groups. To do so, we included trials as a factor in our analyses. A mixed ANOVA revealed a main effect of group ($F_{(1.38, 24.29)}$ = 4.807, p = 0.014, η_p^2 = 0.216), a main effect of trial ($F_{(4.86, 170.03)}$ = 7.084, p < 0.001, η_p^2 = 0.168) and a group x trial interaction ($F_{(9.72, 170.03)}$ = 1.991, p = 0.020, η_p^2 =0.102). Pairwise comparisons revealed a difference between Paired and Paired-Extinction groups (M_{Paired}

– $M_{\text{Paired-Ext}}$ = 15.83, p < 0.001, 95% CI [6.25, 25.42], d = 0.540), and a difference between Unpaired and Paired-Extinction ($M_{\text{Unpaired}} - M_{\text{Paired-Ext}}$ = 21.50, p < 0.001, 95% CI [11.38, 31.62], d = 0.739), and no difference between the Paired and the Unpaired groups ($M_{\text{Paired}} - M_{\text{Unpaired}}$ = -5.667, p =0.430, 95% CI [-5.083, 16.416], d =0.175).

Discussion

In this study, we examined the effect of fear reduction to S1 on fear to SPC-S2. Firstly, we confirmed that SPC-S2 elicits fear as a result of its pairings with S1 (Figure 4.1a). Indeed, the Paired group showed elevated levels of freezing to SPC-S2 on test relative to the Unpaired controls (Figure 4.1d). This comparison demonstrates the SPC effect. We also investigated the reliance of fear to SPC-S2 onto fear to S1. This is achieved by extinguishing fear to S1 via presenting it in the absence of the shock (i.e., extinction training). Only the Paired-Extinction group received S1-extinction subsequent to training but prior to test. The Paired-Extinction group showed lower levels of fear to SPC-S2 relative to the Paired group which did not undergo extinction (Figure 4.1d). This demonstrates that SPC-S2 fear depends on intact fear to S1. Taken together, our results show that fear to SPC-S2 results from its pairings with S1 and if fear to the latter is reduced, fear to the former is disrupted.

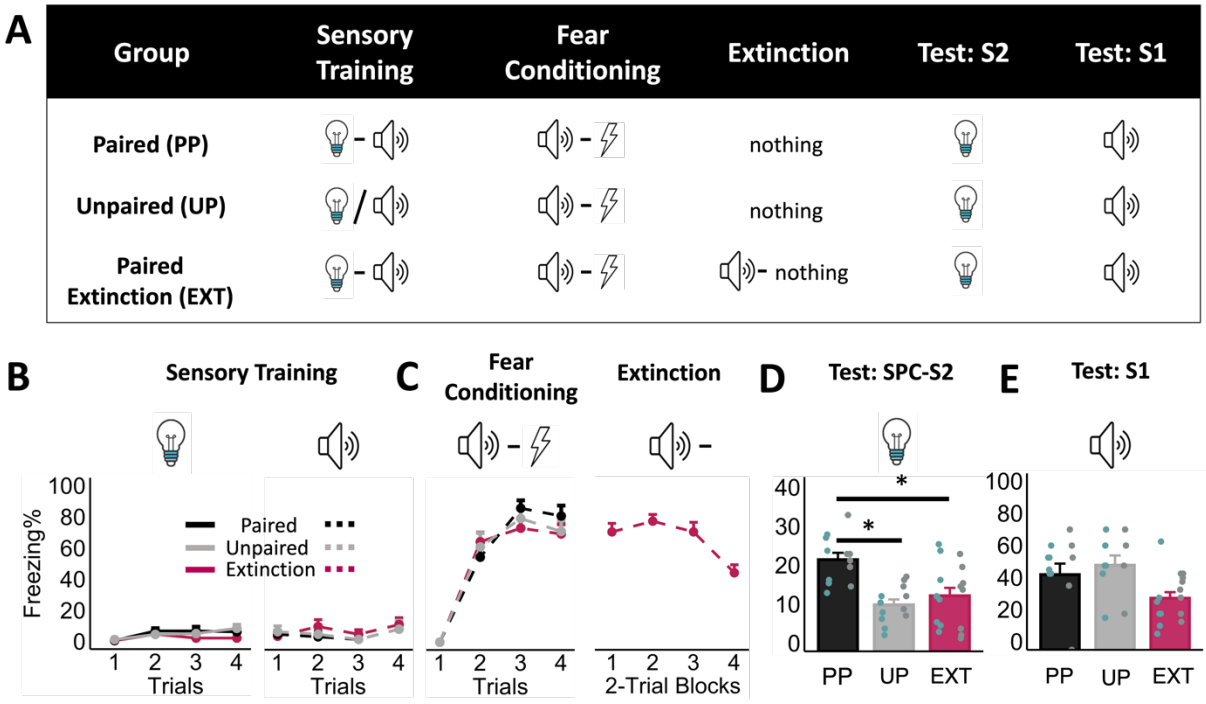

Figure 4.1. Extinction of a first-order fear conditioned stimulus (S1) reduces fear to sensory preconditioned stimulus (SPC-S2) (A) Behavioural protocol. (B) Fear to S2 (solid lines) and S1 (dashed lines) during sensory training. Paired (black) $n = 12$ (6 females, 6 males), Unpaired (grey), $n = 12$ (6 females, 6 males), Paired-Extinction (burgundy), $n = 16$ (8 females, 8 males). (C) Left panel: Acquisition of conditioned fear to S1 across trials. Right panel: Extinction of conditioned fear to S1 across trials. (D) Fear to S2 on test (females green, males grey). Comparison between Paired and Unpaired groups evidence sensory preconditioning effect ($p < 0.001$). Comparison between Paired and Paired-Extinction indicate disruption of sensory preconditioning when S1 memory intact vs extinguished ($p = 0.001$). (E) Fear to S1 on test. All data are mean freezing percent (+ SEM).

Experiment 4.2: Extinction of a first-order fear conditioned stimulus (S1) has no effect on fear to a second-order conditioned stimulus (SOC-S2)

In Experiment 4.2, we aimed to investigate the effect of S1-extinction on the expression of second-order conditioned stimulus (SOC-S2). To do this, we included three groups of rats: Paired (S2→S1), Unpaired (S2/S1) and Paired-Extinction (S2→S1, S1→no_shock; Figure 4.2a). The Paired group was included to show that second-order fear is contingent on S2-S1 pairings while the Unpaired served as a behavioural control as it received explicitly unpaired presentations of S2 and S1. Only the Paired-Extinction group received S1-extinction by presenting S1 in the absence of the shock subsequent to training but prior to test. Comparison between Paired and Unpaired

reveal SOC effect. Comparison between Paired and Paired-Extinction demonstrate the effect of reducing S1-elicited fear on fear to SOC-S2.

Methods

Subjects. Forty-one (21 Males, 20 Females) experimentally naïve Sprague-Dawley WT rats bred in-house and weighing 220-270g (females) and 330-430 (males) were used. Final group numbers were as follows: Group Paired, n =13 (6 females, 7 males); Group Paired-Extinction, n =15 (8 females, 7 males); Group Unpaired, n =13 (6 females, 7 males).

Behavioural procedures and apparatus. As described in Chapter 3 General Methods (Behavioural Apparatus).

Experimental timeline. Days 1, 2 and 4 consisted of context exposure. Day 3 and 5 consisted of fear conditioning and sensory training, respectively. On Days 6 and 7 rats remained in home cages to match the passage of time between sensory training and test with that of cell deletion experiments. Days 8 and 9 consisted of test presentations of the higher-order cue and first-order cue, respectively. For Paired-Extinction group Day 8 consisted of test to the first-order cue (i.e., extinction training) and tests to the sensory preconditioned and first-order cues followed on Days 9 and 10, respectively.

Results

Phase 1 First-Order Conditioning. Conditioned freezing to S1 increased across fear conditioning trials in all groups (Figure 4.2b). A mixed ANOVA revealed no main effect of group ($F_{(2, 35)}$=0.302, p = 0.741 η_p^2=0.016), a main effect of trial ($F_{(3, 114)}$ = 64.01, p < 0.001, η_p^2 = 0.628; Figure 4.2b) and no group x trial interaction ($F_{(6, 114)}$ = 1.402, p = 0.220, η_p^2 = 0.069).

Phase 2 Second-order Conditioning. Conditioned freezing to SOC-S2 increased across trials in the Paired groups and remained low in the Unpaired group (Figure 4.2c, solid lines). A mixed ANOVA revealed a main effect of group ($F_{(2, 38)}$=4.538, p = 0.017 η_p^2=0.193), trial ($F_{(3, 114)}$ = 31.81, p < 0.001, η_p^2 = 0.456) and group x trial interaction ($F_{(6, 114)}$ = 6.271, p < 0.001, η_p^2 = 0.248). Pairwise comparisons showed that Paired groups froze similarly ($M_{\text{Paired}} - M_{\text{Paired-EXT}}$ = -0.239, p = 0.999, 95% CI [-12.235, 12.714], d = 0.008) and higher than the Unpaired group ($M_{\text{Paired}} - M_{\text{Unpaired}}$ = 17.436, p = 0.005, 95% CI [4.524, 30.348], d = 0.648). Fear to S1 remained low across groups (Figure 4.2c, dashed lines). An identical analysis of freezing to S1 revealed no main effect

of group ($F_{(2, 38)}$ = 1.348, p = 0.272, η_p^2 = 0.066), no main effect of trial ($F_{(3, 114)}$ = 1.992, p = 0.119, η_p^2 = 0.050), nor a group x trial interaction ($F_{(6, 114)}$ = 0.508, p = 0.800, η_p^2 = 0.026).

Phase 3 S1 Extinction. Only Paired-Extinction group received non-reinforced presentations of S1, resulting in a reduction in freezing across trials (Figure 4.2d). A mixed ANOVA revealed a main effect of trial as a result of extinction training ($F_{(7, 98)}$ = 12.68, $p < 0.001$, η_p^2 = 0.475).

S2 Test. Conditioned freezing to S2 in the Paired group was higher compared to the Unpaired group, providing evidence for the SOC effect. Extinction of S1 prior to S2 test did not affect freezing to S2 (Figure 4.2e). This was confirmed by one-way ANOVA ($F_{(2, 38)}$ =29.697, $p < 0.001$, η_p^2 = 0.610). Post-hoc analyses with Bonferroni corrections showed that the Paired group froze more to S2 compared to the Unpaired group ($M_{\text{Paired}} - M_{\text{Unpaired}}$ = 28.46, $p < 0.001$, 95% CI [18.05, 38.87], d =2.65), but there was no difference between the Paired and Paired-Extinction groups ($M_{\text{Paired}} - M_{\text{Paired-EXT}}$ =2.09, p = 0.862, 95% CI [-7.70, 11.88], d = 0.179).

S1 Test. To confirm that fear conditioning and extinction were successful we tested for fear to S1 (Figure 4.2f). Freezing to S1 was higher in the two fear conditioned groups that had not undergone extinction (i.e., Paired and Unpaired) compared to Paired-Extinction group which had received S1 extinction. One-way ANOVA revealed main effect of group ($F_{(2, 38)}$ = 30.707, $p <$ 0.001, η_p^2 = 0.618). Post-hoc analyses with Bonferroni corrections confirmed that Paired-Extinction group froze less relative to the Paired and the Unpaired groups ($M_{\text{Paired}} - M_{\text{Paired-EXT}}$ =46.49, $p < 0.001$, 95% CI [30.59, 62.39], d = 3.06; $M_{\text{Unpaired}} - M_{\text{Paired-EXT}}$ =40.33, $p < 0.001$, 95% CI [24.437, 56.23], d = 2.35), while the latter two groups did not differ from each other ($M_{\text{Paired}} - M_{\text{Unpaired}}$= 6.16, p = 0.636, 95% CI [22.61, -10.30], d = 0.321).

Discussion

In this study, we examined the effect of fear reduction to S1 on fear to SOC-S2. Firstly, we confirmed that SOC-S2 elicits fear as a result of its pairings with S1 (Figure 4.2a). Indeed, the Paired group showed elevated levels of freezing to SOC-S2 on test relative to the Unpaired controls (Figure 4.2e). This comparison demonstrates SOC effect. We also investigated the dependency of fear to SOC-S2 onto fear to S1. This is achieved by extinguishing fear to S1 via presenting it in the absence of the shock (i.e., extinction training). Only the Paired-Extinction group received S1-extinction subsequent to training but prior to test. The Paired-Extinction group showed equivalent levels of fear to SOC-S2 relative to the Paired group that did not undergo extinction (Figure 4.2e).

This demonstrates that fear to SOC-S2 is independent of fear to S1. Taken together our results show that fear to SOC-S2 results from its pairings with S1 and if fear to the latter is reduced, fear to the former is unaffected.

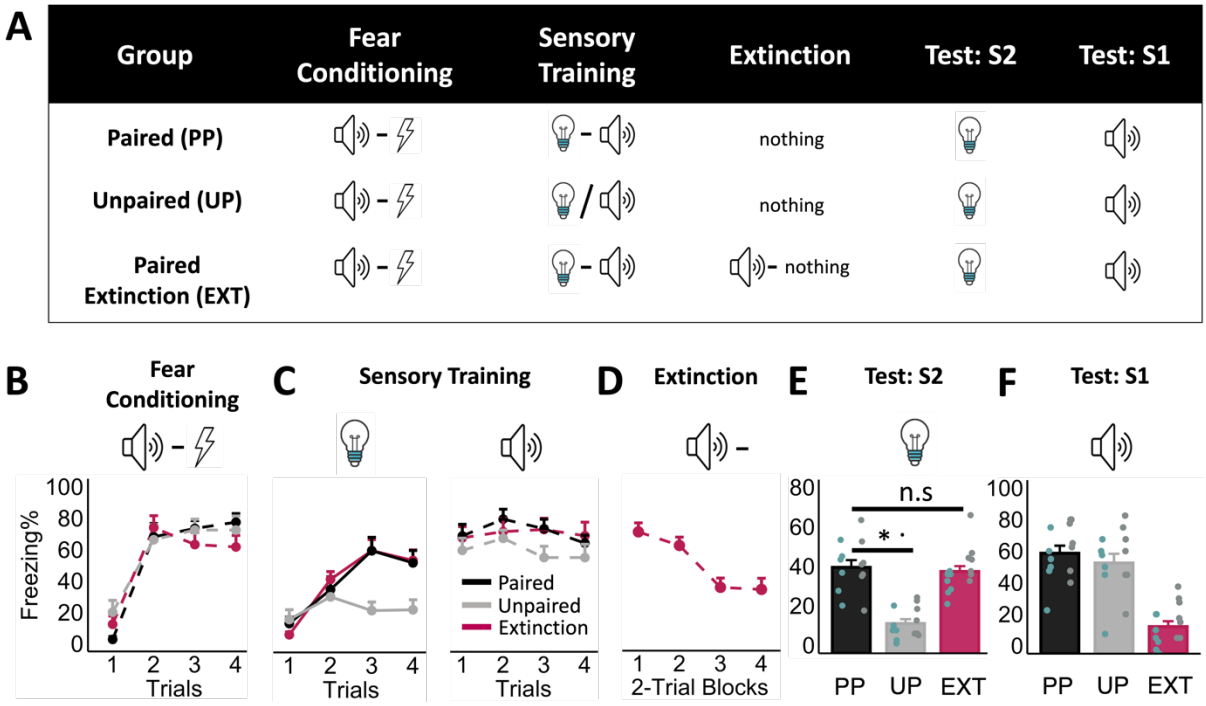

Figure 4.2. Extinction of a first-order fear conditioned stimulus (S1) has no effect on fear to a second-order conditioned stimulus (SOC-S2). (A) Behavioural protocol. (B) Acquisition of conditioned fear to S1 across trials. Paired (black) $n = 13$ (6 females, 7 males), Unpaired (grey), $n = 13$ (6 females, 7 males), Paired-Extinction (burgundy), $n = 15$ (8 females, 7 males). (C) Mean (+SEM) freezing levels to S2 (left panel) and S1 (right panel) during Phase 2. (D) Extinction of conditioned fear to S1 across trials. (E) Fear to S2 on test. Comparison between Paired and Unpaired groups evidence second-order conditioning ($p < 0.001$). Comparison between Paired and Paired-Extinction groups indicate that second-order fear is insensitive to S1 extinction ($p = 0.862$). (F) Fear to S1 on test. Comparison between Paired-Extinction and other groups indicate successful extinction of S1 ($p < 0.001$). All data are mean (+ SEM).

Experiment 4.3: Neurons activated by exposure to S1 in the basolateral amygdala regulate conditioned fear expression

In Experiment 3, we aimed to delete neurons activated by the presentation of a first-order stimulus. In a two-by-two fear discrimination design, all rats received a target cue that was paired with shock and a control cue that was presented non-reinforced (Figure 4.3b). The target group

received cell deletion following presentations of the target stimulus while the control group received cell deletion following control cue presentations. To show that the effect of successful cell deletion depends on intracranial drug but not control infusions we included Daun02 and Vehicle groups, respectively. This resulted in the following groups: Exposure-Daun02, Exposure-Vehicle and Control-Daun02, Control-Vehicle. All groups were tested to target stimulus.

Methods

Subjects. Seventy-four (34 Males, 40 Females) experimentally naïve *fos-LacZ* transgenic rats weighing 220-300g (females) and 350-450g (males) prior to surgery and bred in-house were used. Fourteen rats were excluded from statistical analyses due to incorrect placement. This yielded the following group sizes: Exposure-Daun02, n = 15 (6 females, 9 males) and Exposure-Vehicle, n = 16 (9 females, 7 males), Control-Daun02, n = 15 (9 females, 6 males) and Control-Vehicle n = 14, (7 females, 7 males).

Behavioural apparatus and procedures. See the details about stimuli and experimental chambers in Chapter 3 General Methods (Behavioural Apparatus)**.**

Experiment Timeline. On Days 1-4 rats received fear discrimination training followed by cue reactivation on Day 5 and test on Day 8. Rats were perfused 90-120 minutes after the first test trial. On Days 6 and 7 the rats remained in their home cages (no behavioural training).

Surgery and infusions. All rats are bilaterally implanted with cannulae into the BLA and infused with Daun02 as described in Chapter 3 General Methods (Surgical procedures).

Histology. Cannulae placements (Figure 4.3c) were verified post-mortem as described in Chapter 3 General methods (Histology).

Results

Phase 1 Discrimination Training. Rats learned to discriminate between the reinforced target cue and the nonreinforced control cue across training, freezing higher to target relative to control (Figure 4.3d, left panel). A mixed ANOVA revealed a main effect of cue ($F_{(3, 244)}$= 185.944, $p < 0.001$, $\eta_p^2 = 0.432$), a main effect of trial ($F_{(3, 244)}$= 48.046, $p < 0.001$, η_p^2= 0.372), and a cue x trial interaction ($F_{(3, 244)}$= 58.958, $p < 0.001$, $\eta_p^2 = 0.420$). Post-hoc comparisons with Bonferroni corrections confirmed that rats froze more to target relative to control by the end of training (Last trial block: $M_{\text{Target}} - M_{\text{Control}} = 47.42$, $p < 0.001$, 95% CI [40.29, 54.54], d = 2.37).

Phase 2 Cue reactivation. Rats showed higher levels of freezing to target relative to control cue during reactivation (Figure 4.3c, right panel). A two-way ANOVA revealed a main effect of cue ($F_{(1, 58)}$= 7.149, $p < 0.001$, η_p^2= 0.110), no main effect of drug ($F_{(1, 58)}$= 3.111, p = 0.083, η_p^2= 0.051), and no cue x drug interaction ($F_{(1, 58)}$= 0.974, p = 0.328, η_p^2= 0.017). Post-hoc analyses with Bonferroni corrections showed higher levels of fear to target cue relative to control cue ($M_{\text{Target}} - M_{\text{Control}}$ = 13.33, p = 0.009, 95% CI [3.40, 23.26], d = 0.682).

Test. Target- but not control-evoked cell deletion disrupted conditioned fear to target on test (Figure 4.3e). A two-way ANOVA revealed a main effect of reactivation ($F_{(1, 58)}$= 8.513, p = 0.005, η_p^2= 0.128), a main effect of drug ($F_{(1, 58)}$= 10.190, p = 0.002, η_p^2= 0.149) and a reactivation x drug interaction ($F_{(1, 58)}$= 5.438, p = 0.023, η_p^2= 0.086). Post-hoc analyses with Bonferroni corrections confirmed cell deletion in the target groups ($M_{\text{Target-Daun02}} - M_{\text{Target-Vehicle}}$ = -23.06, $p <$ 0.001, 95% CI [-34.13, -11.99], d =1.53) but not in the control groups ($M_{\text{Control-Daun02}} - M_{\text{Control-Vehicle}}$ = -3.59, p = 0.577, 95% CI [-16.19, -9.41], d = 0.203).

ß-galactosidase expression. Target evoked neural ensemble deletion resulted in fewer activated cells marked by ß-galactosidase expression in the Target-Daun02 group relative to Target-Vehicle (Figure 4.3f). Control groups showed higher ß-galactosidase counts on test relative to target groups, providing evidence for the specificity of Daun02 inactivation technique. A two-way ANOVA detected no main effect of reactivation ($F_{(1, 47)}$= 1.207, p = 0.278, η_p^2 = 0.025), no main effect of drug ($F_{(1, 47)}$= 1.024, p = 0.317, η_p^2 = 0.021) but a reactivation x drug interaction ($F_{(1, 47)}$= 4.428, p = 0.041, η_p^2 = 0.086). Post-hoc analyses with Bonferroni corrections confirmed that Target-Daun02 had fewer ß-galactosidase counts relative to Target-Vehicle ($M_{\text{Target-Daun02}} - M_{\text{Target-Vehicle}}$ = -22.48, p = 0.015, 95% CI [-40.13, -4.83], d =1.12) and relative to Control-Daun02 ($M_{\text{Target-Daun02}} - M_{\text{Control-Daun02}}$ = -21.75, p = 0.047, 95% CI [-43.18, -0.33], d =1.24). Control-Daun02 and Control-Vehicle had equivalent ß-galactosidase counts ($M_{\text{Vehicle}} - M_{\text{Daun02}}$ = -7.39, p = 0.520, 95% CI [-30.84, 16.06], d = 0.429).

Discussion

In this study we found that deletion of neurons in the BLA activated upon presentation of the fear eliciting target cue resulted in lower levels of freezing to that cue on test relative to controls. The lack of drug effect in the control condition show that our effect is specific to presenting the fear conditioned cue and cannot be obtained if another neutral auditory cue is

presented prior to cell deletion. These data show that the Daun02 method is effective in disrupting conditioned fear.

Here we used ß-galactosidase as a proxy for neural activity, and our data show that there are fewer ß-gal positive cells in the BLA in the fear-memory reactivated cell deletion group (i.e., Target-Daun02). This provided a neural (in addition to the behavioural) readout of the effectiveness of the Daun02 deletion method.

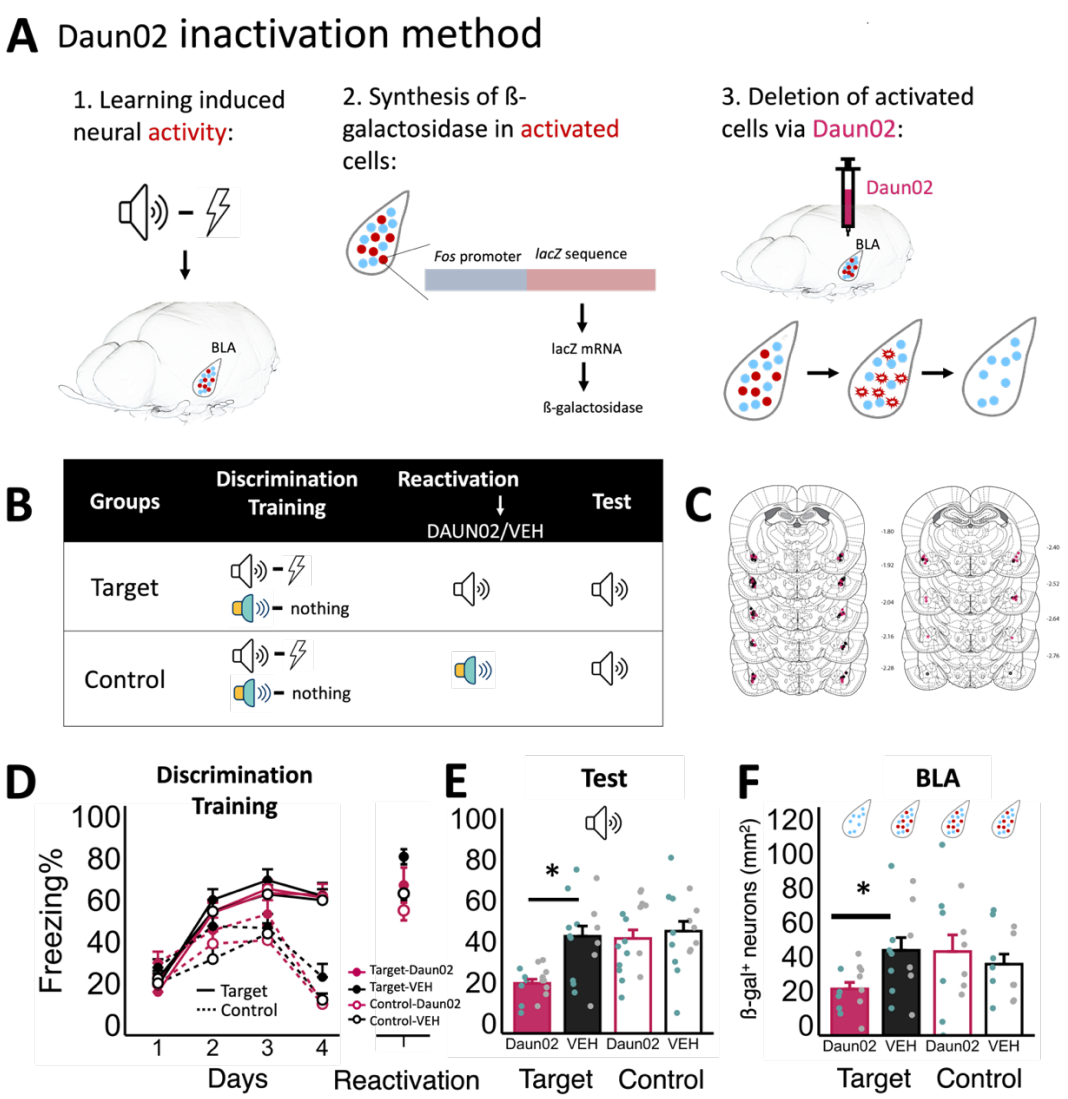

Figure 4.3. Neurons activated by exposure to target in the basolateral amygdala regulate conditioned fear expression. (A) Daun02 inactivation method (Cruz et al., 2013). *lacZ* sequence downstream to immediate early gene *c-Fos,* produces ß-galactosidase. This enzyme catalyzes Daun02 into its active daunorubicin form, which initiates cell death in the cells that were activated as a result of cue exposure. (B) Behavioural protocol. (C) Cannulae placements in the BLA, distances in millimeters from bregma. (D) Left panel: Freezing to target (solid) and control (dashed) cues during discrimination training across days. Right panel: Freezing to reactivated cue. (E) Freezing to target on test. Ensemble deletion following target but not control cue reactivation impairs target memory ($p < 0.001$). (F) ß-galactosidase counts in the BLA. Deletion following target, but not control, reactivation yields fewer ß-gal$^+$ neurons on test, confirming the specificity of Daun02 inactivation method. All data are mean (+SEM).

Experiment 4.4: Neurons in the BLA activated by exposure to S1 regulate fear to SPC-S2

In Experiment 4.4, we sought out to determine whether fear to SPC-S2 depends on fear to S1 using a neuronal disruption approach. We deleted S1-evoked cells in the BLA prior to SPC test to determine if fear expression to a SPC-S2 depends on the ability of S1 to evoke fear. Rats were trained in SPC (Paired and Unpaired groups) following which all groups were exposed to S1 and infused with Daun02 or Vehicle to delete cue-evoked neuronal ensembles or to leave them intact, respectively. This yields the following groups: Paired-Daun02, Paired-Vehicle, Unpaired-Daun02 and Unpaired-Vehicle. Lower levels of freezing to S2 in the Paired-Daun02 compared to the Paired-Vehicle would provide evidence for the dependence of SPC-S2 fear on fear to S1.

Methods

Subjects. Fifty-two (27 Males, 25 Females) experimentally naïve *fos-LacZ* transgenic rats weighing 220-300g (females) and 350-450g (males) prior to surgery and bred in-house were used. Eight rats were excluded from the statistical analyses due to incorrect placement. This yielded following group sizes: Paired-Daun02, n = 11 (6 females, 5 males) and Paired-Vehicle, n = 12 (5 females, 7 males), Unpaired-Daun02, n = 11 (6 females, 5 males) and Unpaired-Vehicle n = 10, (5 females, 5 males).

Behavioural apparatus and procedures. See the details about stimuli and experimental chambers in Chapter 3 General Methods (Behavioural Apparatus)**.**

Experiment Timeline. Days 1, 2 and 6 consisted of context exposure. Days 3 and 4 consisted of fear conditioning and sensory training, respectively. Days 6 and 9 consisted of memory reactivation and test, respectively. Rats were perfused 90-120 minutes following first test trial.

Surgery and infusions. All rats are bilaterally implanted with cannulae into the BLA and infused with Daun02 as described in Chapter 3 General Methods (Surgical procedures).

Histology. Cannulae placements (Figure 4.4b) were verified post-mortem as described in Chapter 3 General methods (Histology).

Results

Phase 1 Sensory Preconditioning. As expected, freezing was low and similar to the SPC-S2 among the four groups (Figure 4.4c, left panel). A mixed ANOVA revealed no main effect of group ($F_{(3, 40)}$= 0.434, p = 0.730, η_p^2= 0.032), a main effect of trial ($F_{(3, 120)}$= 3.289, p = 0.231, η_p^2= 0.076) and no group x trial interaction ($F_{(9, 120)}$= 0.614, p = 0.783, η_p^2= 0.044). Similarly, fear

to S1 was also low and there were no differences among the groups (Figure 4.4c, right panel). An identical analysis of freezing to S1 detected no main effect of group ($F_{(3, 40)}$= 1.049, p = 0.381, η_p^2= 0.073), no main effect of trial ($F_{(9, 120)}$= 0.676, p = 0.569, η_p^2= 0.017) and no group x trial interaction ($F_{(9, 120)}$= 0.617, p = 0.781, η_p^2= 0.044).

Phase 2 First-Order Conditioning. Conditioned freezing to S1 increased across fear conditioning trials in all groups (Figure 4.4d, left panel). A mixed ANOVA revealed no main effect of group ($F_{(3, 40)}$= 0.748, p = 0.530, η_p^2= 0.053), a main effect of trial ($F_{(2.62, 104.59)}$= 75.434, p <0.001, η_p^2= 0.654) and no group x trial interaction ($F_{(9, 120)}$= 0.867, p = 0.556, η_p^2 = 0.061).

Phase 3 Cue reactivation. Freezing to S1 was similar among the groups (Figure 4.4c, right panel). A two-way ANOVA revealed no main effect of group ($F_{(1, 40)}$= 0.453, p = 0.505, η_p^2= 0.011), no main effect of drug ($F_{(1, 40)}$= 1.859, p = 0.180, η_p^2= 0.044) and no group x drug interaction ($F_{(1, 40)}$= 0.396, p = 0.533, η_p^2 = 0.010).

S2 Test. Freezing to SPC-S2 was higher in the Paired-Vehicle relative to Unpaired-Vehicle, providing evidence for the SPC effect. Critically, SPC was disrupted as a result of S1-activated cell deletion (Figure 4.4d). A two-way ANOVA revealed a main effect of group ($F_{(1, 40)}$= 9.893, p = 0.003, η_p^2= 0.198) and a main effect of drug ($F_{(1, 40)}$= 6.160, p = 0.014, η_p^2= 0.141). The group x drug interaction approached significance ($F_{(1, 40)}$= 3.737, p = 0.060, η_p^2= 0.085). Post-hoc analyses adjusted with Bonferroni corrections confirmed that drug groups froze less relative to vehicle groups ($M_{\text{Daun02}} - M_{\text{Vehicle}}$ = -5.758, p = 0.017, 95% CI [-10.448, -1.067], d = 0.756). Post-hoc analyses adjusted with Bonferroni corrections showed that Paired-Daun02 froze less relative to Paired-Vehicle ($M_{\text{Paired-Daun02}} - M_{\text{Paired-Vehicle}}$ = -9.268, p < 0.001, 95% CI [-14.045, -4.490]), d = 1.15).

Discussion

In this experiment, we inactivated S1-evoked cells in the BLA to determine whether expression of fear to SPC-S2 relies on the ability of S1 to evoke fear. Our data showed that S1-evoked cell deletion in BLA disrupts fear to SPC-S2 providing evidence that fear to SPC-S2 relies on a neurobiologically intact S1→shock memory.

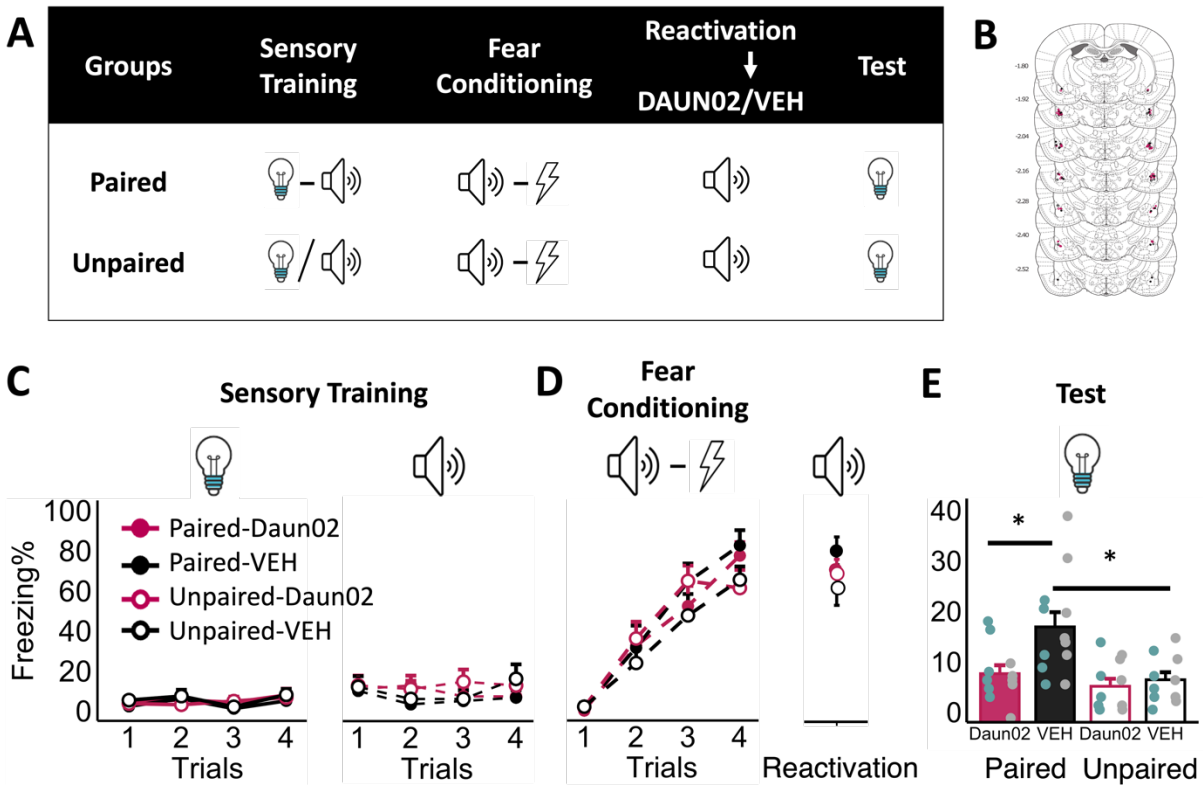

Figure 4.4. Neurons in the BLA activated by exposure to S1 regulate fear to SPC-S2. (A) Behavioural protocol. (B) Cannulae placements in the BLA, distances in millimeters from bregma. (C) Fear to S2 (solid) and S1 (dashed) during sensory training. Paired-Daun02 (filled burgundy) n = 11 (6 females, 5 males), Paired-VEH (filled black) n = 12 (5 females, 7 males), Unpaired-Daun02 (open burgundy) n = 11 (6 females, 5 males), Unpaired-VEH (open black) n = 10 (5 females, 5 males). (D) Acquisition of conditioned freezing to S1 across trials (left panel). Fear to S1 upon reactivation (right panel). (E) Freezing to S2 on Test. S1-evoked neuronal deletion following reactivation disrupts sensory preconditioning *(p = 0.017)*. All data are mean freezing percent (+SEM). Females and males are represented with green and grey dots, respectively.

Experiment 4.5: Neurons in the BLA activated by exposure to S1 do not regulate fear to SOC-S2

In Experiment 4.5, we asked a similar question to that in Experiment 4.4, with the exception that here we used SOC. That is, we deleted S1-evoked cells in the BLA prior to SOC test to determine if fear expression to SOC-S2 depends on the ability of S1 to evoke fear. To do so, rats were trained in SOC (paired and unpaired groups) following which all groups were exposed to S1 and infused with Daun02 or Vehicle to delete cue-evoked neuronal ensembles or to leave them intact, respectively. This yields the following groups: Paired-Daun02, Paired-Vehicle, Unpaired-

Daun02 and Unpaired-Vehicle. Lower levels of freezing to S2 in the Paired-Daun02 compared to the Paired-Vehicle would provide evidence for the dependence of SOC-S2 fear on fear to S1.

Methods

Subjects. Forty-six (20 Males, 26 Females) experimentally naïve fos-LacZ transgenic rats weighing 250-320g (females) and 490-600g (males) prior to surgery and bred in house were used. Six rats were excluded from the statistical analyses due to incorrect placement resulting in following group sizes: Paired-Daun02 n = 11 (6 females, 5 males), Paired-Vehicle n = 10 (5 females, 5 males), Unpaired-Daun02 n = 9 (5 females, 4 males) and Unpaired-Vehicle n = 10 (6 females, 4 males).

Behavioural apparatus and procedures. See the details about stimuli and experimental chambers in Chapter 3 General Methods (Behavioural Apparatus).

Experiment Timeline. Days 1, 2 and 6 consisted of context exposure. Days 3 and 4 consisted of fear conditioning and sensory training, respectively. Days 6 and 9 consisted of memory reactivation and test, respectively. Rats were perfused 90-120 minutes following first test trial.

Surgery and infusions. All rats are bilaterally implanted with cannulae into the BLA and infused with Daun02 as described in Chapter 3 General Methods (Surgical procedures).

Histology. Cannulae placements (Figure 4.5b) were verified post-mortem as described in Chapter 3 General methods (Histology).

Results

Phase 1 First-order Conditioning. Freezing to S1 increased during conditioning trials similarly for all groups (Figure 4.5c). A mixed ANOVA revealed no main effect of group ($F_{(3, 36)}$= 0.374, p = 0.773, η_p^2 = 0.030), a main effect of trial ($F_{(2.63, 84.11)}$= 104.664, p < 0.001, η_p^2= 0.744) and no group x trial interaction ($F_{(9, 108)}$= 0.724, p = 0.687, η_p^2= 0.057).

Phase 2 Second-order Conditioning. Freezing to S2 during conditioning trials increased in the Paired groups relative to the Unpaired groups, which remained low (Figure 4.5d, left panel). A mixed ANOVA detected a main effect of group ($F_{(3, 36)}$= 5.927, p < 0.001, η_p^2= 0.330), a main effect of trial ($F_{(2.44, 77.93)}$= 22.175, p < 0.001, η_p^2= 0.381) and a group x trial interaction ($F_{(2.44, 77.93)}$= 4.713, p < 0.001, η_p^2< 0.282). Post-hoc comparisons adjusted with Bonferroni confirmed higher levels of freezing in the Paired relative to the Unpaired groups ($M_{\text{Paired}} - M_{\text{Unpaired}}$ = 8.365, p < 0.034, 95% CI [0.627, 16.102], d = 0.238). Freezing to S1 during this phase was similar among

the groups (Figure 4.5d, middle panel). An identical analysis did not detect a main effect of group ($F_{(3, 36)}$= 0.862, p = 0.470, η_p^2= 0.067), a main effect of trial ($F_{(3, 108)}$= 0.250, p = 0.860, η_p^2= 0.007), nor a group x trial interaction ($F_{(9, 108)}$= 0.917, p = 0.513, η_p^2= 0.071).

Phase 3 Cue reactivation. Freezing to S1 was similar among the groups during cue reactivation (Figure 4.5d, right panel). A two-way ANOVA detected no main effect of group ($F_{(1, 36)}$= 4.068, p = 0.051, η_p^2= 0.101), no main effect of drug ($F_{(1, 36)}$= 0.680, p = 0.415, η_p^2= 0.019), and no group x drug interaction ($F_{(1, 36)}$= 0.680, p = 0.415, η_p^2= 0.019).

S2 Test. Conditioned freezing to SOC-S2 was higher in the Paired-Vehicle relative to Unpaired-Vehicle, providing evidence for the SOC effect. Freezing to SOC-S2 was not affected by S1-activated neuronal ensemble deletion (Figure 4.5e). A two-way ANOVA revealed a main effect of group ($F_{(1, 36)}$ = 19.271, p < 0.001, η_p^2= 0.349), no main effect of drug ($F_{(1, 36)}$= 0.598, p = 0.444, η_p^2= 0.016) and no group x drug interaction ($F_{(1, 36)}$= 0.614, p = 0.582, η_p^2= 0.009). Pairwise comparisons adjusted with Bonferroni confirmed higher freezing in the Paired groups relative to the Unpaired ($M_{\text{Paired}} - M_{\text{Unpaired}}$ = 13.714, p < 0.001, 95% CI [7.432, 19.995], d = 1.40).

Discussion

In this experiment, we inactivated S1-evoked cells in the BLA to determine whether fear expression to SOC-S2 relies on the ability of S1 to evoke fear. We obtained SOC effect and no effect of drug, providing evidence that the expression of second-order fear does not depend on the integrity of S1→shock memory.

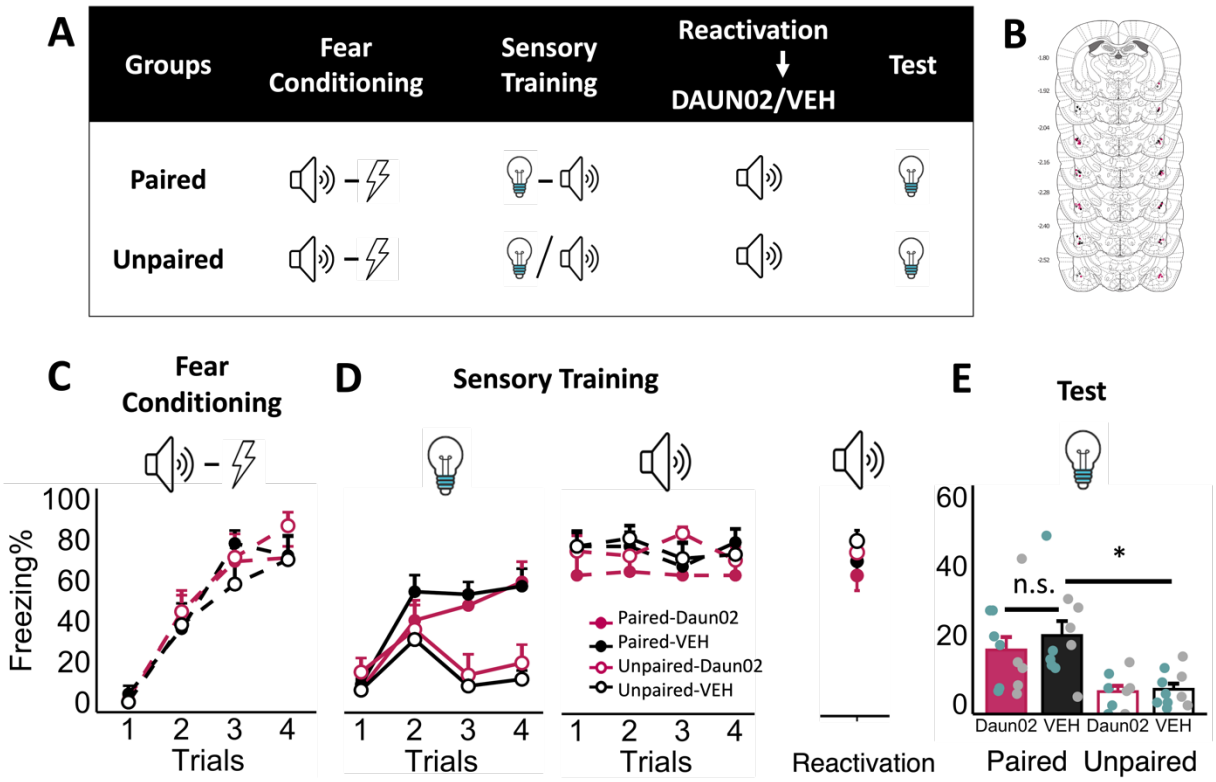

Figure 4.5. Neurons in the BLA activated by exposure to S1 do not regulate fear to SOC-S2. (A) Behavioural protocol. (B) Cannulae placements in the BLA, distances in millimeters from bregma. (C) Acquisition of conditioned freezing to S1. Paired-Daun02 (filled burgundy) n = 11 (6 females, 5 males), Paired-VEH (filled black) n = 10 (5 females, 5 males), Unpaired-Daun02 (open burgundy) n = 9 (5 females, 4 males), Unpaired-VEH (open black) n = 10 (6 females, 4 males). (D) Freezing to SOC-S2 (solid) and S1 (dashed) during sensory training. (D) Acquisition of conditioned freezing to S1 across trials (left panel). Fear to S1 upon reactivation (right panel). (E) Freezing to S2 on Test. S1-evoked neuronal deletion following reactivation disrupts sensory preconditioning *(p = 0.017)*. All data are mean freezing percent (+SEM). Females and males are represented with green and grey dots, respectively.

General Discussion

This chapter investigated the dissociable reliance of higher-order memories on fear to a first-order stimulus behaviourally and neurobiologically. We demonstrated that extinguishing fear to first-order S1 subsequent to training but prior to test leads to a disruption in sensory preconditioning (Experiment 4.1, Figure 4.1d) and leaves second-order conditioning intact (Experiment 4.2, Figure 4.2e). Similarly, when S1-evoked neurons in the BLA were inactivated we showed a disruption in SPC (Experiment 4.4, Figure 4.4d) but not in SOC (Experiment 4.5, Figure 4.5e). These findings replicate Rizley and Rescorla (1972). Importantly and unlike Rizley and Rescorla (1972), in our designs identical training parameters such as number of training trials,

identical training sessions and shock intensity are used, and fear is assessed using freezing as a measure of conditioned responding for both sensory preconditioning (SPC) and second-order conditioning (SOC). The only difference between the two protocols was the order in which first-order conditioning and sensory training took place.

Extinction is an effective technique to investigate the reliance of SPC and SOC type fears onto fear to S1. However, the original S1→shock memory is not unlearned following extinction. (Bouton, 2002). Rather, a new S1→no_shock association is learned, and it interferes with the original memory. Therefore, it is possible for the original S1→shock memory to be restored as a result of passage of time, exposure to shock or exposure to training chamber (Bouton, 2002). Considering these restorative effects, we used a more targeted approach (i.e., Daun02 inactivation method and the *fos-LacZ* rats) that directly *deletes* S1→shock memory. We chose the BLA to target S1→shock memory, as it's shown that a subset of neurons in the BLA are recruited during fear conditioning (Han et al., 2007; Rashid et al., 2016). We showed a reduction in fear to target when target (but not control) cue evoked ensemble was deleted (Experiment 4.3, Figure 4.3e). Our ß-galactosidase counts confirmed cue specific ensemble deletion. There were fewer cells activated on test in the Target-Daun02 relative to controls. This technique enabled us to investigate the reliance of higher-order fear onto S1 evoked neuronal activity in the BLA. Deleting S1-evoked ensembles in the BLA disrupted expression of fear to SPC-S2 but not to SOC-S2 providing further evidence that the former but not the latter depends on intact S1→shock memory.

These findings replicate Rizley and Rescorla (1972) in terms of underlying associative structure supporting SPC and SOC. In both protocols, fear elicited by S2 is contingent on its pairings with S1 as revealed by the comparison between the Paired and the Unpaired groups. However, while expression of fear to SPC-S2 requires the integrity of S1→shock (Experiments 4.1 and 4.4) expression of fear to SOC-S2 does not (Experiments 4.2 and 4.5). These findings provide evidence for the distinct associations that support SPC and SOC effects. As discussed in Chapter 1, SPC is driven by the S-S associations that form between SPC-S2 and S1. On test, SPC-S2 evokes a representation of S1 by virtue of its associations, which in turn leads to the expectation of the shock. Extinction or deletion of S1→shock memory interferes with or blocks the expectation of the shock and results in a decrease in SPC-S2-elicited conditioned fear on test. In contrast to SPC, S-S associations are not the dominant associations that drive SOC effect. Despite identical sensory training that occurs in SPC and SOC one crucial difference defines how behaviour is

governed by different associations in these protocols. That is, in SOC, S2 is paired with S1 *after* it has been fear conditioned while in SPC, this pairing occurs when the stimuli are neutral. Therefore, SOC-S2 becomes associated with the fear elicited by S1 (i.e., S2→fear_state) and subsequent extinction of S1-evoked fear has no effect on the expression of fear to SOC-S2.

The parallels between our behavioural and neurobiological data give insights about how SPC and SOC type memories are represented in the BLA. For example, the disruption obtained in SPC following S1-ensemble deletion suggest an overlap between SPC-S2- and S1-ensembles in the BLA and that the overlapping subset is crucial in driving behaviour. In contrast, expression of fear to SOC-S2 is not dependent on S1-ensemble (Experiment 4.5).

In conclusion, Chapter 4 replicated the behavioural dissociation between two forms of higher-order conditioning that was originally demonstrated by Rizley and Rescorla (1972). Importantly and unlike Rizley and Rescorla (1972), in our design we used exactly the same parameters for both sensory preconditioning and second-order conditioning, and assessed the influence of S1-extinction on the retrieval of these memories in an identical manner. To investigate the reliance of higher-order memories onto S1→shock memory we used the more targeted Daun02 inactivation method. Deletion of BLA S1-ensemble disrupted SPC but spared SOC. Our findings provide neurobiological evidence on the dissociation of SPC and SOC.

Chapter 5. The Lateral Orbitofrontal Cortex regulates higher order conditioning in males but not in females

As discussed in Chapter 1 and demonstrated in Chapter 4, sensory preconditioning *and* second-order conditioning differentially rely on the S2→S1 association for expression of fear to S2. This associative distinction suggests differential neural processing underlying higher-order memories (Parkes & Westbrook, 2010; Holmes et al., 2013). The lateral orbitofrontal cortex (lOFC) emerges as a potential candidate based on its reciprocal anatomical projections to BLA (Carmichael & Price, 1995b; Ghashghaei & Barbas, 2002) and PRh (Hwang et al., 2018), two regions important for higher order conditioning. In addition, lOFC has been directly implicated in learning and expression of *appetitive* SPC (see below). Therefore in this chapter, I examined whether lOFC function is critical for expression of higher-order fear in *aversive* SPC and SOC.

The lateral OFC is mostly studied in appetitive paradigms investigating decision making, credit assignment, economic choice, reward expectation and is rather overlooked in the aversive domain (but see Lay et al., 2020; Morrison & Salzman, 2011). Work investigating lOFC function within the appetitive domain led to theories that restrict its function to keeping track of the value of stimuli (Padoa-Schioppa, 2011; Levy & Glimcher, 2012; Padoa-Schioppa & Conen, 2017). Work that investigates lOFC function in neutral valence situations such as sensory preconditioning has expanded our understanding beyond such value-based framework. Sensory preconditioning requires learning the relationship between two stimuli that do not signal any biologically or motivationally significant outcomes. Research from the Schoenbaum lab showed that neural activity in the lOFC can dissociate the two neutral cues that are paired during Phase 1 of sensory preconditioning, prior to conditioning one of the cues with a reward US (Sadacca et al., 2018). These data showed that value is not necessary for neural activity in the lOFC to keep track of paired associates. Silencing the lateral OFC during S2→S1 pairings (Hart et al., 2020) or prior to test (Jones et al., 2012) disrupted appetitive SPC. Indeed, a variety of tasks that require activity in lOFC highlight its role in keeping track of S-S associations (Pickens et al., 2003; McDannald et al., 2005; Ostlund & Balleine, 2007; McDannald et al., 2014).

Given that the lOFC is involved in learning to associate two neutral events (see above) albeit in the appetitive domain, then there is no reason why the lOFC would not be involved in the same learning in the aversive domain. Therefore, I aimed to determine whether the lOFC is involved in *aversive* sensory preconditioning. If the role of the lOFC is to keep track of S-S associations and use those flexibly, then given that SPC relies on such association (Experiment 4.1), it is expected that inactivation of the lOFC prior to test would disrupt the SPC effect. I extended this investigation of the lOFC in higher order conditioning by also studying its role in aversive SOC. As SOC does not rely on S2→S1 associations for expression of fear to S2 on test, inactivation of the lOFC prior to test should leave SOC intact. I examined the role of the lOFC in SPC and SOC in both male and female rats in separate experiments.

Experiment 5.1: Pharmacological inactivation of lOFC disrupts fear expression to SPC-S2 in male rats

In Experiment 5.1, we aimed to investigate the role of lOFC in expression of fear to a sensory preconditioned stimulus. To do this, we used an identical design to that used in Chapter 4 which included two behavioural conditions: Paired (S2→S1) and Unpaired (S2/S1; Figure 5.1a). A muscimol/baclofen (M/B) mixture ($GABA_{A/B}$ receptor agonist) was used to silence the lOFC bilaterally during the expression test. This yields the following groups: Paired-Drug, Paired-Vehicle, Unpaired-Drug and Unpaired-Vehicle. Comparisons between Paired-Vehicle and Unpaired-Vehicle reflect our behavioural effect, while comparison between Paired-Drug and Paired-Vehicle indicate the effect of lOFC inactivation.

Methods

Subjects. Sixty-four experimentally naïve male Sprague-Dawley WT rats (Envigo Ltd. Indianapolis, USA) were used. The rats weighed 265-340g prior to surgery. Eleven rats were excluded due to incorrect placements yielding the following group sizes: Paired-Drug (n = 14), Paired-Vehicle (n = 13), Unpaired-Drug (n = 12) and Unpaired-Vehicle (n = 14).

Behavioural apparatus and procedures. See the details about stimuli, experimental chambers and procedures in Chapter 3 General Methods (Behavioural Apparatus).

Experiment timeline. Behavioural protocol was the same as the timeline in Experiment 4.1, however, the rats were fear conditioned 24h after sensory training.

Surgery and infusions. All rats were implanted with bilateral cannulae into the lOFC and infused with M/B or vehicle as described in Chapter 3 General Methods (Surgical procedures).

Histology. Cannulae placements (Figure 5.1b) were verified post-mortem as described in Chapter 3 General Methods (Histology).

Results

Phase 1 Sensory Preconditioning. As expected, the average freezing to the neutral S2 and S1 was low and similar among the groups (Figure 5.1C, left panel). A mixed ANOVA for freezing to S2 revealed no main effect of group ($F_{(3, 49)}$= 0.940, p = 0.429, η_p^2= 0.055), a main effect of trial ($F_{(2.65, 129.54)}$= 2.736, p = 0.046, η_p^2= 0.053) and no group x trial interaction ($F_{(7.93, 129.54)}$= 2.736, p = 0.046, η_p^2= 0.053). An identical analysis for freezing to S1 detected no main effect of group ($F_{(3, 49)}$= 0.645, p = 0.590, η_p^2= 0.038), no main effect of trial ($F_{(3, 147)}$= 1.440, p = 0.234,

η_p^2= 0.029) and a group x trial interaction ($F_{(9, 147)}$= 2.188, p = 0.026, η_p^2= 0.118). As seen in Figure 5.1C (right panel) Paired-Drug showed a slightly elevated level of freezing to S1 on Trial 3 relative to the other groups. Importantly there was no difference between Paired-Drug and Paired-Vehicle ($M_{\text{Paired-Drug}} - M_{\text{Paired-Veh}}$ = 1.32, p = 0.93, 95% CI [-4.40, 7.03], d = 0.106) nor between Paired-Vehicle and Unpaired-Vehicle ($M_{\text{Paired-Vehicle}} - M_{\text{Unpaired-Veh}}$ = 1.90, p = 0.83, 95% CI [-3.82, 7.61], d = 0.169; Figure 5.1c).

Phase 2 First-Order Conditioning. Conditioned freezing to S1 increased across trials for all four groups (Figure 5.1D, right panel). A mixed ANOVA revealed no main effect of group ($F_{(3, 49)}$= 0.161, p = 0.922, η_p^2= 0.010), a main effect of trial ($F_{(2.62, 128.12)}$= 272.135, p < 0.001, η_p^2= 0.847) and no group x trial interaction ($F_{(7.84, 128.12)}$= 0.613, p = 0.785, η_p^2= 0.036).

S2 Test. Freezing to S2 was higher in the Paired group with a functional lOFC on test (i.e., Paired-Vehicle) relative to the Unpaired groups, providing evidence for the SPC effect (Figure 5.1E). Inactivation of the lOFC prior to S2 test, however, disrupted the SPC effect (Figure 5.1E). A two-way ANOVA revealed a main effect of group which approached significance ($F_{(1,49)}$ = 3.133, p = 0.083, η_p^2= 0.060), no main effect of drug ($F_{(1, 49)}$ = 1.599, p = 0.212, η_p^2= 0.032) and a group x drug interaction ($F_{(1, 49)}$ = 6.382, p = 0.014, η_p^2= 0.117). Post-hoc comparisons adjusted with Bonferroni showed that Paired-Drug group froze less compared to the Paired-Vehicle group confirming SPC disruption ($M_{\text{Paired-Drug}} - M_{\text{Paired-Vehicle}}$ = -8.526, p = 0.032, 95% CI [-16.10, -0.55], d = 0.954). Paired-Vehicle froze more relative to Unpaired-Vehicle evidencing SPC effect ($M_{\text{Paired-Vehicle}} - M_{\text{Unpaired-Vehicle}}$ = 9.66, p = 0.012, 95% CI [1.68, 17.63], d = 1.08).

S1 Test. Conditioned freezing to S1 was similar among all four groups on drug-free test (Figure 5.1F). A two-way ANOVA detected no main effect of group ($F_{(1, 49)}$=0.012, p = 0.914, η_p^2 < 0.001), no main effect of drug ($F_{(1, 49)}$=0.445, p = 0.508, η_p^2 =0.001), nor group x drug interaction ($F_{(1, 49)}$= 0.027, p = 0.641, η_p^2 < 0.001).

Discussion

In this study we investigated the effect of pharmacological lOFC inactivation on expression of fear to SPC-S2. Inactivating the lOFC disrupted fear to SPC-S2 (Figure 5.1E). Our results revealed a novel role for the lOFC in aversive SPC, showing that a functional lOFC is required for the expression of fear to SPC-S2 stimulus.

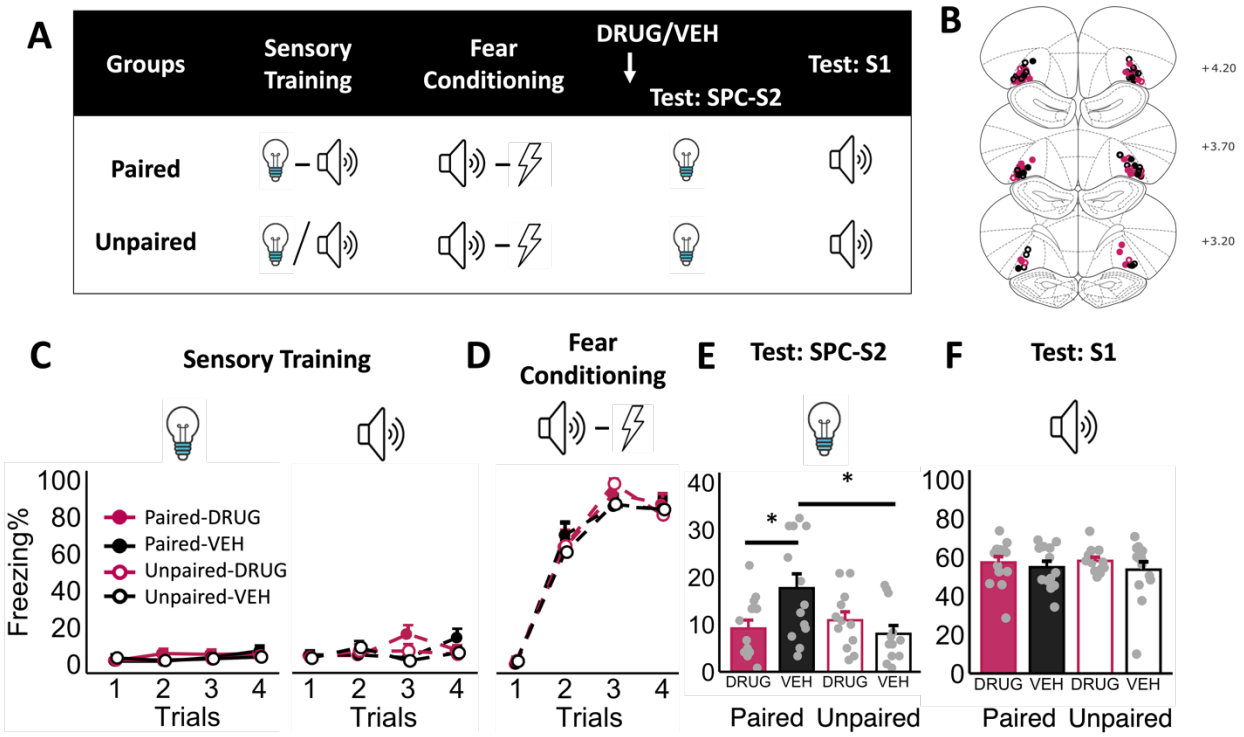

Figure 5.1. Pharmacological inactivation of lOFC disrupts fear expression to SPC-S2 in male rats. (A) Behavioural protocol. (B) Cannulae placements in lOFC, distances from Bregma in mm. (C) Freezing to SPC-S2 (solid lines, left panel) and S1 (dashed lines, right panel) during sensory training (D) Acquisition of conditioned freezing to S1. (E) Freezing to SPC-S2 on test. Inactivation of lOFC disrupts SPC (p = 0.02). (E) Freezing to S1 on test. All data are mean freezing percent + SEM.

Experiment 5.2: Pharmacological inactivation of lOFC enhances fear to SOC-S2 in male rats

Experiment 5.1 revealed a role for the lOFC in the expression of fear to a sensory preconditioned cue. Here, I sought out to determine if the lOFC is also involved in the expression of higher order fear in second order conditioning. We included Paired (S2→S1) and Unpaired (S2/S1; Figure 5.2A) groups to assess our behavioural effect, and drug and vehicle groups to assess the effect of pharmacological lOFC inactivation during the expression test. This resulted in Paired-Drug, Paired-Vehicle, Unpaired-Drug and Unpaired-Vehicle groups. Comparisons between Paired-Vehicle and Unpaired-Vehicle reflect our behavioural effect, while the comparison between Paired-Drug and Paired-Vehicle demonstrate the effect of pharmacological inactivation.

Methods

Subjects. Fifty-six experimentally naïve male Sprague-Dawley WT rats (Envigo Ltd. Indianapolis, USA) weighing 320-370g prior to surgeries were used in this experiment. Fourteen rats were excluded due to incorrect placements resulting in the following group sizes: Paired-Drug (n = 9), Paired-Vehicle (n = 13) groups as experimental groups and Unpaired-Drug (n = 10) and Unpaired-Vehicle (n = 10).

Behavioural apparatus and procedures. See the details about stimuli, experimental chambers and procedures in Chapter 3 General Methods (Behavioural Apparatus).

Experiment timeline. Behavioural protocol was the same as the timeline in Experiment 4.2, however, rats were tested 24h after sensory training.

Surgery and infusions. All rats were implanted with bilateral cannulae into the lOFC and infused with M/B or vehicle as described in Chapter 3 General Methods (Surgical procedures).

Histology. Cannulae placements (Figure 5.1b) were verified post-mortem as described in Chapter 3 General Methods (Histology).

Results

Phase 1 First-Order Conditioning. Conditioned freezing to S1 increased across trials similarly for all four groups (Figure 5.2D). A mixed ANOVA revealed no main effect of group ($F_{(3, 38)}$ = 0.990, p = 0.408, η_p^2 = 0.073), a main effect of trial ($F_{(3, 114)}$ = 212.246, p < 0.408, η_p^2 = 0.848) and no group x trial interaction ($F_{(9, 114)}$ = 0.383, p = 0.942, η_p^2 = 0.029).

Phase 2 Second-Order Conditioning. Conditioned freezing to SOC-S2 increased across trials in the Paired groups but not in the Unpaired (Figure 5.2D, left panel). A mixed ANOVA revealed a main effect of group ($F_{(3, 38)}$ = 6.154, p = 0.002, η_p^2 = 0.327), trial ($F_{(3, 114)}$ = 17.047, p < 0.001, η_p^2 = 0.310) and a group x trial interaction ($F_{(9, 114)}$ = 4.374, p < 0.001, η_p^2 = 0.257). Post-hoc comparisons adjusted with Bonferroni confirmed that Paired groups increased freezing to SOC-S2 relative to the Unpaired ($M_{\text{Paired}} - M_{\text{Unpaired}}$ = 14.92, p < 0.001, 95% CI [9.46, 20.37], d = 0.834). Fear to S1 remained mostly high across trials (Figure 5.2D, right panel). An identical analysis detected no main effect of group ($F_{(3, 38)}$ = 0.471, p = 0.704, η_p^2 = 0.036), a main effect of trial ($F_{(2.68, 102.02)}$ = 3.014, p = 0.039, η_p^2 = 0.074) indicating some extinction of the fear response, and no group x trial interaction ($F_{(8.06, 102.2)}$ = 1.101, p = 0.369, η_p^2 = 0.070).

S2 Test. Conditioned freezing to S2 on test revealed that pharmacological inactivation of lOFC enhances second-order fear. That is, fear to S2 rats without a functional lOFC.(Figure 5.2E). A two-way ANOVA revealed a main effect of group ($F_{(1, 38)} = 124.437$, $p < 0.001$, $\eta_p^2 = 0.766$), a main effect of drug ($F_{(1, 38)} = 0.162$, $p < 0.001$, $\eta_p^2 = 0.219$), and a group x drug interaction ($F_{(1, 38)} = 12.354$ $p < 0.001$, $\eta_p^2 = 0.246$). Pairwise comparisons adjusted with Bonferroni correction confirmed that freezing was higher in the Paired-Drug relative to Paired-Vehicle ($M_{\text{Paired-Drug}} - M_{\text{Paired-Vehicle}} = 18.28$, $p < 0.001$, 95% CI [8.84, 27.72], $d = 1.75$). Freezing in the Paired-Vehicle group was higher than the Unpaired-Vehicle evidencing SOC effect ($M_{\text{Paired-Vehicle}} - M_{\text{Unpaired-Vehicle}} = -0.667$, $p = 0.809$, 95% CI [-6.39, 5.05], $d = 2.19$).

S1 Test. Fear to S1 on test was similar among all four groups (Figure 5.2F). A two-way ANOVA detected no main effect of group ($F_{(1, 38)} = 0.472$, $p = 0.496$, $\eta_p^2 = 0.012$), no main effect of drug ($F_{(1, 38)} = 0.001$, $p = 0.974$, $\eta_p^2 < 0.001$) nor a group x drug interaction ($F_{(1, 38)} = 3.919$, $p = 0.055$, $\eta_p^2 = 0.093$).

Discussion

In this study, we examined the role of the lOFC in the expression of fear to S2 in SOC. Our data showed that inactivating the lOFC enhanced the SOC effect. This is striking given that identical pharmacological treatment of the lOFC led to a disruption in fear expression to a sensory preconditioned S2. This suggests that the lOFC is no involved in fear expression per se, or inactivation of the lOFC prior to either test (SPC or SOC) would yield the same, not opposite, effect.

As mentioned earlier, the lOFC has been implicated in S-S learning. Our data implicate such learning in SPC and not SOC in so far as S2 and S1 are considered. S-S learning, however, may take place during SOC in the form of S2-no_shock. During S2→S1 pairings in SOC, the expected US is not delivered upon presentation of S1. Therefore, while S2 can becomes associated with a state of fear evoked by S1, it can also become associated with the absence of the (expected) shock. In other words, the behavioural expression of fear to SOC-S2 (as observed in the Paired-Vehicle group) is likely due to two opposing associations: S2→fear_state and S2→no_shock. Inactivation of the lOFC prior to test is likely to disrupt the influence of the S-S association over behaviour. That is, removing the fear-inhibiting effect of the S2→no_shock association, resulting

in a facilitation of fear to a second-order conditioned S2. This is further tested in the next study where the opportunity for learning the S2-no_shock associations is eliminated.

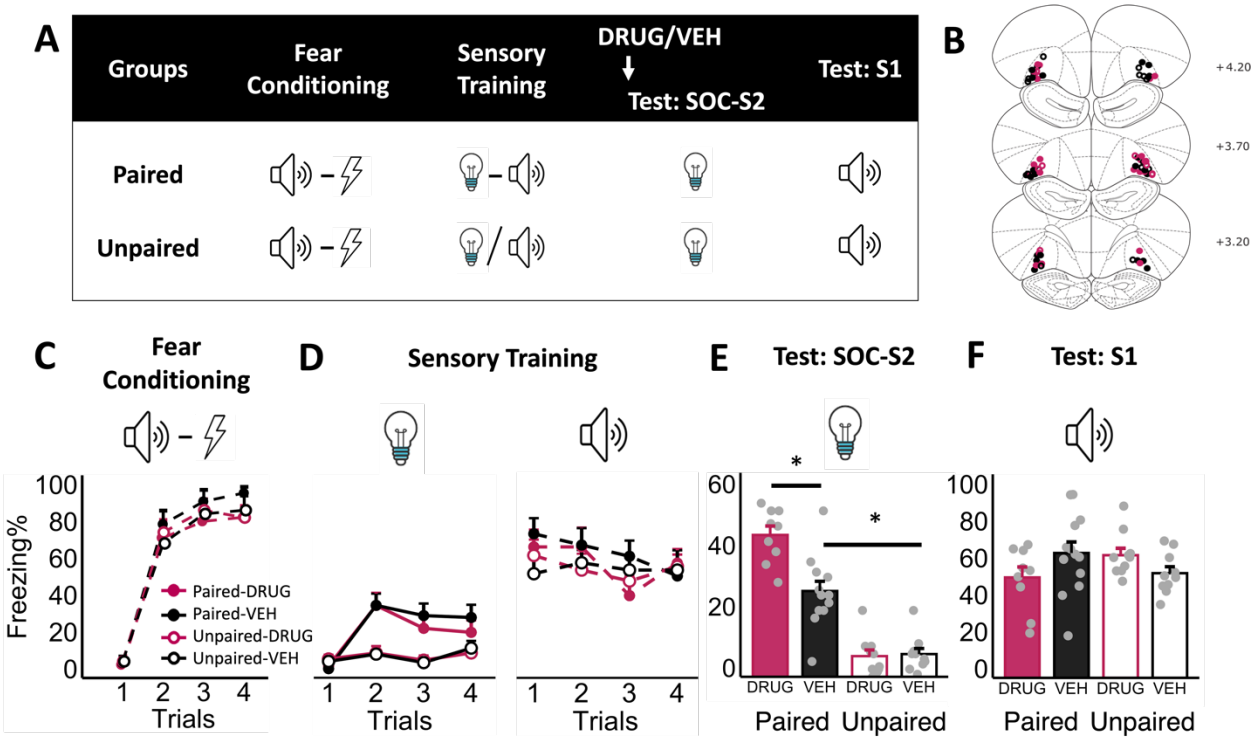

Figure 5.2. Pharmacological inactivation of lOFC enhances fear to SOC-S2 in males (A) Behavioural protocol. (B) Cannulae placements in lOFC, distances from Bregma in mm. (C) Acquisition of conditioned freezing to S1. Paired-DRUG (filled burgundy) $n = 9$, Paired-VEH (filled black) $n = 13$, Unpaired-DRUG (open burgundy) $n = 10$, Unpaired-VEH (open black) $n = 10$. (D) Conditioned freezing to SOC-S2 (solid lines, left panel) and S1 (dashed lines, right panel). (E) Freezing to SOC-S2 on test. Inactivation of lOFC enhances fear to SOC-S2 ($p < 0.001$). (E) Freezing to S1 on test. All data are mean freezing percent + SEM.

Experiment 5.3: Pharmacological inactivation of lOFC has no effect on fear to S2 when second-order conditioning occurred in the presence of shock

In this experiment we aimed to test the hypothesis that the lOFC facilitated fear to SOC-S2 by removing the influence of an S2-no_shock association. To test this hypothesis, we conducted an experiment in which we eliminated the opportunity for a S2-no_shock association to be formed. We did this by delivering the expected shock during Phase 2 of the SOC protocol (i.e., S2→S1→shock). Here, inactivation of the lOFC during S2 test should have no effect over behaviour as S2→no_shock association would not have developed during Phase 2 training. We used a two-group design in which all rats were trained in SOC in the presence of shock and

received either drug or vehicle infusions into the lOFC prior to test. This resulted in Paired-Drug and Paired-Vehicle groups. Comparison between these groups reveal the effect of lOFC inactivation at test in a reinforced second-order conditioning task.

Methods

Subjects. Nineteen (10 males, 9 females) experimentally naïve Sprague-Dawley WT rats (Envigo Ltd. Indianapolis, USA) weighing 253-296 (females) and 310-350 (males) prior to surgery. Group sizes were as follows: Paired-Drug (n = 10), Paired-Vehicle (n= 9).

Behavioural apparatus and procedures. See the details about stimuli and experimental chambers in Chapter 3 – General Methods (Behavioural Apparatus).

Experiment timeline. Behavioural protocol was identical to the timeline in Experiment 5.2 with the exception that Phase 2 consisted of reinforced S2→S1 pairings (i.e., S2→S1→US).

Surgery and infusions. All rats were implanted with bilateral cannulae into the lOFC and infused with M/B or vehicle as described in Chapter 3 General Methods (Surgical procedures).

Histology. Cannulae placements (Figure 5.3B) were verified post-mortem as described in Chapter 3 General Methods (Histology).

Results

Phase 1 First-Order Conditioning. Conditioned freezing to S1 increased across trials similarly for both groups (Figure 5.3C). A mixed ANOVA revealed no main effect of drug ($F_{(1, 17)}$ = 0.012, p = 0.917, η_p^2 < 0.001), a main effect of trial ($F_{(3, 51)}$ = 22.778, p < 0.001, η_p^2 = 0.496) and no drug x trial interaction ($F_{(3, 51)}$ = 0.070, p = 0.976, η_p^2 = 0.004).

Phase 2 Reinforced Second-Order Conditioning. Conditioned freezing to S2 increased across trials similarly for both groups (Figure 5.3D, left panel). A mixed ANOVA revealed no main effect of drug ($F_{(1, 17)}$ = 0.012, p = 0.917, η_p^2 < 0.001), a main effect of trial ($F_{(3, 51)}$ = 16.693, p < 0.001, η_p^2 = 0.496), and no drug x trial interaction ($F_{(3, 51)}$ = 2.203, p = 0.010, η_p^2 = 0.115). Freezing to S1 was similar among the groups (Figure 5.3D, right panel). A similar analysis for freezing to S1 detected no main effect of drug ($F_{(1, 17)}$ = 0.003, p = 0.958, η_p^2 < 0.001), a main effect of trial ($F_{(3, 51)}$ = 8.573, p < 0.001, η_p^2 = 0.335) and no drug x trial interaction ($F_{(3, 51)}$ = 0.262, p = 0.853, η_p^2 = 0.015).

S2 Test. Rats in the two groups froze similarly to S2 on test providing evidence that lOFC inactivation has no effect on fear expression to S2 in reinforced SOC protocol (Figure 5.3E). A t-test revealed no main effect of drug ($t_{(17)}$ = 0.108, p = 0.92, 95% CI [-26.64, 29.51], d = 0.05).

S1 Test. Fear to S1 was similar among the two groups on test (Figure 5.3F). A t-test revealed no main effect of drug ($t_{(17)}$ = 0.181, p = 0.85, 95% CI [-23.98, 28.48], d = 0.083).

Discussion

Using a reinforced SOC design which eliminated the development of a S2→no_shock association, the data show that the lOFC is not necessary for the expression of second-order fear in the reinforced design. Again, the lack of disruptive effect of lOFC inactivation in this reinforced SOC design shows that the lOFC is not involved in fear expression per se. Rather, it likely supports S2→no_shock learning in the classic SOC.

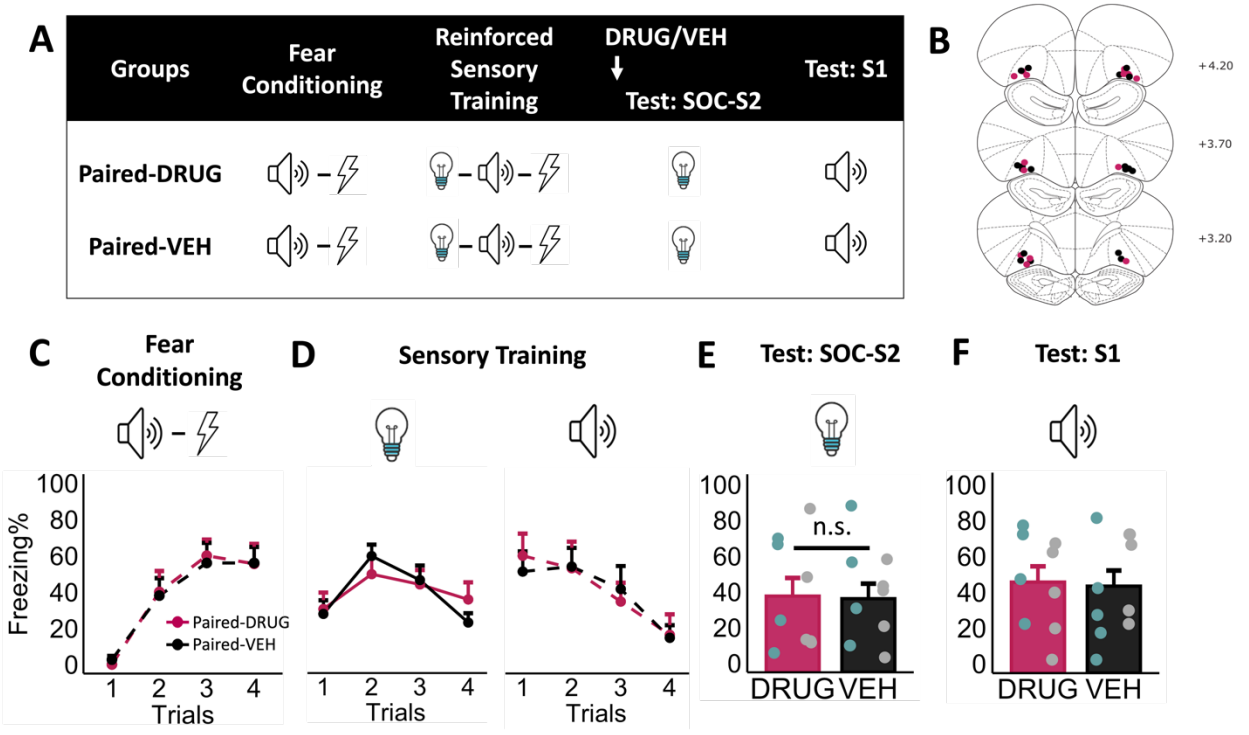

Figure 5.3. Pharmacological inactivation of lOFC has no effect on fear to S2 when second-order conditioning occurred in the presence of shock. (A) Behavioural protocol. (B) Cannulae placements in lOFC, distances from Bregma in mm. (C) Acquisition of conditioned freezing to S1. Paired-DRUG (filled burgundy) n = 9 (4 females, 5 males), Paired-VEH (filled black) n = 10 (5 females, 5 males), (D) Conditioned freezing to SOC-S2 (solid lines, left panel) and S1 (dashed lines, right panel). (E) Freezing to SOC-S2 on test. Inactivation of lOFC has no effect on freezing to SOC-S2 in reinforced second-order conditioning (p = 0.92). (E) Freezing to S1 on test. All data are mean freezing percent + SEM. Females and males are represented with green and grey dots, respectively.

Experiment 5.4a: Pharmacological inactivation of lOFC has no effect on fear to SPC-S2 in female rats

Experiment 5.1 revealed a role for the lOFC in sensory preconditioned fear in males. The aim of the current experiment was to determine if the lOFC serves a similar role in females. We used a 2x2 design in which one factor was the pairing between S2 and S1 (Paired and Unpaired groups) and the other was the drug infusion (Drug and Vehicle groups), yielding the following four groups; Paired-Drug, Paired-Vehicle, Unpaired-Drug and Unpaired-Vehicle. Comparison between Paired-Vehicle and Unpaired-Vehicle reflect the behavioural effect while the comparison between Paired-Drug and Paired-Vehicle show the effect of lOFC inactivation.

Methods

Subjects. Fifty-five experimentally naïve female Sprague-Dawley WT rats (Envigo Ltd. Indianapolis, USA) weighing 250-305g prior to surgeries were used in sensory preconditioning portion of the experiment. Six rats were excluded due to incorrect placements yielding the following group sizes: Paired-Drug (n = 12), Paired-Vehicle (n = 14) groups as experimental groups and Unpaired-Drug (n = 11) and Unpaired-Vehicle (n = 12).

Behavioural apparatus and procedures. See the details about stimuli and experimental chambers in Chapter 3 – General Methods (Behavioural Apparatus).

Experiment timeline. Training was the same as the timeline in Experiment 5.1.

Surgery and infusions. All rats were implanted with bilateral cannulae into the lOFC and infused with M/B or vehicle as described in Chapter 3 General Methods (Surgical procedures).

Histology. Cannulae placements (Figure 5.4a.) were verified post-mortem as described in Chapter 3 General Methods (Histology).

Results

Phase 1 Sensory Preconditioning. Freezing to neutral S2 and S1 was low and similar across groups during sensory training (Figure 5.4a.C, left panel). A mixed ANOVA of freezing to S2 revealed main effect of group ($F_{(3, 45)}$ = 4.512, p = 0.008, η_p^2 = 0.231), a main effect of trial ($F_{(2.37, 106.36)}$ = 4.516, p = 0.005, η_p^2 = 0.091) and no group x trial interaction ($F_{(7.11, 106.36)}$ = 0.700, p = 0.708, η_p^2 = 0.045). Freezing to S1 was similar among the groups (5.4a.C, right panel). An identical analysis of freezing to S1 revealed no main effect of group ($F_{(3, 45)}$ = 0.962, p = 0.419, η_p^2 = 0.060),

no main effect of trial ($F_{(2.38, 106.92)} = 0.894$, $p = 0.446$, $\eta_p^2 = 0.020$) and no group x trial interaction ($F_{(7.13, 106.92)} = 0.894$, $p = 0.446$, $\eta_p^2 = 0.020$).

Phase 2 First-Order Conditioning. Conditioned freezing to S1 increased across trials for all groups (Figure 5.4a.D, right panel). A mixed ANOVA detected no main effect of group ($F_{(3, 45)} = 0.293$, $p = 0.831$, $\eta_p^2 = 0.019$), a main effect of trial ($F_{(3, 135)} = 70.892$, $p < 0.831$, $\eta_p^2 = 0.612$) and no group x trial interaction ($F_{(9, 135)} = 0.523$, $p = 0.856$, $\eta_p^2 = 0.034$).

SPC-S2 Test. Conditioned freezing to S2 in the Paired groups was higher relative to the Unpaired groups, providing evidence for the SPC effect, however, there was no effect of lOFC inactivation in females (Figure 5.4a.E). A two-way ANOVA detected a main effect of group ($F_{(1, 45)} = 13.786$, $p < 0.001$, $\eta_p^2 = 0.234$), no main effect of drug ($F_{(1, 45)} = 0.429$, $p = 0.516$, $\eta_p^2 = 0.008$) and no group x drug interaction ($F_{(1, 45)} = 0.600$, $p = 0.443$, $\eta_p^2 = 0.013$).

Discussion

In this study we showed that lOFC does not modulate fear to SPC-S2 in females unlike the disruption obtained in males (Experiment 5.1). The lack of effect in SPC may be explained by how fear to SPC-S2 accrues and how females express it. As described in Chapter 1, SPC-S2 elicits conditioned fear via mediated or chained associations. In the first scenario, SPC-S2 becomes associated with the US that is signaled by S1 during Phase 2 of SPC. In the second case, SPC-S2 activates the representation of S1 which leads to the expectation of the shock resulting in conditioned fear. Our findings that lOFC inactivation spares fear to SPC-S2 on test suggest that females may be using mediated over chained associations and the expression of the former may not require the lOFC.

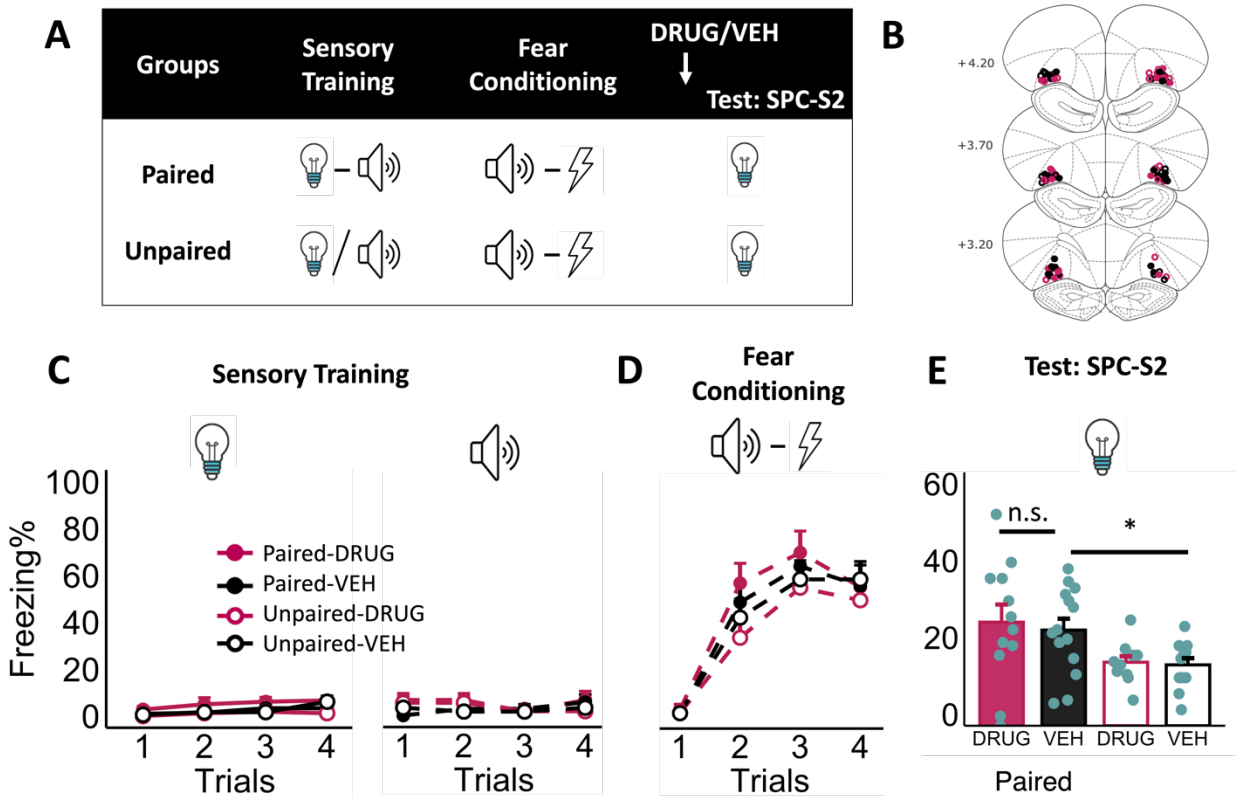

Figure 5.4a. Pharmacological inactivation of lOFC has no effect on fear to SPC-S2 in female rats. (A) Behavioural protocol. (B) Cannulae placements in the lOFC, distances from Bregma in mm. (C) Freezing to SPC-S2 (solid lines, left panel) and S1 (dashed lines, right panel) during sensory training. Paired-DRUG (filled burgundy) n = 12, Paired-VEH (filled black) n = 14, Unpaired-DRUG (open burgundy) n = 11, Unpaired-VEH (open black) n = 12. (D) Acquisition of conditioned freezing to S1. (E) Freezing to SPC-S2 on test. Inactivation of lOFC has no effect on SPC (p = 0.960). (E) Freezing to S1 on test. All data are mean freezing percent + SEM.

Experiment 5.4b: Pharmacological Inactivation of lOFC does not modulate fear to SOC-S2 in female rats

Experiment 5.2 revealed the role of lOFC in second-order fear in males. The aim of this study was to determine if the lOFC serves a similar role in females. Similar to Experiments 5.1 and 5.2, we used a 2x2 design in which one factor was the pairing between S2 and S1 (Paired and Unpaired groups) and the other was the drug infusion (Drug and Vehicle groups). The same set of rats in Experiment 5.4a were used in this experiment with the following groups: Paired-Drug, Paired-Vehicle, Unpaired-Drug and Unpaired-Vehicle. Comparison between Paired-Vehicle and

Unpaired-Vehicle reflect the behavioural effect while the comparison between Paired-Drug and Paired-Vehicle show the effect of lOFC inactivation.

Methods

Subjects. The same set of rats used in Experiment 5.4a were used in second-order conditioning experiment. Four rats were excluded from the analyses due to generalization of fear (for exclusion criteria see Behavioural Procedure) from SPC to SOC phase yielding following group sizes: Paired-Drug (n = 9), Paired-Vehicle (n = 8), Unpaired-Drug (n = 13) and Unpaired-Vehicle (n = 11).

Prior history. Subsequent to the SPC Test in Experiment 5.4a, rats were trained in an SOC protocol using the same stimulus to serve as S1 and a novel stimulus to serve as SOC-S2. First, rats were re-conditioned to S1. Based on S1 re-conditioning performance and behavioural history in SPC phase, they were allocated to Paired and Unpaired SOC groups. That is, equal number of rats in the Paired SOC group had experience with prior paired and unpaired training during SPC. Similarly, equal number of rats in the Unpaired SOC group had experience with paired and unpaired SPC training. Rats that showed more than 20% freezing to SOC-S2 on the first exposure (i.e., first trial of sensory training) were deemed to generalize fear from SPC phase and excluded from the analyses. Finally, rats were tested to SOC-S2 and S1.

Behavioural apparatus and procedure. See the details about stimuli, experimental chambers and procedures in Chapter 3 General Methods (Behavioural Apparatus).

Experiment Timeline. Subsequent to SPC-S2 test in Experiment 5.4a, rats were trained in our classical SOC protocol using the same timeline as in Experiment 5.2.

Surgery and infusions. All rats were implanted with bilateral cannulae into the lOFC and infused with M/B or vehicle as described in Chapter 3 General Methods (Surgical procedures).

Histology. Cannulae placements (Figure 5.4b.B) were verified post-mortem as described in Chapter 3 General Methods (Histology).

Results

Phase 1 Re-training of First-Order Stimulus. Freezing to S1 during re-training remained high (Figure 5.4b.C). A mixed ANOVA revealed no main effect of group ($F_{(3, 38)} = 0.306$, $p = 0.759$, $\eta_p^2 = 0.024$), no trial ($F_{(3, 114)} = 0.290$, $p = 0.833$, $\eta_p^2 = 0.008$), nor group x trial interaction ($F_{(9, 114)} = 0.262$, $p = 0.983$, $\eta_p^2 = 0.020$).

Phase 2 Second-Order Conditioning. Conditioned freezing to S2 increased across trials in the Paired groups but not in the Unpaired groups (Figure 5.4b.D, left panel). A mixed ANOVA of freezing to S2 revealed a main effect of group ($F_{(1, 38)} = 6.139$, $p = 0.001$, $\eta_p^2 = 0.340$), a main effect of trial ($F_{(3, 114)} = 31.413$, $p < 0.001$, $\eta_p^2 = 0.453$), and a group x trial interaction ($F_{(9, 114)} = 4.662$, $p < 0.001$, $\eta_p^2 = 0.269$). Post-hoc comparisons with Bonferroni adjustments confirmed the increase in the Paired relative to the Unpaired across training ($M_{\text{Paired}} - M_{\text{Unpaired}} = 12.72$, $p < 0.001$, 95% CI [8.24, 17.21], $d = 0.880$). Freezing to S1 was similar among the groups (Figure 5.4b.D, right panel). An identical analysis of freezing to S1 revealed no main effect of group ($F_{(1, 38)} = 0.285$, $p = 0.836$, $\eta_p^2 = 0.220$), no main effect of trial ($F_{(2.38, 90.69)} = 0.402$, $p = 0.752$, $\eta_p^2 = 0.011$), nor a group x trial interaction ($F_{(7.16, 90.69)} = 1.496$, $p = 0.158$, $\eta_p^2 = 0.106$).

SOC-S2 Test. Conditioned freezing to SOC-S2 was high in the Paired relative to the Unpaired groups, evidencing second-order conditioning, but this effect was not modulated by lOFC inactivation in females (Figure 5.4b.E). A two-way ANOVA revealed a main effect of group ($F_{(1, 38)} = 24.628$, $p < 0.001$, $\eta_p^2 = 0.393$), no main effect of drug ($F_{(1, 38)} < 0.001$, $p = 0.980$, $\eta_p^2 < 0.001$), and no group x drug interaction ($F_{(1, 38)} = 0.210$, $p = 0.886$, $\eta_p^2 < 0.001$).

S1 Test. Rats froze similarly to S1 on test (Figure 5.4b.F). A two-way ANOVA revealed no main effect of group ($F_{(1, 38)} = 0.596$, $p = 0.445$, $\eta_p^2 = 0.016$), nor drug ($F_{(1, 38)} = 0.014$, $p = 0.907$, $\eta_p^2 < 0.001$) and no group x drug interaction ($F_{(1, 38)} = 2.270$, $p = 0.140$, $\eta_p^2 = 0.056$).

Discussion

In this study, we investigated the effect of lOFC inactivation on expression of fear to SPC-S2 and SOC-S2 in females. Unlike our results in the males (Experiments 5.1 and 5.2), lOFC inactivation had no effect on fear to SPC-S2 and SOC-S2.

The lack of effect in SOC in females as opposed to males may be explained by resorting to our hypothesis on Experiment 5.2 findings, where lOFC inactivation enhances the expression of fear to SOC-S2. In females, the strength of S2→no_shock association that develops during Phase 2 may be weaker relative to the one develops in males in the face of S2→fear_state associations. Therefore, lOFC inactivation and thus rendering S2→no_shock association inaccessible has no observable effect on behaviour.

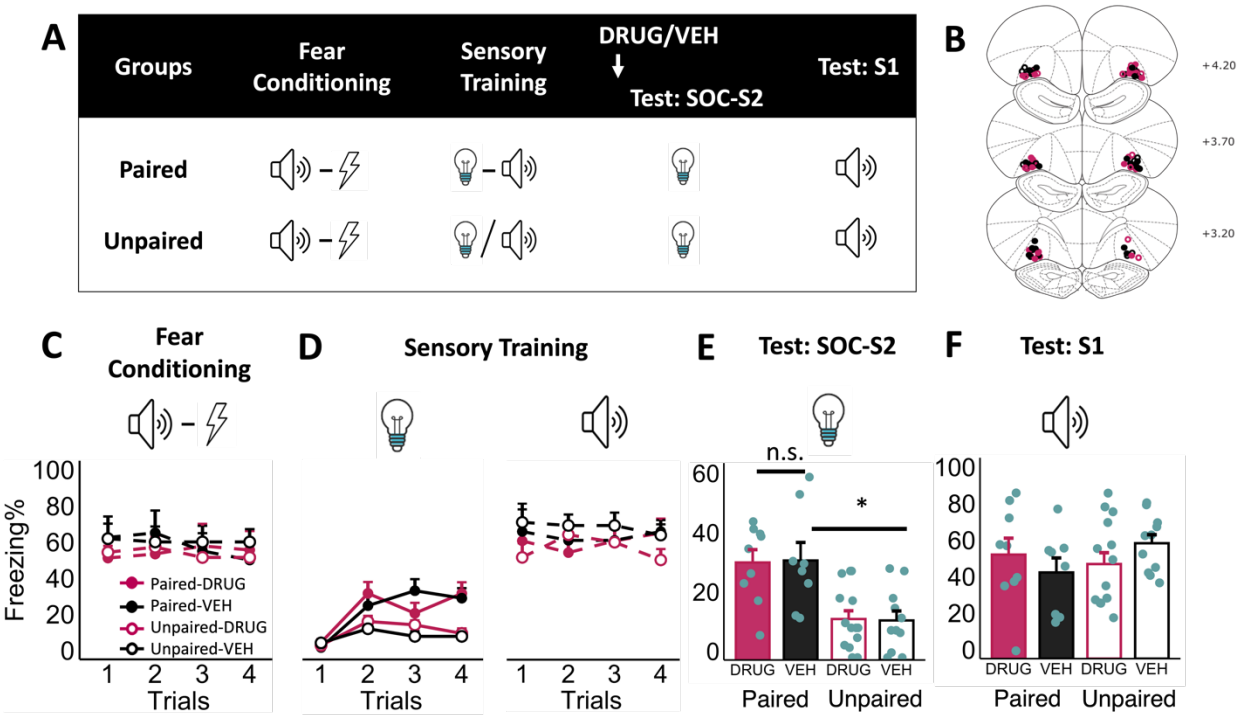

Figure 5.4b. Pharmacological inactivation of lOFC does not modulate fear to SOC-S2 in females (A) Behavioural protocol. (B) Cannulae placements in lOFC, distances from Bregma in mm. (C) Re-conditioning of S1. Paired-DRUG (filled burgundy) n = 9, Paired-VEH (filled black) n = 8, Unpaired-DRUG (open burgundy) n = 13, Unpaired-VEH (open black) n = 11. (D) Conditioned freezing to SOC-S2 (solid lines, left panel) and S1 (dashed lines, right panel). (E) Freezing to SOC-S2 on test. Inactivation of lOFC has no effect on fear to SOC-S2 (p = 1.0). (E) Freezing to S1 on test. All data are mean freezing percent + SEM.

General Discussion

This chapter investigated the role of lateral orbitofrontal cortex (lOFC), a region generally overlooked in fear, in the expression of *aversive* higher-order memories. We demonstrated that inactivation of lOFC via intracranial $GABA_{A/B}$ agonist muscimol/baclofen (M/B) infusions disrupted sensory preconditioning and enhanced second-order conditioning in male rats. Interestingly, females did not show evidence for lOFC control at the time of higher order fear expression. We consider obtained sex differences in terms of the associations that are expressed in the lOFC. Importantly, our data support the role of lOFC in the retrieval of stimulus-stimulus associations in both types of *fear*. This is, to our knowledge, the first evidence that demonstrates the role of lOFC in aversive higher-order conditioning.

Our findings from Experiment 5.1 show that a functional lOFC is necessary for the expression of fear to SPC-S2. These results are in line with those obtained in appetitive SPC, a disruption as a result of pharmacological lOFC inactivation at test (Jones et al., 2012). To investigate how silencing the lOFC contributes to SPC expression we focused on the associative architecture that supports SPC. As discussed in Chapter 1 and shown in Chapter 4, SPC is supported by S-S associations that form between SPC-S2 and S1. Evidence shows that neural activity in the lOFC is necessary for the formation of S2→S1 association in Phase 1. Optogenetic silencing of this region during Phase 1 disrupted the expression of appetitive SPC (Hart et al., 2020). Therefore, pharmacologically silencing lOFC on test impairs the expression of S2→S1 association, and disrupt SPC.

In contrast to a disruption in SPC, we showed that lOFC inactivation *enhances* the expression of fear to SOC-S2. These results clearly show that inactivation of lOFC does not have a general fear suppressing effect. If that were the case the effect of lOFC inactivation would be in the same direction for fear expression in SPC and SOC. To understand the contribution of lOFC to fear expression in SOC, we focused on the S-S associations that may form in SOC given the role of lOFC in the expression of these associations. It is unlikely that lOFC interferes with S2→S1 or an S1 mediated S2→mediated_US associations at test because our data and others' (Holmes et al., 2013; Rescorla, 1973b; Rizley & Rescorla, 1972) have shown that these are not the associations that control behaviour in SOC. Another association that may start forming during sensory training of SOC is the S2→no_shock association. It starts developing as a result of omission of the S1-signalled shock. That is, S1 leads to the expectation of shock but when S1 is preceded by S2, the shock is omitted. Therefore, S2 leads to the expectation of shock omission (i.e., S2→no_shock). Indeed, as discussed in Chapter 1, extended S2→S1 trials strengthen this S2→no_shock association over S2→fear_state and result in low levels of fear expression to S2 (i.e., conditioned inhibition; Herendeen & Anderson, 1968; Holland & Rescorla, 1975b; Yin et al., 1994; Stout et al., 2004). To investigate this possibility, we used a reinforced second-order conditioning design that includes reinforced S2→S1 training (i.e., S2→S1→US). Such reinforced trials eliminate the formation of S2→no_shock associations. Subsequent to S2→S1→US training but prior to test, inactivation of lOFC had no effect on fear to S2 relative to vehicle controls (Experiment 5.4). These results corroborate our hypothesis that classical SOC design results in the formation of two competing associations at test: S2→fear_state and S2→no_shock. Silencing lOFC prior to test

eliminates the retrieval of S2→no_shock association and the expression of S2-fear association prevails, resulting in enhanced fear expression to SOC-S2.

Our female data (Experiments 5.4a and 5.4b) show that inactivation of lOFC did not modulate fear expression to SPC-S2 nor SOC-S2 unlike males. This suggests that females may be using a different strategy during learning or expression of SPC and SOC type fears. For example, in SPC, females may rely on a mediated S2→US association. That is, during Phase 2 while S1 is fear conditioned it may retrieve its S2-associate that enters into association with the US (i.e., S2→US, see Chapter 1). This type of learning may not require lOFC but recruit other regions. For example, the BLA is a potential candidate for the learning and expression of S2→US association, given its role in encoding detailed US representations (Romanski et al., 1993; Paton et al., 2006; Gore et al., 2015). Alternatively, lOFC activity may be necessary during Phase 2, but the expression of S2→US may not require lOFC. These alternatives are discussed in a separate section in Chapter 7. In SOC, our findings implicate two competing associations in males: S2→no_shock and S2→fear_state. These are likely the associations that control behaviour in females as well. However, normal levels of SOC-fear in females relative to enhanced SOC-fear in males suggest that in females, S2→no_shock associations are not as strong as they are in males. More specifically, in males the relative strength of S2→no_shock in the face of S2→fear_state association may be strong enough to result in an observable effect on behaviour when the former is rendered inaccessible via lOFC inactivation. In contrast, S2→no_shock may not be as developed or as strong in females as in males to counter the effect of S2→fear_state, therefore, canceling the former via lOFC inactivation has no effect on fear to SOC-S2. Perhaps extended S2→S1 pairings would lead to a stronger S2→no_shock association in females and lOFC inactivation may reveal a similar pattern to that observed in males. We discuss the mechanisms underlying sex differences in learning in Chapter 7 General Discussion.

Our data provide key insights about lOFC. Firstly, it underlines the importance of sex differences in learning and memory, providing an interesting avenue for research that has been traditionally overlooked (Lebron-Milad & Milad, 2012). Secondly, our data support a more global role for lateral OFC in keeping track of associative relationships between stimuli in guiding behaviour, a role that extends beyond the (acquired) valence or value of these stimuli. We obtained a disruption of SPC-fear and enhancement of SOC-fear, indicating that lOFC does not have a

general role in fear. Rather, our findings demonstrate that lOFC regulates fear based on the S-S associations that enable it to control behaviour distinctly in SPC and SOC.

Chapter 6. Pathway-Specific Inactivation and investigation of neuronal populations in the BLA

As discussed in Chapter 2, the BLA is important for the expression of fear to SPC-S2 and SOC-S2. Chapters 4 and 5 provided evidence that SPC and SOC are differentially regulated in the BLA and the lOFC. Importantly, these two regions have dense reciprocal projections (Carmichael & Price, 1995a). Therefore, one of the aims of this chapter was to characterize the neuronal populations in the BLA to see whether distinct populations are activated as a result of SPC-S2 and SOC-S2 exposure on test, and whether these populations send projections to the lOFC. Next, we investigated the role of BLA→lOFC and lOFC→BLA pathways in the expression of fear to SPC-S2 and SOC-S2.

The lOFC and the BLA are known to be key regions in supporting goal-directed behaviour. In particular, the BLA is involved in forming representations between S1 and the motivationally significant US (Hatfield et al., 1996; Setlow et al., 2002a; Balleine & Killcross, 2006) while the lOFC is shown to be keeping track of these representations in guiding behaviour (Schoenbaum et al., 2000; Pickens et al., 2003; Izquierdo et al., 2004; Ostlund & Balleine, 2007; Murray & Fellows, 2022). Recent evidence demonstrated that the BLA→lOFC, but not lOFC→BLA, pathway is are necessary to use S1-evoked US representations to drive choice behaviour (Lichtenberg et al., 2017). In contrast, lOFC→BLA inputs are required to make predictions about rewarding events (Lucantonio et al., 2015). Less is known about the role of these pathways in fear, however, aberrant lOFC-BLA connectivity is implicated in anxiety disorders in humans and non-human primates (Price & Drevets, 2010; Kim et al., 2011).

Our data showed a neural dissociation between SPC and SOC upon S1→US deletion in the BLA (Chapter 4) and demonstrated a role for the lOFC in aversive SPC and SOC for the first time (Chapter 5). Importantly, we showed that both regions regulate SPC and SOC type of fears in distinct ways. That is, SPC is dependent on S1-evoked neural activity in the BLA while SOC is not and lOFC inactivation disrupts SPC but enhances SOC. Here, we used RNAscope to determine if SPC-S2 and SOC-S2 activate distinct neuronal ensembles in the BLA, as one might expect given

the dissociable reliance of SPC and SOC fear on an S1-activated BLA ensemble. We combined this with a retrograde viral approach to examine whether these ensembles project to the OFC. Next, using DREADDs, we investigated the role of the BLA→lOFC and lOFC→BLA pathways in both types of fear.

Experiment 6.1: SPC-S2 and SOC-S2 activate unique and overlapping neuronal populations that project to the lOFC

In this study, we characterized the neuronal populations in the BLA that are activated as a result of SPC-S2 and SOC-S2 presentation on test. To do this, we used a combined SPC-SOC design, and identified the neurons in an RNAscope in situ hybridization preparation using two c-fos isoforms (i.e., cytoplasmic and nuclear) that differed in their temporal expression profiles. Given the role of lOFC in both types of fear we included a retrograde tracer in the lOFC to investigate BLA→OFC input in fear expression to SPC-S2 and SOC-S2.

Methods

Subjects. Fifteen naïve wild-type male Sprague-Dawley rats bred in-house and weighing 344-484g were used for this experiment. All rats received SPC and SOC training in a within-subjects design. Half the rats were first tested to SPC-S2 while the remaining half were first tested to SOC-S2.

Behavioural apparatus and procedure. See the details about stimuli, experimental chambers, and procedures in Chapter 3 General Methods (Behavioural Apparatus).

Experiment timeline. On Days 1, 2 and 5 rats received context exposure. On Day 3 they received $S2^{SPC}$→S1 pairings. On Day 4 rats received fear conditioning to S1. On Day 6 rats received $S2^{SOC}$→S1 pairings. On Day 7 they received test presentations of SPC-S2 and SOC-S2 in sessions that were 25 minutes apart (test order counterbalanced).

Surgery and Virus. All rats were transfected with retrograde virus in the lOFC as described in Chapter 3 General Methods (Surgical procedures).

Histology. All rats were verified for viral diffusion in the lOFC and brain sections were quantified for viral expression in the cell bodies of the BLA as well as probe detection (Figure 6.1I) post-mortem as described in Chapter 3 General Methods (Histology).

Results

Phase 1 Sensory Training of S2^{SPC}. Freezing to SPC-S2 was low across trials (Figure 6.1C, left panel). A repeated-measures ANOVA detected no main effect of trial ($F_{(3, 56)}$= 1.724, p = 0.173, η_p^2 = 0.085), Freezing to S1 was also low (Figure 6.1C, right panel). An identical analysis for freezing to S1 revealed no main effect of trial ($F_{(3, 56)}$= 1.087, p = 0.362, η_p^2 = 0.055).

Phase 2 Fear Conditioning. Conditioned freezing to S1 increased across trials (Figure 6.1D). A repeated measures ANOVA revealed a main effect of trial ($F_{(3, 56)}$= 31.82, p < 0.001, η_p^2 = 0.630).

Phase 3 Sensory Training of S2^{SOC}. Conditioned freezing to SOC-S2 increased across trials (Figure 6.1E, left panel). A repeated measures ANOVA revealed a main effect of trial ($F_{(3, 56)}$ = 17.05, p < 0.001, η_p^2 = 0.478). Freezing to S1 remained high across trials (Figure 6.1E, right panel). An identical analysis for freezing to S1 revealed no main effect of trial ($F_{(3, 56)}$ = 1.008, p = 0.396, η_p^2 = 0.051).

Test. Freezing to SPC-S2 and SOC-S2 were high while the former was higher than the latter (Figure 6.1F). A paired t-test confirmed higher levels of freezing to SPC-S2 relative to SOC-S2 ($t_{(14)}$ = 14.22, p = 0.03, 95% CI [1.53, 26.92], d = 0.64).

RNAscope quantification. In QuPath, regions of interest are annotated to exclude visibly damaged tissue from the analysis. This resulted in the detection of 57057 cells based on the DAPI channel. In each channel, the signal is characterized across 15 images that were representative of the total 145 images to standardize signal detection criteria across images, as done previously (Secci et al., 2023). The mean fluorescence profiles in retrograde tracer (568nm), cytoplasmic *fos* (488nm) and nuclear *fos* (647nm) channels are examined using histograms. Mean fluorescence intensities are standardized and cells with Z-score >= 3.0 are excluded from the analyses. This yielded 54535 cells. Based on the criteria established in the representative subset of images we used subcellular spot detection tool in Qupath and quantified the signal across images. Each cell is characterized by the presence or absence of signal in each channel. Subsequently, each rat is represented as the total number of cells analyzed and classified into SPC-unique, SOC-unique or SPC ∩ SOC (i.e., overlap). Unique cells showed probe expression uniquely towards that cue, while the overlap cells showed probe expression to both SPC-S2 and SOC-S2 cues. These populations are also classified as lOFC-projecting and non-projecting depending on the presence of retrograde

viral signal, yielding 6 distinct neuronal populations. Similar proportion of BLA cells were activated to SPC-S2 (M_{SPC} = 30.01%, *SEM* = 0.475) or to SOC-S2 (M_{SOC} = 27.97%, *SEM* = 0.404; $t_{(27)}$ = -0.777, p = 0.45, 95% CI [-7.43, 3.35], d = 0.29). A subpopulation projected to the lOFC in SPC-S2 ensemble ($M_{SPC\text{-}OFC}$ = 16.06%, *SEM* =1.34) and SOC-S2 ensemble ($M_{SOC\text{-}OFC}$ = 15.45%, *SEM* =1.24), and the sizes of these subpopulations were similar ($t_{(27)}$ = -0.381, p = 0.71 95% CI [-3.90, 2.68], d = 0.14). We also identified BLA cells uniquely activated to SPC-S2 (i.e., SPC-unique; M = 8.13%, *SEM* = 1.01) or SOC-S2 (i.e., SOC-unique; M = 6.70%, *SEM* = 0.747), and a population of neurons that were activated to SPC-S2 and SOC-S2 (i.e., M = 5.82%, *SEM* = 0.794). Our results show that similar percentage of cells are activated in the BLA to guide SPC and SOC type fears and a subpopulation of these neurons equivalent in size project to the lOFC.

Discussion

In this study, we aimed to characterize the neuronal populations in the BLA that regulate fear to SPC-S2 and SOC-S2. We identified unique and overlapping neurons for SPC and SOC types of fear and found that equivalently sized subpopulation in each ensemble (i.e., SPC-unique, SOC-unique and SPC∩SOC) project to the lOFC (Figure 6.1). These populations provide insights into how the BLA and its connections with the lOFC regulate both types of fear.

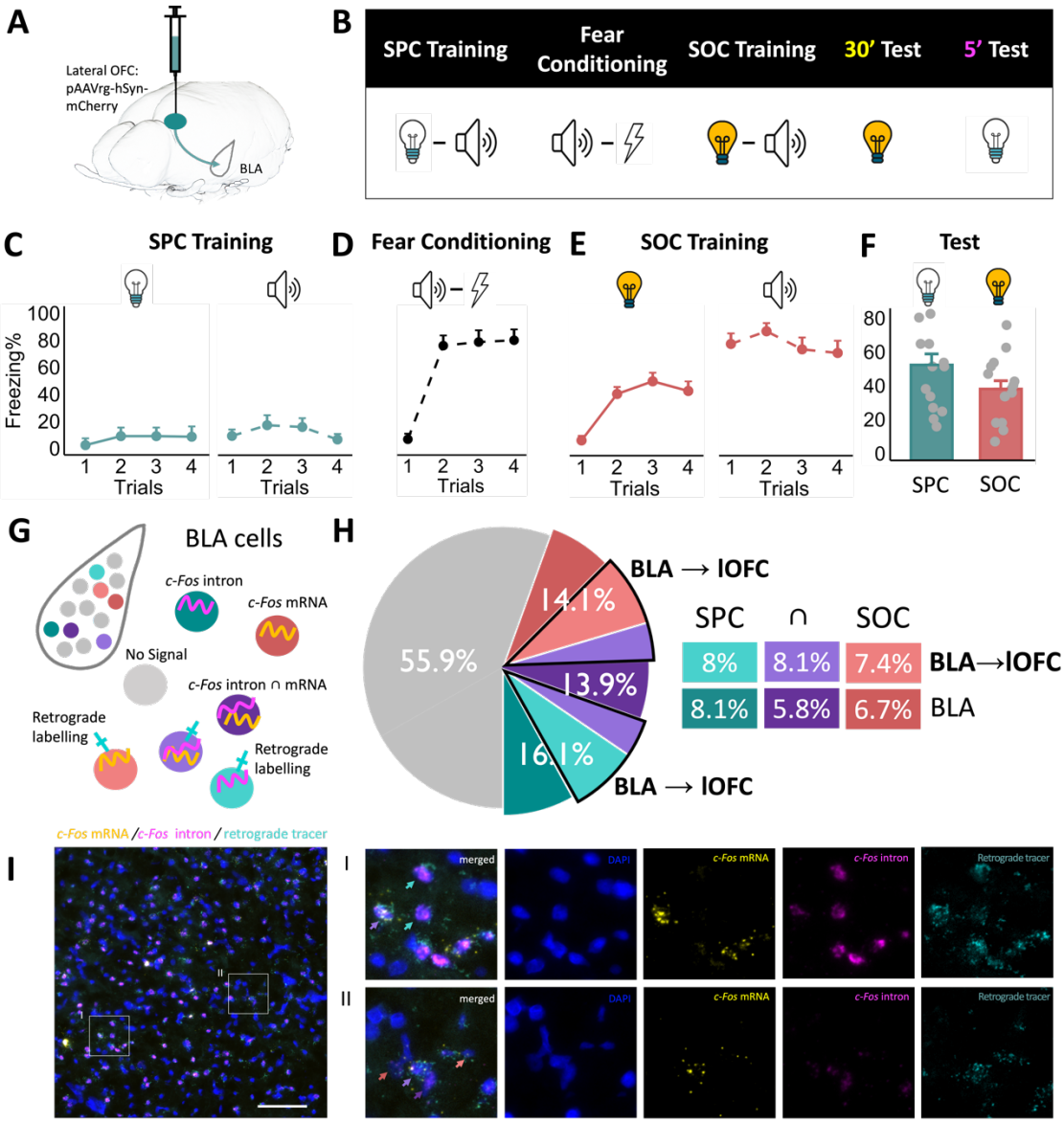

Figure 6.1. SPC-S2 and SOC-S2 activate unique and overlapping populations in the BLA which project to the lOFC. (A) Retrograde virus infusion in the lOFC. (B) Behavioural protocol. (C) Freezing to SPC-S2 (solid lines, left panel) and S1 (dashed lines, right panel). (D) Acquisition of conditioned freezing to S1. (E) Conditioned freezing to SOC-S2 (solid lines, left panel) and S1 (dashed lines, right panel). (F) Freezing to SPC-S2 and SOC-S2 on test. Rats showed similar levels of freezing to SPC-S2 and SOC-S2 with a slight elevation in the former (M_{SPC} -M_{SOC} = 14.22, p = 0.091, 95% CI [-2.41, 30.85], d = 0.64). (G) Characterization of the BLA neurons using RNAscope *in situ* hybridization. (H) SPC-S2 and SOC-S2 activated similar number of neurons on test ($t_{(27)}$ = -0.777, p = 0.45, 95% CI [-7.43, 3.35], d = 0.29) . A subpopulation of these neurons had projections to the lOFC in the SPC-ensemble ($M_{SPC\text{-}OFC}$ = 16.06%, *SEM* =1.34) and SOC-ensemble ($M_{SOC\text{-}OFC}$ = 15.45%, *SEM* =1.24), the size of these populations that showed projections to the lOFC were equivalent ($t_{(27)}$ = -0.381, p = 0.71 95% CI [-3.90, 2.68], d = 0.14). (I) Representative image of BLA cells identified using RNAscope *in situ* hybridization. Colocalization of cytoplasmic and nuclear c-Fos and retrograde labelling is indicated by colored arrows. Scale bar 100µm.

Experiment 6.2a: Inactivation of the BLA→lOFC pathway disrupts fear to SPC-S2

In this study, we investigated the effect of inactivating the BLA→OFC pathway on the expression of fear to SPC-S2. To do this, we included Paired and Unpaired groups to assess the effect of SPC, while hm4Di and the mCherry groups controlled for the effect of DREADDs inactivation. This resulted in the following groups: Paired-hm4Di, Paired-mCherry, Unpaired-hm4Di and Unpaired-mCherry.

Methods

Subjects. Forty-three experimentally naïve wild-type male Sprague-Dawley (Envigo Ltd. Indianapolis, USA) rats weighing 308-385g prior to viral infusion surgeries were used in this experiment. Two rats were excluded due to incorrect cannulae placements. This yielded following group sizes: Paired-hm4Di (n = 10), Paired-mCherry (n = 11), Unpaired-hm4Di (n = 10), Unpaired-mCherry (n = 10).

Behavioural apparatus and procedures. See the details about stimuli, experimental chambers and the procedure in Chapter 3 General Methods (Behavioural Apparatus).

Experiment timeline. Behavioural timeline was same as the timeline in Experiment 5.1.

Surgery, Drugs and Virus. All rats were transfected with hm4Di or the control mCherry DREADD in the BLA, implanted with cannulae bilaterally in the lOFC, and received Clozapine N-Oxide (CNO) infusions as described in Chapter 3 General Methods (Surgical procedures).

Histology. All rats were verified for viral transfection of BLA cell bodies and BLA terminals in the lOFC. (Figure 6.2a.H) and cannulae placements (Figure 6.2a.C) post-mortem as described in Chapter 3 General Methods (Histology).

Results

Phase 1 Sensory Preconditioning. Freezing to SPC-S2 was low across trials and similar among the groups (Figure 6.2a.E, left panel). A mixed ANOVA revealed no main effect of group ($F_{(3, 37)}$ = 0.175, p = 0.913, $\eta_p^2 < 0.014$), a main effect of trial ($F_{(3, 111)}$ = 3.265, p = 0.024, η_p^2 = 0.081), but no group x trial interaction ($F_{(9, 111)}$ = 0.795, p = 0.621, η_p^2 = 0.061). Fear to S1 was low across trials and similar among the groups (Figure 6.2a.E, right panel). An identical analysis for freezing to S1 revealed no main effect of group ($F_{(3, 37)}$= 2.239, p = 0.100, η_p^2 =0.154), no main effect of trial ($F_{(3, 111)}$= 1.129, p = 0.341, η_p^2 = 0.030) and no group x trial interaction ($F_{(3, 111)}$= 0.186, p = 0.906, η_p^2 =0.005).

Phase 2 First-Order Conditioning. Conditioned freezing to S1 increased across trials similarly among the groups (Figure 6.2a.F). A mixed ANOVA revealed no main effect of group ($F_{(3, 37)}$ = 0.144, p = 0.933, η_p^2= 0.012), a main effect of trial ($F_{(2.43, 89.79)}$ = 110.838, p < 0.001, η_p^2 =0.750), and no group x trial interaction ($F_{(7.28, 89.79)}$ = 0.595, p = 0. 799, η_p^2 =0.046).

SPC Test. Conditioned freezing to SPC-S2 was higher in the Paired-mCherry relative to Unpaired-mCherry evidencing SPC effect. Silencing the BLA→OFC pathway prior to test disrupted the expression of fear to SPC-S2, as indicated by lower levels of freezing in the Paired-hm4Di relative to the Paired-mCherry (Figure 6.2a.G). A two-way ANOVA revealed a main effect of training ($F_{(1, 37)}$= 9.922, p = 0.003, η_p^2 =0.212), a main effect of virus ($F_{(1, 37)}$= 4.352, p = 0.044, η_p^2 =0.105) and training x virus interaction ($F_{(1, 37)}$ = 5.502, p = 0.025, η_p^2 = 0.130). Post-hoc analyses with Bonferroni corrections confirmed the disruption of SPC as a result of BLA→OFC pathway inactivation evidenced by lower levels of fear in the Paired-hm4Di group relative to Paired-mCherry ($M_{\text{Paired-hm4Di}} - M_{\text{Paired-mCherry}}$ = -14.235, p = 0.016, 95% CI [-26.31, -2.16], d = 1.17) while Paired-mCherry froze more than the Unpaired-mCherry evidencing the SPC effect ($M_{\text{Paired-mCherry}} - M_{\text{Unpaired-mCherry}}$ = 17.65, p = 0.006, 95% CI [5.68, 29.73], d= 1.81). There was no difference between the Paired-hm4Di and Unpaired-hm4Di groups confirming completely abolished SPC effect ($M_{\text{Paired-hm4Di}} - M_{\text{Unpaired-hm4Di}}$ = 2.58, p = 0.943, 95% CI [-9.76, 14.94], d = 0.240).

Discussion

In this study, we investigated the role of BLA→OFC pathway in fear to SPC-S2. Silencing this pathway using DREADDs prior to test disrupted fear to SPC-S2, providing evidence that BLA input to the lOFC is required for sensory preconditioned fear. Moreover, our data provided evidence that this disruption was complete in the sense that it reduced responding to the level of the unpaired control.

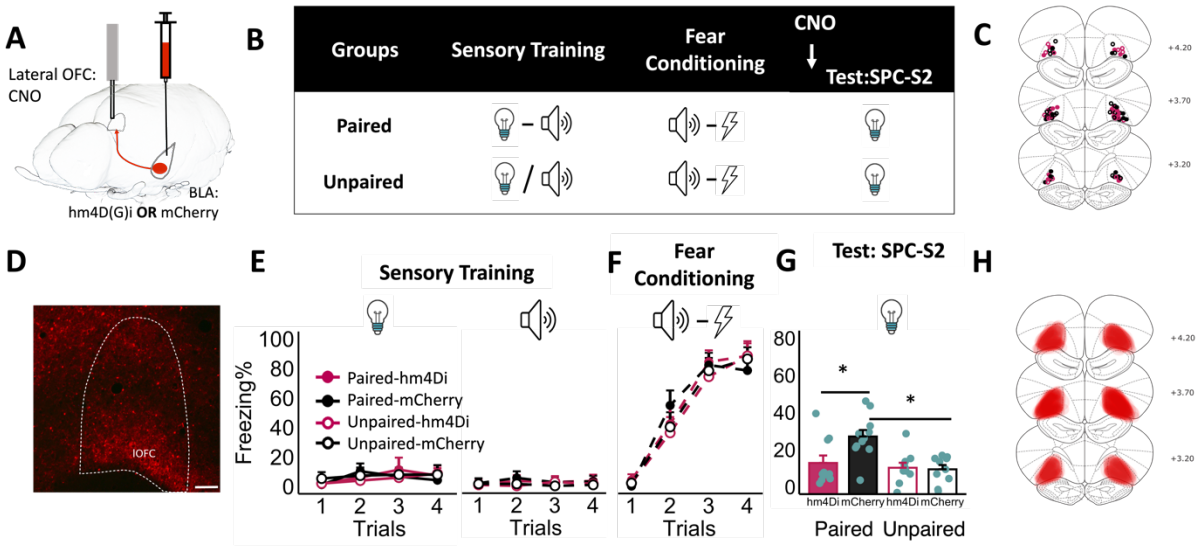

Figure 6.2a. Inactivation of the BLA→lOFC pathway disrupts fear to a SPC-S2. (A) Depiction of viral infusion and cannulation. (B) Behavioural protocol. (C) Cannulae placements in the lOFC, distances from Bregma in mm. (D) Image represents BLA terminals in the lOFC. Scale bar 100µm. (E) Freezing to SPC-S2 (solid lines, left panel) and S1 (dashed lines, right panel) during sensory training. Paired-hm4Di (filled burgundy) n = 10, Paired-mCherry (filled black) n = 11, Unpaired-hm4Di (open burgundy) n = 10, Unpaired-mCherry (open black) n = 10. (F) Acquisition of conditioned freezing to S1. (G) Freezing to SPC-S2 on test. Chemogenetic inactivation of BLA→lOFC pathway disrupts freezing to SPC-S2 (p = 0.016). (H) Viral diffusion in the BLA. All data are mean freezing percent + SEM.

Experiment 6.2b: Inactivation of the BLA→lOFC pathway attenuates fear to SOC-S2

In this study, we investigated the effect of inactivating the BLA→OFC pathway on the expression of fear to SOC-S2. To do this, we trained the rats in SOC protocol subsequent to Experiment 6.2a. Rats were counterbalanced for their prior experience in SPC (see Prior History under Methods). Paired and Unpaired groups were included to assess the effect of SPC, while hm4Di and mCherry groups controlled for the effect of virus. This resulted in the following groups: Paired-hm4Di, Paired-mCherry, Unpaired-hm4Di and Unpaired-mCherry.

Methods

Subjects. Thirty-five rats were used in this experiment with the following group numbers: Paired-hm4Di (n = 11), Paired-mCherry (n = 10), Unpaired-hm4Di (n = 8), Unpaired-mCherry (n = 6).

Prior history. Subsequent to the SPC Test in Experiment 6.2a, rats were trained in an SOC protocol using the same stimulus to serve as S1 and a novel stimulus to serve as SOC-S2. First, rats were re-conditioned to S1. Based on S1 re-conditioning performance and behavioural history in SPC phase, they were allocated to Paired and Unpaired SOC groups. That is, equal number of rats in the Paired SOC group had experience with prior paired and unpaired training during SPC. Similarly, equal number of rats in the Unpaired SOC group had experience with paired and unpaired SPC training. Rats that showed more than 20% freezing to SOC-S2 on the first exposure (i.e., first trial of sensory training) were deemed to generalize fear from SPC phase and excluded from the analyses. Finally, rats were tested to SOC-S2 and S1.

Behavioural apparatus and procedure. See the details about stimuli, experimental chambers and procedures in Chapter 3 – General Methods (Behavioural Apparatus).

Experiment timeline. Behavioural timeline was the same as the timeline in Experiment 5.4b.

Surgery, Drugs and Virus. All rats were transfected with inhibitory hm4Di or the control mCherry DREADD in the BLA, implanted with cannulae bilaterally in the lOFC, and received CNO infusions as described in Chapter 3 General Methods (Surgical procedures)..

Histology. All rats were verified for viral transfection of BLA cell bodies and BLA terminals in the lOFC (Figure 6.2b.I) and cannulae placements (Figure 6.2b.C) post-mortem as described in Chapter 3 General Methods (Histology).

Results

Phase 1 Re-training of First-Order Stimulus. Conditioned freezing to S1 remained high and similar among the groups (Figure 6.2b.E). A mixed ANOVA revealed no main effect of group ($F_{(3, 31)}$= 1.250, p = 0.309, η_p^2 = 0.108), no main effect of trial ($F_{(2.21, 68.36)}$= 2.636, p = 0.069, η_p^2 =0.079) and no group x trial interaction ($F_{(6.62, 68.36)}$ = 0.890, p = 0.515, η_p^2 = 0.079).

Phase 2 Second-Order Conditioning. Conditioned freezing to SOC-S2 increased across trials in the Paired groups relative to the Unpaired groups (Figure 6.2b.F, left panel). A mixed ANOVA revealed a main effect of group ($F_{(3, 31)}$= 4.534, p < 0.001, η_p^2 =0.305), a main effect of trial ($F_{(2.48, 76.82)}$= 16.846, p < 0.001, η_p^2 =0. 352) and a group x trial interaction ($F_{(7.44, 76.82)}$= 6.898, p = 0.011, η_p^2 =0.213). Post-hoc comparisons with Bonferroni adjustments confirmed higher levels of freezing in the Paired relative to the Unpaired ($M_{\text{Paired}} - M_{\text{Unpaired}}$ = 23.33, p < 0.001, 95% CI [14.86, 31.81], d = 0.939). Fear to S1 was similar among the groups (Figure 6.2b.F, right panel).

An identical analysis of fear to S1 revealed no main effect of group ($F_{(1, 23)}$= 0.792, p = 0.511, η_p^2 =0.094), a main effect of trial indicating some reduction of the fear response due to non-reinforcement ($F_{(3, 23)}$= 4.359, p = 0.007, η_p^2 =0.159) and no group x trial interaction ($F_{(9, 69)}$= 0.929, p = 0.506, η_p^2 =0.108).

SOC Test. Freezing to SOC-S2 was lower in the Paired-hm4Di group relative to the Paired-mCherry group providing evidence that silencing the BLA→lOFC pathway prior to test attenuated the expression of fear to SOC-S2. The Paired-mCherry group froze more in comparison to the Unpaired groups, confirming the presence of a SOC effect (Figure 6.2b.G). A two-way ANOVA revealed a main effect of training ($F_{(1, 31)}$= 85.931, p < 0.001, η_p^2 = 0.735), a main effect of virus ($F_{(1, 31)}$= 6.155, p = 0.016, η_p^2 =0.175) and a training x virus interaction ($F_{(1, 31)}$= 6.834, p = 0.014, η_p^2 = 0.181). Pairwise comparisons adjusted with Bonferroni corrections confirmed the reduction in fear to SOC-S2 as a result of BLA→OFC inactivation ($M_{\text{Paired-hm4Di}} - M_{\text{Paired-mCherry}}$ = -13.370, p = 0.004, 95% CI [-22.91, -3.83], d = 1.36) and the SOC effect ($M_{\text{Paired-mCherry}} - M_{\text{Unpaired-mCherry}}$ = -30.71, p < 0.001, 95% CI [20.21, 40.93], d = 3.30). The Paired-hm4Di froze more relative to Unpaired-hm4Di, suggesting incomplete disruption of SOC ($M_{\text{Paired-hm4Di}} - M_{\text{Unpaired-hm4Di}}$ = 17.20, p < 0.001, 95% CI [7.04, 27.35], d = 2.51).

S1 Test. Freezing to S1 was similar across groups (Figure 6.2b.H). A two-way ANOVA revealed no main effect of training ($F_{(1, 25)}$= 2.064, p = 0.163, η_p^2 =0.076), no main effect of virus ($F_{(1, 25)}$= 0.081, p = 0.778, η_p^2 =0.003) and no training x virus interaction ($F_{(1, 25)}$= 0.774, p = 0.388, η_p^2 = 0.030).

Discussion

In this study, we investigated the role of BLA→OFC pathway in expression of fear to SOC-S2. Silencing this pathway using DREADDs prior to test attenuated fear to SOC-S2 providing evidence that BLA input to the lOFC is critical for second-order fear. That is, while silencing the BLA→lOFC pathway reduced fear to SOC-S2 on test, it did not do so completely as fear to SOC-S2 was greater compared to the unpaired control.

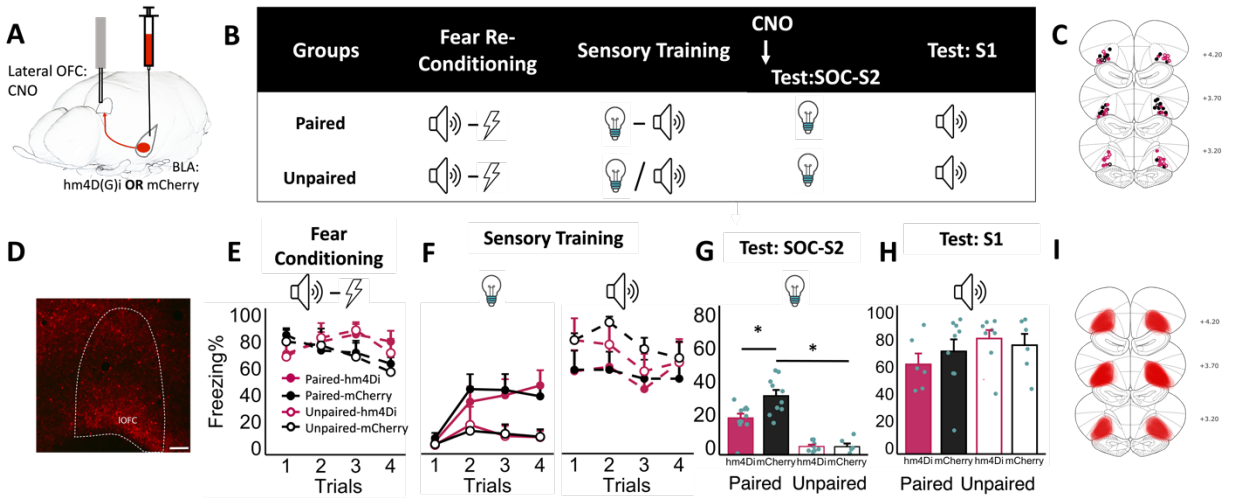

Figure 6.2b. Inactivation of the BLA→lOFC pathway disrupts fear to a SOC-S2. (A) Depiction of viral infusion and cannulation. (B) Behavioural protocol. (C) Cannulae placements in the lOFC, distances from Bregma in mm. (D) Image represents lOFC terminals in the BLA. Scale bar 100µm. (E) Freezing to S1 during fear (re)conditioning. Paired-hm4Di (filled burgundy) n = 11, Paired-mCherry (filled black) n = 10, Unpaired-hm4Di (open burgundy) n = 8, Unpaired-mCherry (open black) n = 6. (F) Freezing to SOC-S2 (solid lines, left panel) and S1 (dashed lines, right panel) during sensory training. (G) Freezing to SOC-S2 on test. Chemogenetic inactivation of BLA→lOFC pathway disrupts freezing to SOC-S2 (p = 0.004). (H) Freezing to S1 on test. (I) Viral diffusion in the lOFC. All data are mean freezing percent + SEM.

Experiment 6.3a: Intact BLA→lOFC pathway in fear to SPC-S2: A control study

To ensure that the effects reported in Experiment 6.2a are not due to the hm4Di alone, but rather due to pathway inhibition as a result of CNO infusion in hm4Di-transfected rats, we conducted the same study as that reported in 6.2a but in the absence of the ligand (CNO). We infused all rats with vehicle prior to test, thus leaving the BLA→OFC pathway intact. We expect there would be no difference between the paired groups irrespective of viral infusion (hm4Di vs mCherry). There were four groups in this study: Paired-hm4Di, Paired-mCherry, Unpaired-hm4Di and Unpaired-mCherry.

Methods

Subjects. Forty-three experimentally naïve wild-type male Sprague-Dawley (Envigo Ltd. Indianapolis, USA) rats weighing 317-377g prior to viral infusion surgeries were used in this experiment. Four rats were excluded due to incorrect cannulae placements. This yielded the following group sizes: Paired-hm4Di (n = 10), Paired-mCherry (n = 10), Unpaired-hm4Di (n = 8), Unpaired-mCherry (n = 11).

Behavioural apparatus and procedure. See the details about stimuli, experimental chambers and the procedure in Chapter 3 – General Methods (Behavioural Apparatus).

Experiment timeline. Behavioural timeline was the same as the timeline in Experiment 6.2a.

Surgery, Drugs and Virus. All rats were transfected with hm4Di DREADD or the mCherry control in the BLA, implanted with cannulae bilaterally in the lOFC, and received vehicle infusions as described in Chapter 3 General Methods (Surgical procedures).

Histology. All rats were verified for viral transfection of BLA cell bodies and BLA terminals in the lOFC. (Figure 6.3a.H) and cannulae placements (Figure 6.3a.C) post-mortem as described in Chapter 3 General Methods (Histology).

Results

Phase 1 Sensory Preconditioning. Freezing to SPC-S2 was low and similar among the groups across trials (Figure 6.3a.E, left panel). A mixed ANOVA revealed no main effect of group ($F_{(3, 35)}$= 0.214, p = 0.887, η_p^2 < 0.001), a main effect of trial ($F_{(3, 105)}$= 3.273, p = 0.024, η_p^2 = 0.086), but no group x trial interaction ($F_{(9, 105)}$= 0.378, p = 0.944, η_p^2 =0.031). Freezing to S1 was similar among the groups across trials (Figure 6.3a.E, right panel). An identical analysis for freezing to S1 revealed no main effect of group ($F_{(1, 35)}$= 1.404, p = 0.258, η_p^2 =0.108), no main effect of trial ($F_{(3, 105)}$= 1.750, p = 0.162, η_p^2 =0.048) and no group x trial interaction ($F_{(9, 105)}$= 0.386, p = 0.940, η_p^2 =0.032).

Phase 2 First-Order Conditioning. Conditioned freezing to S1 increased across trials similarly for all groups (Figure 6.3a.F). A mixed ANOVA revealed that no main effect of group ($F_{(3, 35)}$= 0.860, p = 0.471, η_p^2 = 0.069), a main effect of trial ($F_{(3, 105)}$= 110.164, p < 0.001, η_p^2 =0.759) and no group x trial interaction ($F_{(3, 105)}$= 0.548, p = 0.836, η_p^2 =0.045).

SPC Test. Freezing to SPC-S2 was higher in the Paired groups relative to the Unpaired, while Paired groups did not differ from each other (Figure 6.3a.G). A two-way ANOVA revealed a main effect of training ($F_{(1, 35)}$= 10.948, p = 0.002, η_p^2 =0.238), no main effect of virus ($F_{(1, 35)}$= 0.452, p = 0.502, η_p^2 = 0.013), and no training x virus interaction ($F_{(1, 35)}$= 10.426, p = 0.518, η_p^2 =0.012). These results further confirmed that reduction in SPC fear in Experiment 6.2a was due to CNO-induced inactivation of the BLA→lOFC pathway as the effect was absent in vehicle-treated rats.

Discussion

In this study, we aimed to show that the disruption obtained in Experiment 6.2a is due to pathway inhibition as a result of CNO infusion in hm4Di-transfected rats. Therefore, vehicle infusions that leave the pathway intact would have no effect. As predicted, we found no difference between hm4Di and mCherry groups. These results provide supporting evidence that SPC disruption obtained in Experiment 6.2a is due to CNO-induced BLA→OFC pathway inactivation.

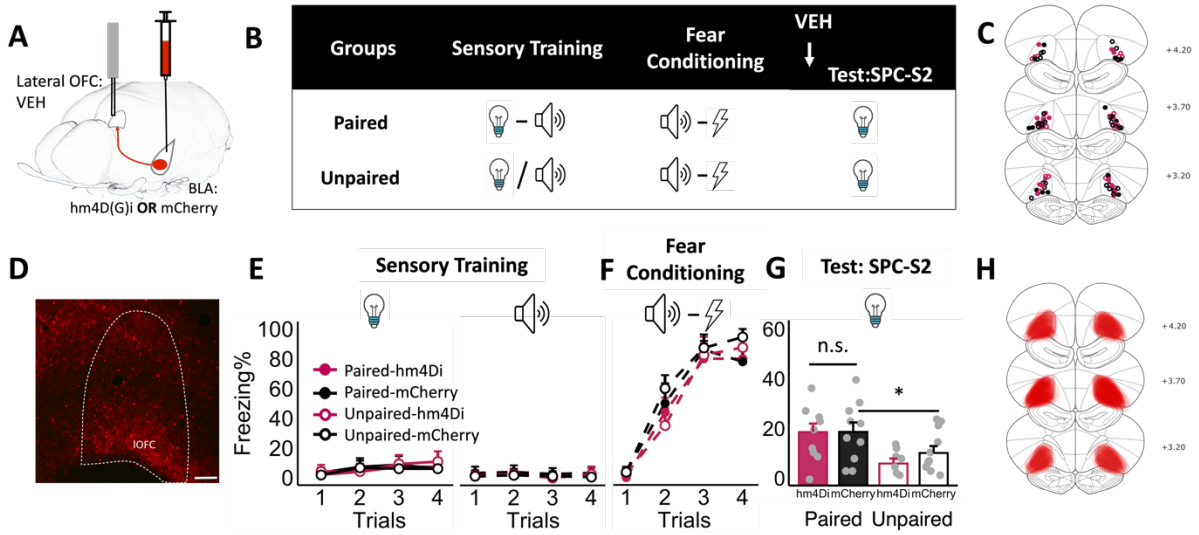

Figure 6.3a. Intact BLA→lOFC pathway in fear to a SPC-S2: A control study. (A) Depiction of viral infusion and cannulation. (B) Behavioural protocol. (C) Cannulae placements in the lOFC, distances from Bregma in mm. (D) Image represents BLA terminals in the lOFC. Scale bar 100µm. (E) Freezing to SPC-S2 (solid lines, left panel) and S1 (dashed lines, right panel) during sensory training. Paired-hm4Di (filled burgundy) n = 10, Paired-mCherry (filled black) n = 10, Unpaired-hm4Di (open burgundy) n = *8*, Unpaired-mCherry (open black) n = 11. (F) Acquisition of conditioned freezing to S1. (G) Freezing to SPC-S2 on test. Vehicle infusions leave BLA→lOFC pathway intact and therefore have no effect on freezing to SPC-S2 (p = 0.999). (H) Viral diffusion in the lOFC. All data are mean freezing percent + SEM.

Experiment 6.3b: Intact BLA→lOFC pathway in fear to SOC-S2: A control study

To show that the effects obtained in Experiment 6.2b are due to CNO-induced pathway inactivation in the hm4Di-transfected rats, and not hm4Di alone, we conducted the same study as that reported in Experiment 6.2a but in the absence of the CNO. We infused all the rats with vehicle prior to test, thus leaving BLA→lOFC pathway intact. We expect there would be no difference

between the Paired groups, irrespective of hm4Di or mCherry. There were four groups in this study: Paired-hm4Di, Paired-mCherry, Unpaired-hm4Di and Unpaired-mCherry.

Methods

Subjects. Forty-one rats were used in this study. Six rats generalized sensory preconditioned fear to SOC-S2 (see Prior History) and were therefore excluded from the analyses. This resulted in the following group numbers: Paired-hm4Di (n = 8), Paired-mCherry (n = 10), Unpaired-hm4Di (n = 7), Unpaired-mCherry (n = 10).

Prior history. Subsequent to the SPC Test in Experiment 6.3a, rats were trained in an SOC protocol using the same stimulus to serve as S1 and a novel stimulus to serve as SOC-S2. First, rats were re-conditioned to S1. Based on S1 re-conditioning performance and behavioural history in SPC phase, they were allocated to Paired and Unpaired SOC groups. That is, equal number of rats in the Paired SOC group had experience with prior paired and unpaired training during SPC. Similarly, equal number of rats in the Unpaired SOC group had experience with paired and unpaired SPC training. Rats that showed more than 20% freezing to SOC-S2 on the first exposure (i.e., first trial of sensory training) were deemed to generalize fear from SPC phase and excluded from the analyses. Finally, rats were tested to SOC-S2 and S1.

Behavioural apparatus and procedure. See the details about stimuli, experimental chambers, and procedures in Chapter 3 – General Methods (Behavioural Apparatus).

Experiment timeline. Behavioural timeline was the same as the timeline in Experiment 6.2b.

Surgery, Drugs and Virus. All rats were transfected with hm4Di or the control mCherry DREADD in the BLA, implanted with cannulae bilaterally in the lOFC, and received vehicle infusions as described in Chapter 3 General Methods (Surgical procedures)..

Histology. All rats were verified for viral transfection of BLA cell bodies and BLA terminals in the lOFC (Figure 6.3b.I) and cannulae placements (Figure 6.3b.C) post-mortem as described in Chapter 3 General Methods (Histology).

Results

Phase 1 Re-training of First-Order Stimulus. Conditioned freezing to S1 was similar among the groups across trials (Figure 6.3b.E). A mixed ANOVA revealed no main effect of group ($F_{(3, 31)}$= 0.386, p = 0.764, η_p^2 =0.036), no main effect of trial ($F_{(3, 93)}$= 1.541, p = 0.209, η_p^2 = 0.048) and no group x trial interaction ($F_{(9, 93)}$ = 1.553, p = 0.141, η_p^2 = 0.131).

Phase 2 Second-Order Conditioning. Freezing to SOC-S2 increased in the Paired groups across trials relative to the Unpaired, while the latter remained low (Figure 6.3b.F, left panel). A mixed ANOVA revealed a main effect of group ($F_{(3, 31)}$= 6.227, p = 0.002, η_p^2 = 0.376), a main effect of trial ($F_{(3, 93)}$ = 14.932, p < 0.001, η_p^2 = 0.325) and a group x trial interaction ($F_{(6, 93)}$ = 2.798, p = 0.006, η_p^2 = 0.213). Post-hoc comparisons with Bonferroni adjustments confirmed higher levels of freezing in the Paired relative to the Unpaired ($M_{\text{Paired}} - M_{\text{Unpaired}}$ = 19.59, p < 0.001, 95% CI [12.76, 26.42], d = 0.959). Freezing to S1 was similar among the groups (Figure 6.3b.F, right panel). An identical analysis for fear to S1 revealed no main effect of group ($F_{(3, 20)}$= 1.199, p = 0.336, η_p^2 < 0.153), no main effect of trial ($F_{(3, 60)}$= 0.903, p = 0.445, η_p^2 = 0.043) and no group x trial interaction ($F_{(9, 60)}$= 0.652, p = 0.748, η_p^2 = 0.089).

SOC-S2 Test. Freezing to SOC-S2 was similar in Paired-hm4Di and Paired-mCherry groups while Paired groups froze more relative to the Unpaired providing evidence for SOC effect (Figure 6.3b.G). A two-way ANOVA revealed a main effect of training ($F_{(1, 31)}$= 34.312, p < 0.001, η_p^2 = 0.526), no main effect of virus ($F_{(1, 31)}$= 0.023, p = 0.881, η_p^2 < 0.001) and no training x virus interaction ($F_{(1, 31)}$= 3.236, p = 0.082, η_p^2 = 0.095).

S1 Test. Freezing to S1 was similar among the groups (Figure 6.3b.H). A two-way ANOVA revealed no main effect of training ($F_{(1, 22)}$= 0.127, p = 0.725, η_p^2 =0.006), no main effect of virus ($F_{(1, 22)}$ < 0.001, p = 0.980, η_p^2 < 0.001) and no training x virus interaction ($F_{(1, 22)}$ = 0.037, p = 0.849, η_p^2 < 0.001).

Discussion

In this study, we aimed to show that the effects obtained in Experiment 6.2b were due to CNO infusion that results in pathway inactivation in the hm4Di transfected rats, and not driven by hm4Di alone. We showed that there was no difference between the hm4Di and the mCherry groups when infused with vehicle. These results provide supporting evidence that SOC disruption obtained in Experiment 6.2b is due to CNO-induced BLA→OFC pathway inactivation.

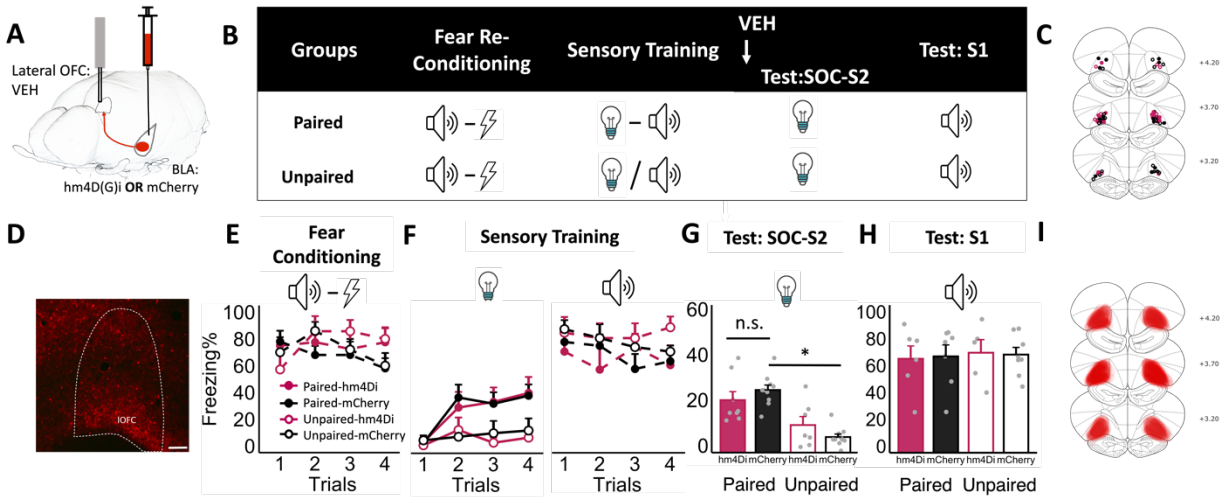

Figure 6.3b. Intact BLA→lOFC pathway in fear to SOC-S2: A control study (A) Depiction of viral infusion and cannulation. (B) Behavioural protocol. (C) Cannulae placements in the lOFC, distances from Bregma in mm. (D) Image represents BLA terminals in the lOFC. Scale bar 100µm. (E) Freezing to S1 during fear (re)conditioning. Paired-hm4Di (filled burgundy) n = 8, Paired-mCherry (filled black) n = 10, Unpaired-hm4Di (open burgundy) n = 7, Unpaired-mCherry (open black) n = 10. (F) Freezing to SOC-S2 (solid lines, left panel) and S1 (dashed lines, right panel) during sensory training. (G) Freezing to SOC-S2 on test. Vehicle infusions leave BLA→lOFC pathway intact and therefore have no effect on freezing to SOC-S2 (p = 0.639). (I) Viral diffusion in the lOFC. All data are mean freezing percent + SEM.

Experiment 6.4a: Inactivation of the lOFC→BLA pathway disrupts fear to a SPC-S2

In this study, we investigated the role of lOFC→BLA pathway in expression of fear to SPC-S2. To do this, we used DREADDs in a manner identical to Experiment 6.2a with the following groups: Paired-hm4Di, Paired-mCherry, Unpaired-hm4Di, Unpaired-mCherry.

Methods

Subjects. Thirty-nine experimentally naïve Sprague-Dawley (Envigo Ltd. Indianapolis, USA) male rats weighing 310-410g prior to viral infusion surgeries were used in this experiment. Two rats were excluded due to incorrect cannulae placements. This yielded following group sizes: Paired-hm4Di (n = 10), Paired-mCherry (n = 9), Unpaired-hm4Di (n = 10), Unpaired-mCherry (n = 8).

Behavioural apparatus and the procedure. See the details about stimuli, experimental chambers, and the procedure in Chapter 3 – General Methods (Behavioural Apparatus).

Experiment timelines. Behavioural protocol was identical to the timeline used in Experiment 6.2a.

Surgery, Drugs and Virus. All rats were transfected with hm4Di DREADD or mCherry control in the lOFC, implanted with bilateral cannulae into the BLA and infused with CNO as described in Chapter 3 General Methods (Surgical procedures).

Histology. All rats were verified for viral transfection of lOFC cell bodies and lOFC terminals in the BLA (Figure 6.4a.H) and cannulae placements (Figure 6.4a.C) were verified post-mortem as described in Chapter 3 General Methods (Histology).

Results

Phase 1 Sensory Preconditioning. Freezing to SPC-S2 was low and similar among the groups (Figure 6.4a.E, left panel). A mixed ANOVA revealed no main effect of group ($F_{(3, 33)}$ = 0.687, p = 0.566, η_p^2= 0.059), a main effect of trial ($F_{(3, 99)}$ = 4.133, p = 0.008, η_p^2= 0.111), and no group x trial interaction ($F_{(9, 99)}$ = 1.221, p = 0.291, η_p^2= 0.100). Similarly, freezing to S1 was low and there were no differences among the groups (Figure 6.4a.E, right panel). An identical analysis of fear to S1 revealed no main effect of group ($F_{(3, 33)}$ = 0.667, p = 0.579, η_p^2= 0.057), no main effect of trial ($F_{(2.36, 77.76)}$ = 0.468, p = 0.646, η_p^2= 0.014) and no group x trial interaction ($F_{(7.07, 77.76)}$ = 0.076, p= 0.582, η_p^2= 0.068).

Phase 2 First-Order Conditioning. Conditioned freezing to S1 increased across fear conditioning trials in all groups (Figure 6.4a.F). A mixed ANOVA revealed no main effect of group ($F_{(3, 33)}$ = 0.782, p = 0.513, η_p^2= 0.067), a main effect of trial ($F_{(2.58, 85.37)}$ = 139.803, p < 0.001, η_p^2= 0.809) and no group x trial interaction ($F_{(7.76, 85.37)}$ = 1.075, p = 0.388, η_p^2= 0.089).

SPC Test. Freezing to SPC-S2 was reduced in Paired-hm4Di relative to Paired-mCherry as a result of pathway inactivation (Figure 6.4a.G). The latter group froze more in comparison to the Unpaired groups, providing evidence for SPC effect (Figure 6.1f). A two-way ANOVA revealed a main effect of training ($F_{(1, 33)}$ = 50.952, p < 0.001, η_p^2= 0.606), a main effect of virus ($F_{(1, 33)}$ = 9.617, p = 0.003, η_p^2= 0.225) and a training x virus interaction ($F_{(1, 33)}$ = 4.798, p = 0.035, η_p^2 = 0.127). Post-hoc analyses with Bonferroni adjustment confirmed disruption of fear to SPC-S2 in the Paired-hm4Di relative to the Paired-mCherry ($M_{\text{hm4Di}} - M_{\text{mCherry}}$ = -18.882, p = 0.008, 95% CI [-32.122, -5.643], d = 1.38). The latter group froze more relative to the Unpaired-mCherry group evidencing an SPC effect ($M_{\text{Paired}} - M_{\text{Unpaired}}$ = 33.287, p < 0.001, 95% CI [19.72, 46.85], d = 2.98). The Paired-hm4Di froze more than the Unpaired-hm4Di ($M_{\text{Paired-hm4Di}} - M_{\text{Unpaired-hm4Di}}$ = 17.66, p = 0.003, 95% CI [5.17, 30.14], d = 1.85).

Discussion

In this study, we investigated the role of lOFC→BLA pathway in expression of fear to SPC-S2. Inactivating this pathway using DREADDs prior to test disrupted SPC (Figure 6.4a.G) and did so completely by reducing responding to the levels of the unpaired controls. These findings demonstrate that lOFC inputs to the BLA are necessary for sensory preconditioned fear. These findings demonstrate that lOFC inputs to the BLA are necessary for sensory preconditioned fear.

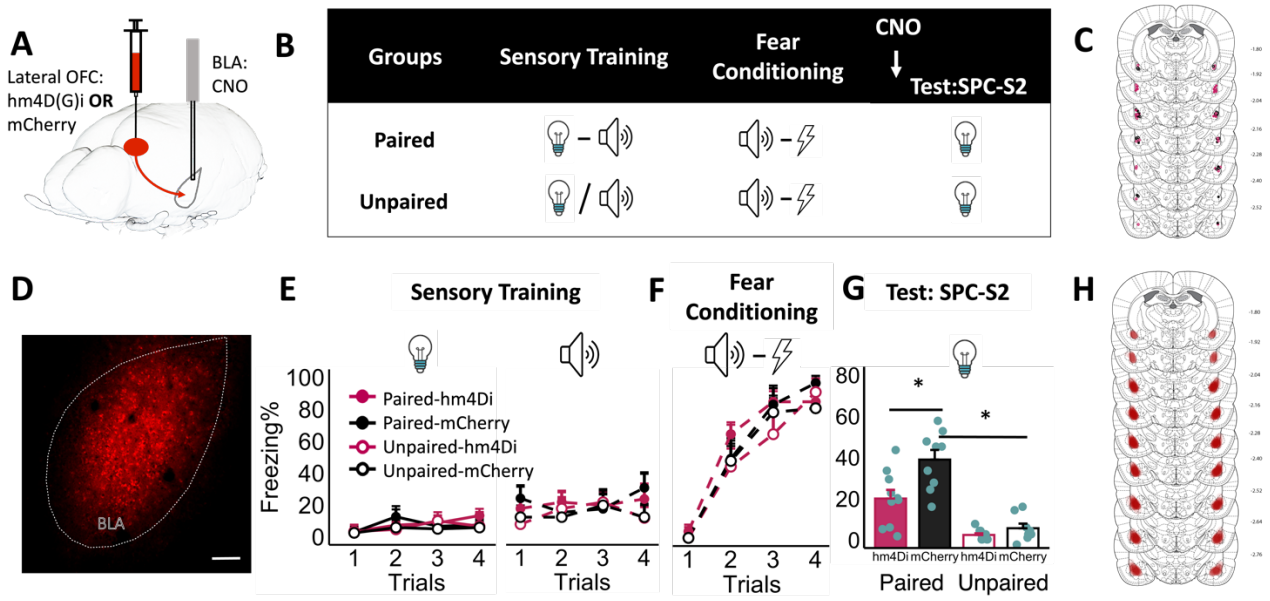

Figure 6.4a. Inactivation of the lOFC→BLA pathway disrupts fear to a SPC-S2. (A) Depiction of viral infusion and cannulation. (B) Behavioural protocol. (C) Cannulae placements in the BLA, distances from Bregma in mm. (D) Image represents lOFC terminals in the BLA. Scale bar is 100µm. (E) Freezing to SPC-S2 (solid lines, left panel) and S1 (dashed lines, right panel) during sensory training. Paired-hm4Di (filled burgundy) n = 10, Paired-mCherry (filled black) n = 9, Unpaired-hm4Di (open burgundy) n = 10, Unpaired-mCherry (open black) n = 8. (F) Acquisition of conditioned freezing to S1. (G) Freezing to SPC-S2 on test. Chemogenetic inactivation of lOFC→BLA pathway disrupts freezing to SPC-S2 (p = 0.008). (H) Viral diffusion in the BLA. All data are mean freezing percent + SEM.

Experiment 6.4b: Inactivation of the lOFC→BLA pathway spares fear to SOC-S2

In this study, we investigated the role of lOFC→BLA pathway in expression of fear to SOC-S2. To do this, we used DREADDs in a manner identical to Experiment 6.2b with the following groups: Paired-hm4Di, Paired-mCherry, Unpaired-hm4Di, Unpaired-mCherry.

Methods

Subjects. Forty rats were used in this experiment. One rat generalized sensory preconditioned fear to SOC-S2, therefore, excluded from the analyses. This yielded the following group sizes: Paired-hm4Di (n = 9), Paired-mCherry (n = 9), Unpaired-hm4Di (n = 12), Unpaired-mCherry (n = 9).

Prior history. Subsequent to the SPC Test in Experiment 6.3a, rats were trained in an SOC protocol using the same stimulus to serve as S1 and a novel stimulus to serve as SOC-S2. First, rats were re-conditioned to S1. Based on S1 re-conditioning performance and behavioural history in SPC phase, they were allocated to Paired and Unpaired SOC groups. That is, equal number of rats in the Paired SOC group had experience with prior paired and unpaired training during SPC. Similarly, equal number of rats in the Unpaired SOC group had experience with paired and unpaired SPC training. Rats that showed more than 20% freezing to SOC-S2 on the first exposure (i.e., first trial of sensory training) were deemed to generalize fear from SPC phase and excluded from the analyses. Finally, rats were tested to SOC-S2 and S1.

Behavioural apparatus and procedure. See the details about stimuli and experimental chambers in Chapter 3 – General Methods (Behavioural Apparatus).

Experiment timeline. Behavioural timeline was the same as the timeline in Experiment 6.2b.

Surgery, Drugs and Virus. All rats were transfected with hm4Di DREADD or mCherry control in the lOFC, implanted with bilateral cannulae into the BLA and infused with CNO as described in Chapter 3 General Methods (Surgical procedures).

Histology. All rats were verified for viral transfection of lOFC cell bodies and lOFC terminals in the BLA (Figure 6.4b.I) and cannulae placements (Figure 6.4b.C) post-mortem as described in Chapter 3 General Methods (Histology).

Results

Phase 1 Re-training of First-Order Stimulus. Conditioned freezing to S1 remained high among the groups (Figure 6.4b.E). A mixed ANOVA revealed no main effect of group ($F_{(3, 35)}$ = 0.150, p =0.929, η_p^2= 0.013), no main effect of trial ($F_{(3, 105)}$ = 1.283, p =0.284, η_p^2= 0.036) nor a group x trial interaction ($F_{(9, 105)}$ = 0.796, p = 0.621, η_p^2 = 0.064).

Phase 2 Second-Order Conditioning. Conditioned freezing to SOC-S2 increased in the Paired groups relative to the Unpaired groups which remained low (Figure 6.4b.F, left panel). A

mixed ANOVA revealed a main effect of group ($F_{(3, 35)}$ = 9.756, $p < 0.001$, η_p^2= 0.456), a main effect of trial ($F_{(3, 105)}$ = 22.622, $p < 0.001$, η_p^2= 0.393), and a group x trial interaction ($F_{(3, 105)}$ = 3.555, $p < 0.001$, η_p^2= 0.234). Post-hoc comparisons with Bonferroni adjustments confirmed higher freezing in the Paired relative to the Unpaired groups ($M_{\text{Paired}} - M_{\text{Unpaired}}$ = 19.56, $p < 0.001$, 95% CI [13.24, 25.88], d = 0.982). Freezing to S1 was similar across the groups (Figure 6.4b.F, right panel). An identical analysis of freezing to S1 revealed no main effect of group ($F_{(3, 35)}$ = 0.130, p = 0.288, η_p^2= 0.100), a main effect of trial indicating some reduction of the fear response due to non-reinforcement ($F_{(3, 105)}$ = 5.143, p = 0.003, η_p^2= 0.128) but no group x trial interaction ($F_{(9, 105)}$ = 0.378, p = 0.944, η_p^2= 0.031).

SOC-S2 Test. Freezing to SOC-S2 was equivalent between Paired-hm4Di and Paired-mCherry groups suggesting that silencing lOFC→BLA pathway prior to test had no effect on expression of fear to SOC-S2 (Figure 6.4b.G). Paired-mCherry froze more relative to the Unpaired groups, providing evidence for the SOC effect. A two-way ANOVA revealed a main effect of training ($F_{(1, 35)}$= 15.014, $p < 0.001$, η_p^2 =0.300), no main effect of virus ($F_{(1, 35)}$= 0.067, p = 0.798, η_p^2 = 0.002) and no training x virus interaction ($F_{(1, 35)} < 0.001$, p = 0.988, $\eta_p^2 < 0.001$).

S1 Test. Freezing to S1 was similar across all groups on test (Figure 6.4b.H). A two-way ANOVA revealed no main effect of training ($F_{(1, 35)}$= 0.023, p = 0.882, η_p^2 <0.001), no main effect of virus ($F_{(1, 35)}$= 0.011, p = 0.918, $\eta_p^2 < 0.001$) and training x virus interaction ($F_{(1, 35)}$= 0.600, p = 0.444, η_p^2 =0.017).

Discussion

In this study, we aimed to determine whether of lOFC→BLA pathway is involved in the expression of fear to SOC-S2. Silencing this pathway using DREADDs prior to test had no effect on expression of fear to SOC-S2 (Figure 6.4b.G). These findings demonstrate that lOFC inputs to the BLA are not necessary for second-order fear.

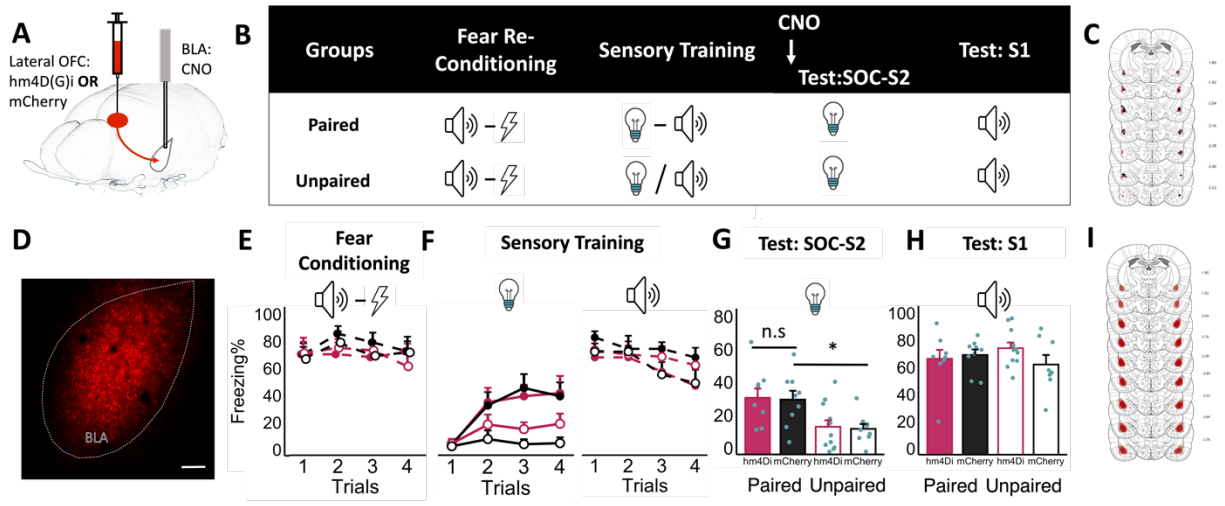

Figure 6.4b. Inactivation of the lOFC→BLA pathway has no effect on fear to a SOC-S2. (A) Depiction of viral infusion and cannulation. (B) Behavioural protocol. (C) Viral diffusion in the BLA (D) Image represents lOFC terminals in the BLA. Scale bar 100µm. (E) Freezing to S1 during fear (re)conditioning (F) Freezing to SOC-S2 (solid lines, left panel) and S1 (dashed lines, right panel) during sensory training. Paired-hm4Di (filled burgundy) n = 9, Paired-mCherry (filled black) n = 9, Unpaired-hm4Di (open burgundy) n = 12, Unpaired-mCherry (open black) n = 9. (F) Acquisition of conditioned freezing to S1. (G) Freezing to SOC-S2 on test. Chemogenetic inactivation of lOFC→BLA pathway has no effect on freezing to SOC-S2 (p = 0.993). (H) Freezing to S1 on test. (I) Cannulae placements in the BLA, distances from Bregma in mm. All data are mean freezing percent + SEM.

Experiment 6.5a: Intact lOFC→BLA pathway in fear to SPC-S2: A control study

In this study, we aimed to show that the effects obtained in Experiment 6.4a are due to CNO-induced pathway inactivation in the hm4Di-transfected rats and not driven by the viral construct. Therefore, we used the same design as Experiment 6.4a but left lOFC→BLA pathway intact via vehicle infusions prior to SPC-S2 test. This resulted in the following groups: Paired-hm4Di, Paired-mCherry, Unpaired-hm4Di, Unpaired-mCherry.

Methods

Subjects. Forty-two experimentally naïve Sprague-Dawley (Envigo Ltd. Indianapolis, USA) male rats weighing 320-470g prior to viral infusion surgeries were used in this experiment. Ten rats were excluded due to incorrect cannulae placements. This yielded the following group sizes: Paired-hm4Di (n = 12), Paired-mCherry (n = 13), Unpaired-hm4Di (n = 9), Unpaired-mCherry (n = 8).

Behavioural apparatus and the procedure. See the details about stimuli, experimental chambers, and the procedure in Chapter 3 General Methods (Behavioural Apparatus).

Experiment timeline. Behavioural timeline was same as the timeline in Experiment 6.2a.

Surgery, Drugs and Virus. All rats were transfected with hm4Di DREADD or mCherry control in the lOFC, implanted with bilateral cannulae into the BLA and infused with vehicle as described in Chapter 3 General Methods (Surgical procedures)..

Histology. All rats were verified for viral transfection of lOFC cell bodies and lOFC terminals in the BLA (Figure 6.5a.H) and cannulae placements (Figure 6.5a.C) post-mortem as described in Chapter 3 General Methods (Histology).

Results

Phase 1 Sensory Preconditioning. Freezing to SPC-S2 was low and similar across groups (Figure 6.5a.E, left panel). A mixed ANOVA revealed no main effect of group ($F_{(3, 38)}$= 0.469, p = 0.706, η_p^2 =0.036), a main effect of trial ($F_{(2.40, 91.19)}$ = 7.065, p < 0.001, η_p^2 = 0.157) but no group x trial interaction ($F_{(7.20, 91.19)}$ = 1.050, p =0.403, η_p^2 = 0.077). Freezing to S1 was low and similar across groups (Figure 6.5a.E, right panel). An identical analysis for freezing to S1 revealed no main effect of group ($F_{(3, 38)}$= 1.927, p = 0.142, η_p^2 =0.132), trial ($F_{(2.44, 92.72)}$ = 1.606, p = 0.201, η_p^2 =0. 041), nor a group x trial interaction ($F_{(7.32, 92.72)}$ = 1.715, p = 0.112, η_p^2 =0.119).

Phase 2 First-Order Conditioning. Conditioned freezing to S1 increased across trials similarly among the groups (Figure 6.5a.F). A mixed ANOVA revealed no main effect of group ($F_{(3, 38)}$ = 0.617, p = 0.609, η_p^2 = 0.047), a main effect of trial ($F_{(1.84, 70.02)}$= 153.900, p < 0.001, η_p^2 = 0.802) and no group x trial interaction ($F_{(5.53, 70.02)}$= 0.530, p = 0.769, η_p^2 = 0.040).

SPC-S2 Test. Freezing to SPC-S2 was higher in the Paired relative to the Unpaired groups. As expected, vehicle infusions had no effect on freezing and hm4Di groups showed equivalent levels of fear as the mCherry controls (Figure 6.5a.G). A two-way ANOVA revealed a main effect of training ($F_{(1, 38)}$ = 15.00, p < 0.001, η_p^2= 0.283), no main effect of virus ($F_{(1, 38)}$ = 1.180, p = 0.284, η_p^2= 0.030) and no training x virus interaction ($F_{(1, 38)}$ = 0.675, p = 0.417, η_p^2= 0.018).

Discussion

In this study, we aimed to show that in the absence of CNO infusions, hm4Di-transfected rats would show normal fear to an SPC-S2. As expected, vehicle infusions had no effect on fear expression to SPC-S2 (Figure 6.5a.G). These results provide supporting evidence that SPC

disruption obtained in Experiment 6.4a are due to CNO-induced inactivation of lOFC→BLA pathway.

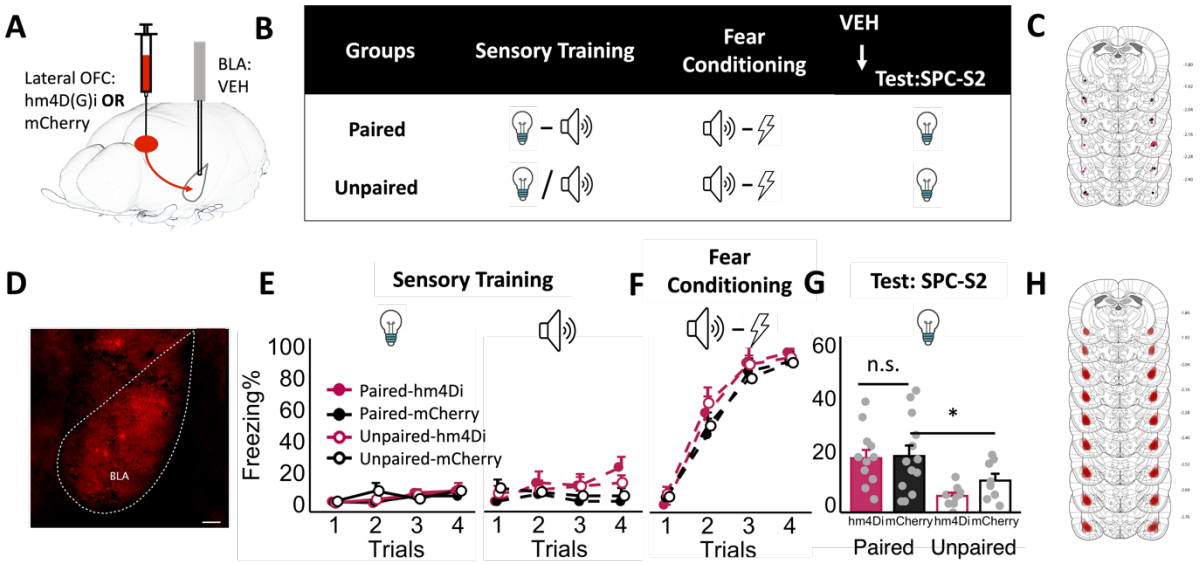

Figure 6.5a. Intact lOFC→BLA pathway has no effect on fear to a SPC-S2. (A) Depiction of viral infusion and cannulation. (B) Behavioural protocol. (C) Cannulae placements in the BLA, distances from Bregma in mm. (D) Image represents lOFC terminals in the BLA. Scale bar 100µm. (E) Freezing to SPC-S2 (solid lines, left panel) and S1 (dashed lines, right panel) during sensory training. Paired-hm4Di (filled burgundy) n = 12, Paired-mCherry (filled black) n = 13, Unpaired-hm4Di (open burgundy) n = 9, Unpaired-mCherry (open black) n = 8. (F) Acquisition of conditioned freezing to S1. (G) Freezing to SPC-S2 on test. Vehicle infusions leave lOFC→BLA pathway intact and therefore, have no effect on freezing to SPC-S2 (p = 0.997). (H) Viral diffusion in the BLA. All data are mean freezing percent + SEM.

Experiment 6.5b: Intact lOFC→BLA pathway in fear to SOC-S2: A control study

In this study, we sought out to confirm similar performance when the lOFC→BLA pathway is left intact as there was no effect of pathway silencing in Experiment 6.4b. To do this, we used the same design as Experiment 6.4b and left lOFC→BLA pathway intact via vehicle infusions prior to SOC-S2 test. This resulted in the following groups: Paired-hm4Di, Paired-mCherry, Unpaired-hm4Di, Unpaired-mCherry.

Methods

Subjects. Thirty-nine rats were used in this experiment. Two rats generalized sensory preconditioned fear to SOC-S2, and therefore, excluded from the analyses. This yielded the

following group sizes: Paired-hm4Di (n = 10), Paired-mCherry (n = 12), Unpaired-hm4Di (n = 8), Unpaired-mCherry (*n* = 7).

Prior history. Subsequent to the SPC Test in Experiment 6.3a, rats were trained in an SOC protocol using the same stimulus to serve as S1 and a novel stimulus to serve as SOC-S2. First, rats were re-conditioned to S1. Based on S1 re-conditioning performance and behavioural history in SPC phase, they were allocated to Paired and Unpaired SOC groups. That is, equal number of rats in the Paired SOC group had experience with prior paired and unpaired training during SPC. Similarly, equal number of rats in the Unpaired SOC group had experience with paired and unpaired SPC training. Rats that showed more than 20% freezing to SOC-S2 on the first exposure (i.e., first trial of sensory training) were deemed to generalize fear from SPC phase and excluded from the analyses. Finally, rats were tested to SOC-S2 and S1.

Behavioural apparatus and procedure. See the details about stimuli and experimental chambers in Chapter 3 General Methods (Behavioural Apparatus).

Experiment timeline. Behavioural timeline was same as the timeline in Experiment 6.3b.

Surgery, Drugs and Virus. All rats were transfected with hm4Di DREADD or mCherry control in the lOFC, implanted with bilateral cannulae into the BLA and infused with vehicle as described in Chapter 3 General Methods (Surgical procedures).

Histology. All rats were verified for viral transfection of lOFC cell bodies and lOFC terminals in the BLA (Figure 6.5b.I) and cannulae placements (Figure 6.5b.C) post-mortem as described in Chapter 3 General Methods (Histology).

Results

Phase 1 Re-training of First-Order Stimulus. Freezing to S1 remained high and similar across groups (Figure 6.5b.E). A mixed ANOVA revealed no main effect of group ($F_{(3, 33)} = 0.287$, $p = 0.596$, $\eta_p^2 = 0.009$), a main effect of trial indicating an increase in fear across trials ($F_{(9, 99)} = 4.419$, $p = 0.006$, $\eta_p^2 = 0.118$) and no group x trial interaction ($F_{(3, 99)} = 1.508$, $p = 0.156$, $\eta_p^2 = 0.121$).

Phase 2 Second-Order Conditioning. Conditioned freezing to SOC-S2 increased across trials in the Paired groups relative to the Unpaired groups, while the latter remained low (Figure 6.5b.F, left panel). A mixed ANOVA revealed a main effect of group ($F_{(3, 33)} = 11.447$, $p < 0.001$, $\eta_p^2 = 0.510$), a main effect of trial ($F_{(2.62, 86.30)} = 20.010$, $p < 0.001$, $\eta_p^2 = 0.378$) and a group x trial

interaction ($F_{(7.85,\ 86.30)}$ = 4.464, p < 0.001, η_p^2= 0.289). Post-hoc comparisons adjusted with Bonferroni confirmed higher levels of freezing in the Paired relative to the Unpaired groups ($M_{\text{Paired}} - M_{\text{Unpaired}}$ = 15.46, p < 0.001, 95% CI [9.88, 21.03], d = 0.918). Freezing to S1 was similar across the groups (Figure 6.5b.F, right panel). An identical analysis revealed no main effect of group ($F_{(3,\ 33)}$ = 0.390, p = 0.760, η_p^2 = 0.034), a main effect of trial ($F_{(3,\ 99)}$ = 2.784, p = 0.045, η_p^2= 0.078) and no group x trial interaction ($F_{(9,\ 99)}$ = 0.69, p = 0.716, η_p^2= 0.059).

SOC-S2 Test. Freezing to SOC-S2 was equivalent in the Paired-hm4Di and the Paired-mCherry groups, while the latter froze more relative to the Unpaired groups (Figure 6.5b.G). A two-way ANOVA revealed a main effect of training ($F_{(1,\ 33)}$ = 30.202, p<0.001, η_p^2= 0.478), no effect virus ($F_{(1,\ 33)}$ = 0.264, p = 0.610, η_p^2= 0.008) and no training x virus interaction ($F_{(1,\ 33)}$ = 0.010, p = 0.920, η_p^2 < 0.001).

S1 Test. Freezing to S1 was similar among the groups (Figure 6.5b.H). A two-way ANOVA did not detect main effects of training ($F_{(1,\ 33)}$ = 0.290, p = 0.594, η_p^2 = 0.009), virus ($F_{(1,\ 33)}$ = 0.002, p = 0.963, η_p^2 < 0.001), nor training x virus interaction $F_{(1,\ 33)}$ = 0.448, p = 0.508, η_p^2=0.014).

Discussion

In this study, we aimed to confirm similar performance when lOFC→BLA is left intact to that in Experiment 6.4b, given that silencing lOFC→BLA via CNO infusions had no effect on fear to SOC-S2. As expected, vehicle infusions had no effect on fear expression to SOC-S2, providing support for the results obtained in Experiment 6.4b that lOFC inputs to the BLA are not necessary for second-order fear.

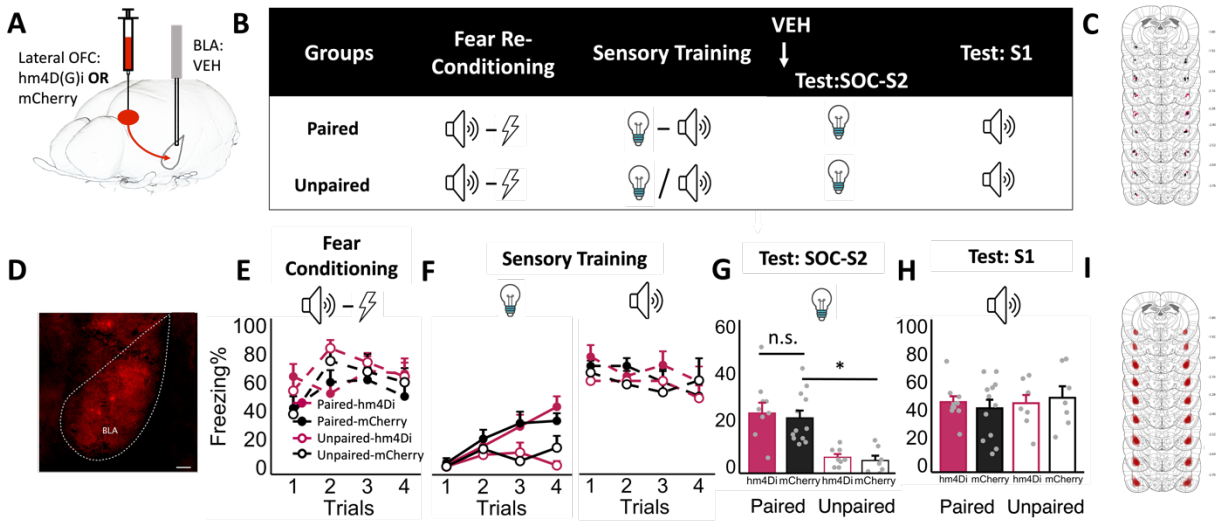

Figure 6.5b. Intact lOFC→BLA pathway has no effect on fear to a SOC-S2. (A) Depiction of viral infusion and cannulation. (B) Behavioural protocol. (C) Cannulae placements in the BLA, distances from Bregma in mm. (C) Image represents lOFC terminals in the BLA. Scale bar 100µm. (E) Freezing to S1 during fear (re)conditioning. Paired-hm4Di (filled burgundy) n = 10, Paired-mCherry (filled black) n = 12, Unpaired-hm4Di (open burgundy) n = 8, Unpaired-mCherry (open black) n = 7. (F) Freezing to SOC-S2 (solid lines, left panel) and S1 (dashed lines, right panel) during sensory training. (G) Freezing to SOC-S2 on test. Vehicle infusions leave lOFC→BLA pathway intact and therefore have no effect on freezing to SOC-S2 (p = 0.997). (I) Viral diffusion in the BLA. All data are mean freezing percent + SEM.

General Discussion

This chapter investigated how distinct and overlapping SPC- and SOC-activated neural populations in the BLA regulate fear to SPC-S2 and SOC-S2 in communication with the lOFC. We identified three main ensembles in the BLA: a unique SPC ensemble, a unique SOC ensemble and an overlapping ensemble that showed activity as a result of both types of stimuli (Figure 6.1H). We showed that, half the cells in each ensemble had projections to the lOFC as evidenced by retrograde viral signal. Subsequently, we showed that silencing BLA→lOFC pathway disrupt fear to SPC-S2 and attenuate fear to SOC-S2. These data show that neuronal populations in the BLA regulate fear to SPC-S2 and SOC-S2 while the BLA inputs to the lOFC are necessary for the expression both types of fear. In contrast, silencing the reverse pathway, lOFC→BLA, disrupted expression of fear to SPC-S2 but not to SOC-S2. Importantly, we confirmed that the effects obtained in pathway studies were due to CNO-induced pathway inactivation in the hm4Di-transfected groups, as control studies with vehicle infusions did not show any differences between hm4Di and the mCherry groups.

As discussed in Chapters 2 and shown in Chapters 4 and 5, SPC is supported by neural activity in the BLA and the lOFC. Our RNAscope findings confirmed a population of neurons in the BLA that are engaged subsequent to SPC-S2 test and half of these neurons had projections to the lOFC that were crucial for the expression of SPC-fear (Experiment 6.2a). Research in the appetitive domain show that BLA→lOFC function as providing a stimulus evoked US (S1→US) representation to the lOFC (Schoenbaum et al., 2003a; Lichtenberg et al., 2017). Indeed, fear to SPC-S2 critically depends on S1→US memory in the BLA (Experiments 4.1 and 4.4). Therefore, silencing BLA inputs to the lOFC interferes with S1→US memory. This interpretation necessitates the activation of S1→US memory on tests with SPC-S2 despite the absence of S1 delivery. This activation occurs associatively, given S2→S1 learning. That is, test presentations of SPC-S2 retrieves a representation of its S1-associate, which in turn activates S1→US ensemble in the BLA. Our RNAscope data suggest that S2→S1 association is somehow activated in the BLA given SPC-S2 evoked neuronal activity at test. We reasoned that the lOFC provides S2→S1 association to the BLA for two reasons. Firstly, evidence show that neural activity in the lOFC is crucial for S2→S1 learning, optogenetic inhibition during this phase disrupted appetitive SPC (Hart et al., 2020). Given that the cues are neutral during S2→S1 pairings in SPC, the role of lOFC would be equally crucial in an aversive design as it is in an appetitive design. Secondly, evidence shows that the lOFC provides information to the BLA about complex associative relationships (Schoenbaum et al., 2000; Lucantonio et al., 2015; Sias et al., 2021). Supporting our reasoning, silencing lOFC inputs to the BLA (Experiment 6.4a) disrupted the expression of fear to SPC-S2. Taken together, our pathway inactivation data show that projections between the lOFC and the BLA are crucial in the expression of fear to SPC-S2 and suggest that BLA→lOFC pathway gates S1→US association while lOFC→BLA is involved in providing S2→S1 association, suggesting that the pathways form a circuit.

Our RNAscope findings revealed a unique SOC ensemble and an overlapping SPC ∩ SOC ensemble. Importantly, half of these populations had projections to the lOFC. Silencing this BLA→lOFC population attenuated fear to SOC-S2 suggesting that the BLA sends inputs to the lOFC about S2→fear_state associations. Albeit attenuated, SOC was still partially supported given higher levels of fear in the Paired-hm4Di relative to the Unpaired-hm4Di. This may be due to SOC-unique ensemble supporting behaviour via S2→fear_state associations. Alternatively, this effect may be due to higher levels of fear in the Paired groups to begin with. That is, although

S2→fear_state association is suppressed via BLA→lOFC silencing, responding may not go down to the level of the Unpaired groups.

In contrast to BLA→lOFC pathway, silencing lOFC→BLA had no effect on fear to SOC-S2. As discussed above, lOFC is shown to provide complex associative relationships to the BLA and it would be predicted to provide S-S associations given its role in encoding such associations (Hart et al., 2020; Sadacca et al., 2018). Therefore, one of the predictions about this pathway was that it may be gating S→no_US association that we uncovered in Experiment 5.2. Our pathway inactivation (Experiment 6.4b) did not show evidence for the role of lOFC→BLA in the expression S2→no_US association. This effect might be mediated by some polysynaptic projections from the lOFC to the BLA or in other cortical areas that are involved in S-S learning (Hart et al., 2020). These possibilities are further discussed in Chapter 7.

In this chapter, we investigated the BLA ensembles and identified unique populations for SPC and SOC, and a population that guides both types of fear (i.e, SPC ∩ SOC). In each of these populations equivalently sized subpopulations showed projections to the lOFC. We investigated the role of these lOFC-projecting cells in fear to SPC-S2 and SOC-S2 using inhibitory DREADDs to target BLA terminals in the lOFC. We showed that BLA→lOFC is important for the expression of both types of fear while, lOFC→BLA is necessary for fear expression to an SPC-S2, but not to an SOC-S2. We consider these findings in terms of the associations that guide behaviour. BLA→lOFC provides S1→US association in SPC and S2→fear_state association in SOC, while lOFC→BLA provide information about the associative relations between stimuli, which is S-S in nature.

Chapter 7 General Discussion

The present thesis investigated the behavioural and neural basis of two types of higher-order fear conditioning: sensory preconditioning (SPC) and second-order conditioning (SOC). In a series of behavioural and neural manipulation studies, we show that an SPC-S2 and an SOC-S2 elicit fear as a result of their pairings with S1, but once learned, they differentially depend on S1 for fear expression. To elaborate, extinguishing fear to S1 disrupted fear to an SPC-S2 but not an SOC-S2 (Experiments 4.1 and 4.2). Chemogenetic deletion of BLA neurons activated by the previously fear conditioned S1 disrupted fear to an SPC-S2 but not to an SOC-S2. We further probed the neurobiological basis of SPC and SOC by silencing neural activity in lOFC. Our results

showed that pharmacological inactivation of the lOFC disrupted expression of fear to SPC-S2 (Experiment 5.1) but enhanced fear expression to SOC-S2 (Experiment 5.2) in male rats but had no effect in female rats. We investigated the neuronal populations in the BLA that engage in the expression of fear to SPC-S2 and SOC-S2 and identified overlapping and unique neuronal populations. Importantly, a subpopulation of these ensembles had projections to the lOFC and silencing this BLA→lOFC pathway disrupted both types of fear. Silencing the reverse, lOFC→BLA pathway disrupted SPC but had no effect on SOC. Our findings delineate neurobiological structures differentially supporting SPC and SOC types of fear and characterize the role of lOFC in the fear circuit.

Dependence of SPC and SOC on first-order conditioning

Sensory preconditioning and second-order conditioning critically depend on pairings between S2 and S1 during sensory training. As a result of such pairings, fear conditioned S1 imbue fear to S2 in both tasks. Although S2 elicits fear by virtue of its associations with S1 in both tasks, extinguishing fear to S1 subsequent to training but prior to test reduce expression of fear to an SPC-S2 but not to an SOC-S2 (Rizley & Rescorla, 1972). Put differently, fear to SPC-S2 is dependent on intact fear to S1 while fear to SOC-S2 persists even when fear to S1 is reduced. As discussed in Chapters 1 and 4, this difference is due to *how* S2 is imbued with fear. That is, sensory training between SPC-S2 and S1 occur when the stimuli are neutral, forming an S-S association (i.e., S2→S1 association). Fear accrues to SPC-S2 by virtue of its associations with S1 *after* the latter is fear conditioned. Similarly, when S1 no longer reliably evokes fear as a result of extinction, nor does its S2-associate. In contrast, sensory training between SOC-S2 and S1 occur when the latter is already fear conditioned, and S1-evoked fear accrues to SOC-S2 *during* sensory training (i.e., S2→fear_state; Holland, 1977; Rescorla, 1979; Gewirtz & Davis, 2000; Winterbauer & Balleine, 2005). As a result, SOC-S2 is directly linked with the (S1-evoked) fear_state and its expression is not dependent on the fear eliciting properties of S1.

Our neurobiological investigations with the *fos-LacZ* rats revealed the same pattern: expression of fear to an SPC-S2 relied on intact S1-activated neuronal ensemble in the BLA whilst fear to an SOC-S2 did not. As discussed in Chapter 4, disruption of SPC as a result of S1-ensemble deletion suggests an overlapping neuronal population between SPC-S2- and S1-ensembles in the BLA and that this ensemble is crucial in driving behaviour. In contrast, intact SOC-fear following

S1-ensemble deletion suggests that SOC is driven by a subset of neurons in the BLA (Parkes & Westbrook, 2010) that are distinct from the S1-ensemble. These data show parallels with our behavioural findings: reduction in conditioned fear to S1 behaviourally via extinction or by direct deletion of S1-ensemble in the BLA similarly disrupt sensory preconditioned fear while having no effect on second-order conditioned fear. Therefore, neural representations of SPC and SOC type fears in the BLA reflect the nature of associations that guide behaviour and are distinct from each other.

Our examination of the BLA neurons using RNAscope (Experiment 6.1) confirmed neuronal populations that engage in the expression of both types of fear and showed unique SPC and SOC populations as well as an overlapping SPC $\cap$ SOC population. The overlapping population may be engaged due to stimulus similarity (i.e., same modality) and the functional importance of these overlapping neurons remains to be determined. More importantly, the presence of unique populations provide evidence that SPC and SOC type fears are regulated by distinct populations in the BLA in line with our *fos-LacZ* findings. Furthermore, we showed that these unique SPC and SOC populations in the BLA had projections to the lOFC and silencing the BLA→lOFC pathway disrupted SPC *and* SOC (Experiments 6.2a and 6.2b).

The role of BLA→lOFC pathway in SPC and SOC

Fear expression to SPC-S2 and SOC-S2 engage distinct neuronal populations in the BLA. However, both of these populations send inputs to the lOFC, which are crucial for the expression of both types of fear (Experiments 6.2a and 6.2b). In SPC, we reason that BLA→lOFC pathway provides S1→US information to the lOFC and blocking the reach of this information disrupts fear to SPC-S2. The reason is several folds. Firstly, SPC critically depends on the integrity of S1→US association (Experiments 4.1 and 4.4; Holmes et al., 2013; Rizley & Rescorla, 1972). The BLA is a key region for the learning and the expression (Experiment 4.3) of S1→US association across domains (aversive: LeDoux et al., 1990; Davis & Smith, 1992; Killcross et al., 1997; appetitive: Hatfield et al., 1996; Pickens et al., 2003; Murray & Izquierdo, 2007). Furthermore, the lOFC is involved in the integration of associative information acquired in separate phases of training (Experiment 5.1; Jones et al., 2012; Panayi & Killcross, 2018; Parkes et al., 2018) and it is

important for S2→S1 learning (Hart et al., 2020). Therefore, lOFC is a great candidate to receive S1→US information from the BLA to support the expression of fear to SPC-S2 on test.

The interpretation above suggests that S1→US association is somehow activated in the BLA during SPC test, despite the absence of S1. This can occur associatively, S2 presentation activates the representation of its S1-associate, hence the S1-ensemble in the BLA. In line with prior evidence which showed that the BLA is crucial for the expression of SPC-fear (Parkes & Westbrook, 2010), we identified an SPC-ensemble in the BLA (Experiment 6.1) and demonstrated that this SPC-ensemble overlaps with the S1 ensemble (Experiment 4.4). Although the extent of this overlap be it complete or partial remains to be determined they are crucial in driving SPC. By virtue of this overlap, presentation of SPC-S2 on test likely activates SPC-ensemble as well as the S1-ensemble, and in turn these neurons send S1→US association to the lOFC. Therefore, silencing the BLA→lOFC pathway disrupts fear to SPC-S2.

Silencing BLA→lOFC pathway also disrupt SOC, however, unlike in SPC the information sent to the lOFC is not S1→US association. This is because the expression of fear to SOC-S2 proceeds irrespective of the status of S1-evoked fear, SOC is driven by S2→fear_state associations (Experiments 4.2 and 4.5). Neuronal activity in the BLA is necessary for the acquisition and expression of these associations (Lay et al., 2018; Parkes & Westbrook, 2010). Therefore, SOC-ensembles that we identified in the BLA (Experiment 6.1) signal S2→fear_state associations. Silencing the subset of neurons that send projections to lOFC interfere with signalling S2→fear_state information and disrupt SOC. Despite reduced, SOC was not *completely* disrupted. That is, rats that received BLA→lOFC pathway inactivation still showed SOC behaviourally. This residual SOC effect can be attributed to high levels of fear in the Paired groups and that silencing the pathway cannot suppress conditioned fear to the level of the Unpaired groups. Alternatively, S2→fear_state association may be partitioned between the two subsets of the SOC-ensemble: BLA→lOFC neurons and those that do not send projections to the lOFC. In this manner, the latter likely supports the effect to a lower degree when the BLA→lOFC pathway is silenced.

The role of lOFC in higher-order fear expression

Our BLA→lOFC pathway inactivation results showed that the BLA inputs to the lOFC are required for the expression of fear to SPC-S2 *and* SOC-S2, although these inputs support fear

based on distinct, S1→US and S2→fear_state associations, respectively. So, what is the contribution of lOFC in regulation of fear to SPC-S2 and SOC-S2? Lateral OFC is shown to be crucial for integrating associative information in various settings including appetitive SPC but not SOC (Hart et al., 2020; Howard et al., 2020; Izquierdo et al., 2004; Jones et al., 2012; Lichtenberg et al., 2017; McDannald et al., 2005, 2014; Ostlund & Balleine, 2007; Panayi & Killcross, 2018; Parkes et al., 2018; Wang et al., 2020). Higher-order conditioning provide an excellent testbed to dissect lOFC function in integrating associative information because identical training episodes experienced in reverse order produce two distinct effects, which are supported by differential associative and neural structures. We showed that a functional lOFC is necessary for the expression of fear in SPC *and* SOC in males but not in females. The sex differences that we obtained in lOFC control are particularly intriguing and are discussed separately.

The role of lOFC was opposite in the two tasks: disrupting SPC and enhancing SOC. This opposing role suggests that the lOFC does not have a general role in fear regulation. If it did, then it should have disrupted both types of higher-order fear. Furthermore, these opposing effects also rule out state-dependency (between drug-free training to testing under the influence of the drug) as a potential explanation of our findings. Rather, these findings are best explained considering the role of lOFC in supporting the differential associations that form in SPC and SOC.

As described above, sensory preconditioning is supported by S-S associations formed between SPC-S2 and S1 during Phase 1 of SPC training (Rizley & Rescorla, 1972; Holmes et al., 2013; Experiment 4.1 and 4.4). Lateral OFC neurons respond to SPC-S2 and S1 during Phase 1 (Sadacca et al., 2018) before either of the cues signal any motivationally relevant outcomes. Importantly, inactivation of lOFC during Phase 1 (Hart et al., 2020) or prior to test (Jones et al., 2012) disrupted the expression of appetitive SPC. These data provide evidence that the lOFC is critical for associating S2 and S1 in Phase 1 and for using this information on test to evoke appetitive approach to S2. Our results using an aversive SPC design are in congruence with the data reported using the appetitive design, and therefore silencing the lOFC on test likely disrupts aversive SPC because the S2→S1 associations are not available to guide behaviour (Experiment 5.1). That is, the lOFC allows for S2 to evoke a representation of the fear conditioned S1 on test and thus engage defensive (freezing) behaviours (but see Future Directions for an alternative on the role of lOFC in the retrieval of mediated associations).

Our results showing a facilitation of SOC following lOFC inactivation on test are in line with the S-S account of lOFC function. That is, lOFC tracks the possible S-S associations that form during training and guide behaviour accordingly. Although our behavioural (and neural) data suggest that S2→S1 associations do not guide fear responding in SOC, an alternative S-S association between S2 and the *absence* of the US can be developing during Phase 2 of SOC. To elaborate, during S2→S1 pairings in Phase 2, presentations of S1 would evoke the expectation of the US, which is not delivered allowing for S2→no_US associations to develop. Indeed, conditioned inhibition, that is, learning that a stimulus signals the absence of an expected outcome uses trial types that are identical to those used in SOC (Herendeen & Anderson, 1968; Stout et al., 2004). Behaviourally, the effect of S2→no_US associations on test is undermined by the strength of S2→fear_state associations. However, inactivating the lOFC leave S2→fear_state association to be the sole controller of behaviour. Thus, lOFC inactivation facilitates SOC-fear. We tested this hypothesis in a reinforced SOC design, delivering the S1-signalled US during sensory training (i.e., S2→S1→US; Experiment 5.3) and therefore eliminating the formation of S2→no_US association. There was no effect of lOFC inactivation on fear expression to reinforced SOC-S2 providing supporting evidence for our hypothesis about the role of lOFC in the expression of S2→no_US associations.

The role of lOFC in higher-order fear expression in females

Strikingly, pharmacological inactivation of the lOFC had no effect on sensory preconditioned or second-order conditioned fear in females compared to males. Here we discuss potential strategies used by females to explain sex-dependent lOFC control in higher-order fear and consider the evidence that show sex-dependent neural activity in fear regulation.

In Chapter 5, we discussed that the differential lOFC control over SPC in males and females can be attributed to the distinct associations that support SPC, and the role of lOFC in the retrieval of these associations. In Chapter 2 we described two accounts that support SPC: chaining and mediation. In chaining, S2 elicits responding on test as a result of integrating S2→S1 and S1→US associations. That is, presentation of S2 retrieves a representation of its S1-associate, which retrieves a representation of the US (i.e., S2→S1→US) and therefore leads to conditioned responding. In mediation, S1 mediates a link between associatively retrieved S2 and the US during

Phase 2. That is, while S1 fear conditioned, it retrieves a representation of its S2-associate, which forms an association with the US (i.e., S2→US). Neither our male nor female data can resolve whether the lOFC is involved in chaining of associations or the retrieval of mediated associations because test inactivation can be disrupting either of these processes. However, behavioural control of chaining or mediation stands out as a possibility that can explain why in males inactivation of lOFC or its inputs to the BLA disrupt SPC unlike females. We interpret the SPC disruption obtained in males to be more in line with the chaining account given the role of lOFC in learning and the retrieval of S-S associations between S2 and S1 (Hart et al., 2020; Jones et al., 2012). If indeed sex differences emerge as a result of SPC being differentially supported by these two accounts between the sexes, females would be relying on mediated S2→US association. Notably, in an appetitive SPC task evidence show that in males *and* females a functional lOFC is required to learn S2→S1 association in Phase 1 (Hart et al., 2020), suggesting that sex differences emerge either in Phase 2 or at test. One way to investigate whether females rely on mediation over chaining is to inactivate lOFC during Phase 2 and test when the lOFC is intact. If lOFC is important for mediation we would obtain a disruption in SPC. Alternatively, lOFC may only be important for the retrieval of S2→US and the PRh, a region shown to be important for mediated learning (Wong et al., 2019) may be providing this information. Targeting lOFC and PRh connectivity by contralaterally inactivating these regions prior to Phase 2 or test would provide evidence for the role of lOFC in the retrieval of mediated associations.

In second-order conditioning, our lOFC inactivation studies (Experiments 5.2 and 5.3) in males provided evidence that there are two competing associations at test: S2→fear_state associations and S2→no_US associations. Expression of the latter required a functional lOFC in males but not in females. Regardless of lOFC inactivation prior to test, females showed similar levels of fear to SOC-S2 in the classical and the reinforced SOC design. This can be explained in two ways. First is the possibility that the formation of S2→no_US association in females does not require the lOFC for its expression. Notably, Fam and colleagues (2023) showed the role of IL in developing S2→no_US associations using both males and females and did not report sex differences. Perhaps the sex differences we obtained point to a sex-dependent lOFC control in fear regulation. Second, the manifestation of S2→no_US association may require longer training in females, making them reliant on the S2→fear_state association in Experiment 5.4b. Indeed, evidence from tasks other than SOC show that females require more training to learn a contingency

that is in competition with a learned stimulus-outcome relationship (Lay et al., 2020; Baran et al., 2010; Delamater et al., 2017). For example, in overexpectation females need longer training when two stimuli previously paired with a US are subsequently paired in compound with the same US (Lay et al., 2020) or when a stimulus paired with the US is later presented non-reinforced and thus paired with the absence of the US (i.e., extinction, Baran et al., 2010; Delamater et al., 2017). Importantly, such paradigms that require adaptive downregulation of behaviour in a dynamic environment are critically dependent on cortical structures such as the lOFC (Takahashi et al., 2013) and the IL (Morgan et al., 1993).

Indeed, lOFC and IL are cortical structures that are implicated in learning to inhibit fear 2024-03-22 10:45:00 AM. There is evidence in humans (Mak et al., 2009) and rodents (Baran et al., 2010; Cover et al., 2014) that show sex-dependent effects in cortical control. For example, in an fMRI study that measured neural activity during emotion regulation, Mak and colleagues (2009) showed more prefrontal activity including dorsolateral and lateral orbitofrontal cortices in males during negative emotion regulation relative to females, while medial orbitofrontal gyrus showed more activity in females relative to males. Authors interpret these findings as females taking a more affective processing strategy during negative emotion regulation while males engage regions involved in cognitive processing. That is, females used a strategy to regulate their emotional response that involved activity in the amygdala while males engaged regions that are involved in updating the emotional value of the stimuli such as the lOFC. These findings are in line with our proposal that females may be relying on mediated S2→US in SPC and S2→fear_state associations in SOC. These associations engage neural substrates involved in fear processing such as the BLA and may be bypassing activity in the lOFC in females in contrast to males.

Sex differences in fear regulation are clinically well documented in humans (Bangasser & Cuarenta, 2021). For example, women are more likely to suffer from fear regulation problems including post-traumatic stress disorder (PTSD; Kessler et al., 1995; Tolin & Foa, 2006), panic disorder (de Jonge et al., 2016) and generalized anxiety disorder (Howell et al., 2001). Research in preclinical animal models is key to understand the neural substrates underlying sex differences in fear regulation (Bangasser & Cuarenta, 2021). This body of work generally focus on first-order fear conditioning and its extinction because inability downregulate fear is a marker of fear regulation problems. The neural substrates implicated in these tasks show parallels between humans and rodents (Gilboa et al., 2004; Quirk et al., 2003; Knapska et al., 2012). However, work

that report sex differences in animal models do not consistently show the same pattern as that seen in humans (Shansky, 2015). There are reports of females showing impaired extinction retention relative to males, suggesting that they rely more on the original fear memory rather than the extinction memory (Baran et al., 2009). There is also work that showed no difference between males and females (Gruene et al., 2015) and those that showed a facilitation in the expression of extinction in females (Milad et al., 2009). More specifically, Milad et al., (2009) showed that female rats that underwent extinction training during proestrus phase while estradiol and progesterone levels were higher showed better extinction recall on test. Indeed, mounting evidence show that estradiol modulate fear extinction (Rivas-Arancibia & Vazquez-Pereyra, 1994; Yuan & Chambers, 1999; Zeidan et al., 2011) and presumably does so via its influence on gene expression and learning-induced plasticity in the IL, strengthening its inputs to the amygdala (for review, Cover et al., 2014). In our studies we used freely cycling female rats and sex differences only emerged in the studies that involved investigations probing lOFC function. We interpret these sex differences based on the associations that guide behaviour in females relative to males. That is, when a fear-linked S2→fear_state (i.e., SOC) or S2→US (i.e., meditated SPC) association guides behaviour, the inhibitory S2→no_fear or the S2→S1 association that forms in the lOFC does not downregulate behaviour in females as shown in SOC and SPC experiments, respectively. Perhaps estradiol modulates the learning of the lOFC-dependent associations similar to its function in extinction learning. Future work will provide evidence delineating the conditions under which sex differences emerge in cortical control over fear regulation.

The role of lOFC→BLA pathway in higher-order fear expression

Thus far, the BLA (Experiment 4.4, Qureshi et al., 2023), the PRh (Holmes et al., 2013, 2018; Iordanova et al., 2009; Wong et al., 2019; Qureshi et al., 2023) and the lOFC are shown to have a role in associating S2 and S1 in SPC (Experiment 5.3; Hart et al., 2020). More specifically, we showed that silencing lOFC→BLA pathway (Experiment 6.4a) disrupts fear to SPC-S2. Given the role of lOFC in forming S-S associations, we reason that lOFC provides S2→S1 information to the BLA. These lOFC inputs may be converging on the SPC-ensembles that we identified in the BLA, which, in turn gates the expression of fear to SPC-S2. In combination with our BLA→lOFC findings, projections between the lOFC and the BLA form a circuit in regulating SPC. The

contribution of the PRh in regulating fear to an SPC-S2 and its interactions with the pathways between the BLA and the lOFC remains to be determined.

How does the lOFC communicate with other brain areas to exert the effect of S2→no_US associations in the fear circuit? As described in Chapter 6 Discussion, lOFC sends inputs to the BLA about complex relationships between stimuli (Lucantonio et al., 2015). Therefore, one possibility is that the lOFC sends S2→no_US information directly to the BLA, thereby suppressing the expression of S2→fear_state association. Our lOFC→BLA pathway inactivation had no effect on the expression of fear to SOC-S2 (Experiment 6.4b), ruling out this possibility. Perhaps the lOFC regulates the expression S2→no_US association via another brain region. Indeed, a similar form of learning that involves the omission of an expected aversive outcome, that is extinction, is regulated via the connections between the infralimbic cortex (IL) and the BLA, (for review, Sah & Westbrook, 2008; Herry et al., 2010). Subsequent to extinction learning (i.e., S→no_US association), IL inputs to the amygdala (the BLA, the ICM or the CeA) downregulate conditioned fear via inhibitory neurons in the BLA or the ICM. A functional lOFC could therefore be modulating IL inputs to the amygdala to express S2→no_US association and downregulate fear to SOC-S2 (Chang & Grace, 2018). A recent study showed that chemogenetically inactivating IL prior to Phase 2 of SOC or test, facilitated fear expression to SOC-S2 implicating a role in learning and the expression of S2→no_US associations (Fam et al., 2023). However, the mechanism underlying lOFC and the IL interaction in facilitating SOC-fear is currently unknown and remains to be investigated.

Theoretical considerations about the lOFC function

One of the most influential theories about lOFC function has considered it in terms of value computation. Namely, the economic choice hypothesis posits that lOFC calculates the utility of available options in real time (Padoa-Schioppa, 2011). That is, lOFC is involved in constructing subjective value for stimuli by integrating the dimensions across which the value varies and compares this value across stimuli to drive choice behaviour. Support for this theory came from electrophysiology studies that showed neural correlates of value in the lOFC (Gottfried et al., 2003; Padoa-Schioppa & Assad, 2006; Schoenbaum & Eichenbaum, 1995; Zhou et al., 2021). This role of lOFC is mainly investigated in the appetitive domain, using reward US. Our studies using

aversive US provide evidence that lOFC is important to drive behaviour when stimuli signal *negative* value. According to economic choice hypothesis, inactivation of the lOFC would result in a deficit to retrieve this negative value in both SPC and SOC, failing to capture the facilitation effect we obtained in the latter. There is also evidence from the appetitive domain that is at odds with the main function of lOFC being value computation as conceived by this theory (Gardner et al., 2017, 2018, 2020). Importantly, Gardner and colleagues (2017, 2018, 2020) sought to provide *causal* evidence for this type of value computation using a task analogous to that used in monkeys which paved the way for the economic choice hypothesis. They showed that lOFC is not required for economic choice in a well-established task space but choosing between novel cue pairs required lOFC (Gardner et al., 2020). Moreover, data from sensory preconditioning tasks clearly demonstrate that value need not be present to obtain neural activity in the lOFC (Hart et al., 2020; Sadacca et al., 2018). Taken together, our lOFC inactivation data and those reported here provide evidence that the role of lOFC is not restricted to value computation and highlight its role in the learning and expression of S-S associations.

Another theory, the cognitive map hypothesis consider lOFC function in tracking the associative network provided by downstream regions and updating future expectancies in guiding behaviour (Schoenbaum & Setlow, 2001; Delamater, 2007; Stalnaker et al., 2015; Gardner & Schoenbaum, 2021). Lateral OFC also contributes to this map by forming S-S associations and thus, the theory accounts for the evidence obtained in SPC tasks (Jones et al., 2012; Sadacca et al., 2018; Hart et al., 2020). Our lOFC inactivation data are more in line with this framework, given its emphasis on the lOFC function in the formation of S-S associations. That is, in SPC, inactivation of lOFC blocks the retrieval of S2→S1 association resulting in a disruption, while in SOC, it blocks the retrieval of S2→no_US association resulting in an enhancement.

Our results have several implications. First, we showed that the lOFC role is not restricted to appetitive domain and provided evidence that it regulates fear. Second, this fear regulation is not a general role in suppressing or enhancing fear, rather a regulation based on the associative architecture that supports fear to stimuli, hence why we obtained opposing effects in SPC and SOC upon lOFC inactivation. Third, these results show that lOFC control in fear is based specifically on S-S associations, which form between S2 and S1 in SPC, and S2 and the absence of the US in SOC.

Future Directions

The disruption in SPC obtained as a result of S1→US memory deletion (Experiment 4.4) suggests an overlap between SPC- and S1-ensembles. However, whether the extent of this overlap is a complete or partial one is unknown. A study that uses the same RNAscope approach with an RNA probe targeting S1-activated cells in the BLA could be used to visualize whether SPC- and S1-ensembles overlap completely or partially. A complete overlap would suggest that cortical inputs (i.e., the lOFC, the PRh and the RSC) about S2→S1 association converge onto the S1-ensemble in the BLA. On the other hand, a partial overlap would suggest that the overlapping subset is crucial in driving SPC.

We identified an overlapping SPC ∩ SOC ensemble in our RNAscope study that could be engaged as a result of stimulus similarity between SPC-S2 and SOC-S2. This raises the question of whether the overlap neurons have a role in guiding SPC and SOC types of fear. We can answer this question using the Daun02 inactivation technique and either delete SPC or SOC ensemble and test for the deleted and the non-deleted ensemble in different groups of rats. If the overlap neurons support fear, deletion of one ensemble (e.g., SPC) would impair fear to the deleted as well as to the non-deleted cue (i.e., SOC). In contrast, if the overlapping neurons are only recruited as a result of stimulus similarity and have no functional role in guiding fear, deletion of an ensemble would have no effect on the expression of fear to non-deleted cue.

The role of SPC and SOC ensembles in the BLA that showed projections to the lOFC are crucial in supporting SPC and SOC. One question pertains to whether these BLA terminals synapse on the same or different lOFC neurons in expressing SPC and SOC type fears. This can be addressed using an RNAscope approach to identify SPC and SOC ensembles in the lOFC and visualize whether BLA terminals labeled with a trans-synaptic viral vector (Xu et al., 2020) converge on similar or distinct ensembles in the lOFC. Although silencing BLA inputs in the lOFC disrupted both types of fear, inactivating the lOFC produced a disruption in SPC and a facilitation in SOC. A cell-type specific staining in the lOFC subsequent to SPC and SOC retrieval would reveal the nature of the neuronal populations in the lOFC that are involved in regulating these opposing effects.

Our pharmacological lOFC and chemogenetic lOFC→BLA pathway inactivation studies focused on the role of neural structures in the expression of fear to SPC-S2. These disruptive effects can be explained in two ways. On the one hand, lOFC could be involved in the retrieval of

S1-mediated S2→US association that form in Phase 2 of SPC. That is, during S1→US pairings S1 retrieves a representation of its S2-associate and enables a mediated association between SPC-S2 and the US. On the other hand, lOFC could be involved in chaining of S2→S1 and S1→US associations (i.e., S2→S1→US). Our studies cannot differentiate between these two accounts. To determine whether lOFC is involved in the retrieval of mediated S2→US or chained S2→S1→US associations we can target lOFC function during Phase 2, when mediation occurs. Inactivation of lOFC is shown to have no effect on fear conditioning to S1 (Sun & Chang, 2022). Therefore, any effects obtained would be attributed to lOFC role in mediated learning. For example, if lOFC is important for mediation, silencing lOFC via antagonizing NMDA receptors or agonizing GABA receptors prior to Phase 2 would disrupt expression of SPC on test. Alternatively, lOFC inactivation could spare mediated learning but impair the retrieval of mediated S2→US association. The PRh is shown to be crucial for mediated learning (Wong et al., 2019) and it could be sending inputs about mediated S2→US association to the lOFC for its retrieval. This possibility can be investigated by contralateral inactivation of the PRh and the lOFC prior to test.

In contrast to SPC, lOFC inactivation facilitated SOC and did so by blocking the effect of S2→no_US association that competes with S2→fear_state association on test. We showed that the former association is not signalled by lOFC→BLA pathway and suggested that lOFC modulates signaling between the IL and the BLA. As it is also proposed by Fam and colleagues (2023), targeting IL→BLA and BLA→IL pathways prior to Phase 2 and test will provide causal evidence for the competition between S2→no_US and S2→fear_state associations at these time points. Next, the mechanisms underlying lOFC modulation of these pathways need to be addressed. The role of lOFC→IL and IL→lOFC pathways can be investigated during Phase 2 and test to delineate the interactions between these substrates in the formation of S2→no_US association.

Conclusion

The present thesis showed that the expression SPC and SOC type fears differentially rely on fear to a first-order S1 stimulus, behaviourally and neurobiologically. Despite being conditioned by S1 in an identical manner, SOC and SPC control behaviour distinctly based on whether S1 is fear conditioned before or after conditioning SOC-S2 and SPC-S2, respectively.

Specifically, we showed that SPC type fear is dependent on intact S1-ensemble in the BLA, while SOC is not. We found equivalently sized neuronal populations in the BLA that were activated by SPC and SOC on test, and a subset of these neurons showed projections to the lOFC. We identified the role for the lOFC, a region generally understudied in fear, in regulating both types of fear via S-S associations. Specifically, silencing the lOFC resulted in a disruption in SPC and a facilitation of SOC. We pursued BLA→lOFC pathway and showed that silencing the neuronal populations that we identified disrupt both types of fear, while lOFC→BLA was crucial for the expression of fear to SPC but not SOC. Together, the thesis work highlights the role of the BLA, the lOFC and their connectivity in supporting higher-order fear.

The ability to identify cues that signal danger is crucial for survival. This ability often relies on integrating relationships between events and traumatic episodes that are acquired at different time points. The way in which events are associated with a traumatic episode necessitate distinct therapeutic approaches to target the core event underlying fear regulation problems that arise post-trauma. Higher-order conditioning tasks are excellent tools to delineate the neural substrates underlying similarly acquired fears' distinct control over behaviour.

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