PhD Proposal

The genetic and epigenetic substrates of second-order fear conditioning in the basolateral complex of the amygdala

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**Introduction**

Post-traumatic stress disorder (PTSD) is one of the most common mental disorders, affecting 7-9% of populations worldwide (REF). It typically develops as a response to one or more traumatic events including warfare, natural disasters, sexual assault or other threats to the life and/or safety of a person. Re-exposure to trauma-related reminders often leads to severe distress and recurring memories. These reminders include cues that had been present at the time of the traumatic events, but may also include cues that were not present. For example, a person with PTSD might encounter a trauma-related cue in an environment not previously associated with the trauma. As a result, any novel features of this environment may subsequently become incorporated into the existing trauma memory, thereby imbuing the new environment with the capacity to provoke panic, distress and avoidance.

Animal models are used to study the processes through which cues present at the time of an aversive event come to elicit fear. In a traditional fear conditioning paradigm, laboratory rodents are exposed to pairings of an initially neutral stimulus (e.g. a noise) and an innate source of danger (e.g. brief but aversive foot shock) (REF). After these pairings, subsequent presentations of the conditioned noise elicit a range of reactions that are indicative of fear in people, including changes in cardiovascular (e.g. changes in blood pressure and heart rate), endocrine (e.g. release of adrenocorticotropic hormone), and behavioural responses (e.g. freezing, potentiated startle) (REF).

Animals also learn to fear new associates of already-conditioned stimuli. This process is referred to as second-order fear conditioning. In a typical laboratory procedure, rats are exposed to pairings of a neutral stimulus (e.g. a light) and the already-conditioned noise from the example above (REF). When subsequently presented with the light alone, rats show conditioned fear responses despite the light having not been paired with a foot shock. Recent work in our laboratory has demonstrated this effect and shown that it is conditional on learning that occurs in each stage of training. Rats exposed to noise-shock pairings in stage 1 and light-noise pairings in stage 2 froze more during test presentations of the light alone relative to controls (REF LAB). Pavlov described this type of learning as second-order, to distinguish it from learning about cues that signal innate sources of danger, which he described as first-order (REF).

Much is now known about the neural substrates of first-order fear conditioning, including the critical role of the amygdala. This conditioning requires gene transcription and *de novo* protein synthesis in the basolateral amygdala (BLA) (REF). Further, signaling pathways upstream of gene transcription, involving extracellular-related kinase and mitogen activated protein kinase (ERK/MAPK) and calcium calmodulin dependent kinase II and IV (CaMKII/IV) signaling pathways are also heavily implicated in this process (REF). In addition, it has been also shown that first-order conditioning requires DNA methylation, a key regulator of the transcriptional processes that underlie fear memory consolidation (REF).

Despite what has been elucidated with respect to first-order fear conditioning, little is known about the neural mechanisms underlying second-order conditioning in the BLA. Work in our laboratory has shown that, while requiring CaMKII/IV signaling, DNA methylation and gene transcription, second-order conditioning does not require *de novo* protein synthesis in the BLA, and does not require ERK/MAPK signaling (REF).

The current research stems from these findings. Its general goal is to advance our understanding of how second-order fear is consolidated in the BLA. It has two specific aims. The first is to identify the genes and signaling pathways that regulate second-order conditioning. The second is to identify the epigenetic substrates of second-order conditioning in the basolateral complex of the amygdala.

**Background**

Pavlovian fear conditioning in laboratory rodents is the most widely used protocol for studying the substrates of learning and memory in the mammalian brain. In a standard protocol, rats are exposed to pairings of an innocuous auditory or visual stimulus (conditioned stimulus [CS]) and brief but aversive foot shock (unconditioned stimulus [US]). After one or a few such pairings, subsequent presentations of the CS elicit a range of reactions that are indicative of fear in people. These reactions are taken to imply formation of a CS-US association that is retrieved during presentations of the tone and expressed in the form of defensive behaviours.

Studies using this protocol have shown that the amygdala plays a critical role in the conditioning of defensive reactions to a CS that signals danger (Fendt & Fanselow, 1999; Maren, 2011). It consists of 13 nuclei that form anatomically, morphologically and functionally distinct clusters: the basolateral complex (BLA) consists of lateral, basolateral and basomedial nuclei, whereas the central nucleus (CeA) is divided into lateral (CeL) and medial sub-terrirotories (CeM). According to the accepted model, sensory information about the CS and US is processed in regions of the thalamus and cortex, and converges in the BLA. This convergence leads to activation of N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors at specific synapses in the BLA, opening of calcium (Ca2+) channels and influx of Ca2+ to post-synaptic neurons (McKernan and Shinnick-Gallagher, 1997, Rogan et al, 1997). The Ca2+ influx activates multiple second messengers, including protein kinases A (PKA) and C (PKC), extracellular signal-regulated kinase (ERK), mitogen-activated protein kinase (MAPK) and calmodulin-dependent kinases II (CaMKII) and IV (CaMKII/IV). These, in turn, promote phosphorylation of transcription factors such as CREB (Schafe and LeDoux 2000; Schafe et al. 2001, Schafe et al, 2005, Ou and Gean 2009). Phosphorylated CREB (pCREB) binds to the promoter region of genes, resulting in gene expression and synthesis of new proteins. These proteins are required for the cellular processes that effectively stabilize, or consolidate, the CS-US association (Johansen et al, 2011). Specifically, they play a critical role in changing neurotransmission at recently activated synapses, such as through changes in the size, shape and number of dendritic spines (REF). These changes are such that BLA neurons and intra-amygdala pathways are more readily activated by subsequent presentations of the CS. Critically, these pathways terminate in CeA: once activated, CeA neurons then co-ordinate defensive reactions, including autonomic, endocrine and freezing, via their projections to specific regions of the midbrain and hypothalamus (REF).

As discussed above, the formation of a CS-US association involves a dialogue between genes and synapses in the BLA. The genes implicated in associative formation can be divided into two groups. The first group, immediate early genes (IEGs), are induced rapidly, decay within 1-2 hours, and regulate transcriptional processes that are critical for stabilization of the CS-US association (REF). *C-fos* and *egr1* are two IEGs that have been implicated in such processes. Rosena et al. (1998) compared the levels of amygdala *c-fos* and *egr1* expression among rats with differing levels of fear conditioning. Groups either received a shock several minutes after exploring a novel context (fear conditioning), immediately upon placement in a novel context (shock group), or that were exposed to the novel context in the absence of shock (context group). They found that *c-fos* is elevated in the amygdala 30-40 minutes later in rats in both the fear conditioning and shock groups, but not the context group. In contrast, the level of *egr1* expression at the same time point was uniquely elevated in only the fear conditioning treatment group. The researchers concluded that, within the amygdala, *c-fos* is activated by a range of environmental stimuli, whereas *egr1* is specifically activated by the formation of a context conditioned fear memory (Campeau et al, 1991, Malkani and Rosen, 2000, Malkani et al., 2004).

Three IEGs, *Arc*, *Homer1a*, and *bdnf* have also been shown to be upregulated after fear conditioning. Importantly all three are critically involved in associative learning-induced synaptic structural changes. *Arc* expression is increased in the LA from 30-180 minutes after tone-shock pairings (REF). Further, modulation of *Arc* signaling in the LA impairs consolidation of the long-term tone-shock fear memory; relative to controls, rats that received a pre-conditioning LA infusion of an *Arc* antisense oligodeoxynucleotide froze less during test presentations of the tone (Ploski et al, 2008). Similarly, Mahan et al. (2012) demonstrated that *homer1a* expression increased in amygdala two hours after fear learning. Moreover, it was shown that up-regulation of this gene is dependent on the increase in expression of *bdnf*. The role of the latter in fear conditioning has been extensively studied. Specifically, fear memory consolidation leads to significant increase in *bdnf* exon I and III-containing mRNA in amygdala (Rattiner et al. 2004, Ou & Gean, 2006, Ou & Gean, 2009).

Other IEGs, such as *Per1*, *Egr2*, and *Nr4a2* have also shown changes resulting from fear conditioning. *Per1* is critically involved in circadian rhythm pathways, whereas *Nr4a2* and *Egr2* encode a protein that acts as a transcription factor. Using a combination of microarray technology and qRT-PCR, Ploski et al. (2010) demonstrated that all three genes were upregulated in the LA as a consequence of fear conditioning, with the highest expression at 30-minutes post-conditioning.

Aside from IEGs, a second group of genes implicated in associative memory formation show peak activation between three and eight hours after fear conditioning. At three hours after fear conditioning, *Rnf39*, a gene involved in long term potentiation (LTP), and *Sat1*, a gene responsible for acetylation of polyamines, have both been shown to be up-regulated (REF). Similarly, *Praja1* has been shown to be up-regulated at six hours. This gene encodes E3 ubiquitin ligase Praja1, and is involved in neuronal plasticity (Stork 2001). In contrast, between two and four hours post-conditioning, some genes have been shown to be down-regulated. These include: *gephyrin*, a gene involved in anchoring glycine and γ-aminobutyric acid type A (GABAA) receptors at inhibitory synapses, and *RC3/neurogranin*, which encodes a postsynaptic protein that regulates the availability of calmodulin (Ressler et al., 2002). Both genes were shown to return to baseline levels at eight hours after conditioning.

The past decade has witnessed rapid developments in genomic high-throughput technologies and cytogenetic techniques, including microarrays and sequencing. These developments have made it possible to assess the complex gene networks implicated in learning and memory generally, and fear learning and memory, specifically. In one of the earliest applications of these technologies, the transcriptional changes following fear conditioning were assessed using microarrays and qPCR (Mei, 2005). Within the amygdala, as many as 222 genes exhibited altered expression from 0.5-24 hours in rats after receiving CS-US pairings, relative to controls who received explicitly unpaired presentations of the CS and US. Specifically, 123 of these genes were upregulated following CS-US pairings, 99 were downregulated. Importantly, 45% of the entire gene set were related to cell signalling processes or structural and cell adhesion processes. Later, using the same technique, Keeley and coauthors (2006) showed that upregulation of genes in amygdala 30 minutes after pairings of the CS and US (relative to naïve animals) was partially correlated to associative learning and partially correlated to non-associative components of fear conditioning. Ploski and coauthors (2010), identified the genes that changed their expression in LA in response to fear conditioning. Overall, 25 genes were found to be up-regulated 30 minutes after fear conditioning. The expression level of the most significantly regulated genes were then also evaluated at 90 and 180 minutes after fear learning. It was demonstrated that *Egr2, Arc, Nr4a2* and *Per1* revealed the highest expression at 30 minutes, while *Rnf39* and *Sat1* peaked at 180 minutes. In order to distinguish the genes that were regulated by fear learning rather than exposure to tone or shock alone, various controls were used. It was shown that 5 IEG (*Arc, Fos, Egr1, Egr2, Egr4*) were uniquely upregulated following fear conditioning (relative to animals that received exposure to the tone or shock only). It was also demonstrated that the identified genes are downstream to ERK/MAPK signalling pathway.

**Epigenetic Mechanisms in First-Order Fear Conditioning**

Recent evidence shows that consolidation of a CS-US fear memory also requires an array of epigenetic mechanisms in the BLA. These mechanisms regulate access to DNA through post-translational modifications of chromatin structure, thereby permitting robust and temporally specific regulation of transcriptional processes (for reviews, see Day and Sweatt, 2011, Vogel-Ciernia and Wood, 2014). Firstly, the access to DNA can be regulated through chemical modifications of amino-terminal histone tails, including methylation, phosphorylation and acetylation. These are associated with either enhancement or suppression of transcriptional activity. Secondly, remodeling of chromatin is also possible through direct modification of DNA. This involves the addition of methyl groups onto 5'-C-phosphate-G-3' sites (CpG islands) of DNA, and is generally known to suppress gene activity. Finally, addition, deletion or shifting nucleosomes along the DNA strand have been also shown to alter the structure of chromatin and hence change the patterns of gene expression (for review, see Zovkic et al., Kwapis & Wood, 2013).

Among histone modifications, acetylation is the most well-characterized regulator of gene expression. In one set of studies, inhibitors of enzymes promoting histone acetylation, called histone acetyltransferases (HAT) disrupted fear memory formation (REF). For example, Maddox (2013a) and coauthors showed that infusion of the histone acetyltransferase inhibitor, garcinol, into the rat LA resulted in significantly lower levels of histone H3 acetylation and disrupted consolidation and reconsolidation of fear memory. Conversely, a second set of studies have shown that inhibitors of histone deacetylases (HDAC), enzymes that reduce or reverse histone acetylation, facilitate fear memory consolidation in the amygdala (Monsey et al, 2011, Maddox et al, 2013a, 2013b). That is, it was shown that intra-LA infusions of the histone deacetylase inhibitor, TSA, led to elevated level of H3 acetylation and enhanced long-term memory after auditory fear conditioning (Monsey et al, 2011).

Another type of epigenetic modification, that does not involve changing of histones structures, is DNA methylation. Two lines of evidence illustrate the role of this epigenetic modification in consolidation of a CS-US fear memory. First, expression of DNA methyltransferases (DNMTs), enzymes promoting DNA methylation, is increased in the amygdala following fear conditioning (Monsey et al, 2011, Lay et al, 2018). Second, blocking the activity of these enzymes impaired fear memory consolidation (Maddox et al, 2014).

**Role for BLA in Second-Order Fear Conditioning**

As noted above, animals and people also learn to fear cues that signal learned sources of danger. Pavlov described this conditioning as second-order, to distinguish it from learning about cues that signal innate sources of danger (such as the tone paired with shock), which he described as first-order (REF). In the past decade, research in our laboratory has examined the role of the BLA in encoding and consolidating second-order conditioned fear. This work has shown that, as is the case for first-order conditioned fear, the encoding and consolidation of second-order fear requires neuronal activity in the BLA. For example, Parkes and Westbrook (2010) showed that inhibiting activity in the BLA prior to S2-S1 pairings (via infusions of the GABA agonist, muscimol) prevents the acquisition of fear to S2. Similarly, Lay and coauthors (2018) demonstrated that infusions of the sodium-channel blocker, bupivacaine, into the BLA after S2-S1 pairings resulted in low levels of freezing to the second-order, but not first-order, CS during its subsequent tests.

Within the BLA, many of the cellular and molecular processes required for the encoding and consolidation of second-order conditioned fear are also required for the encoding and consolidation of first-order conditioned fear. That is, like first-order conditioned fear, the encoding of second-order conditioned fear requires activation of NMDA receptors in the BLA (Parkes & Westbrook, 2010). Pre-training infusions of an NMDA receptor antagonist, ifenprodil, prevent freezing to S2 at test. Further, consolidation of this fear also requires CaMKII/IV signaling, gene expression and DNA methylation in the BLA (Lay et al., 2018). Rats that received post-training infusions of a CaMKII/IV inhibitor, KN-62, a transcriptional inhibitor, actinomycin-D, or the DNMT inhibitors, Rg108 and 5-AZA froze less when tested to the S2 alone compared to controls that received the same infusions several hours after the second-order conditioning session.

Our laboratory has also shown that, within the BLA, the processes required for consolidation of the two types of conditioned fear differ in critical respects. Firstly, unlike consolidation of first-order conditioned fear, consolidation of second-order fear occurs independently of ERK/MAPK and PKA/PKC signaling in the BLA. Here, rats that received post-training BLA infusions of the MEK inhibitor, U0126, or the PKA/PKC inhibitor, H7, froze at the same level as controls infused with vehicle alone (or that received the drug infusion several hours later (REF) when they were tested with the light. Secondly, and most importantly, unlike consolidation of first-order fear, consolidation of second-order fear occurs independently of *de novo* protein synthesis in the BLA. Rats that received post-training BLA infusions of the protein synthesis inhibitor, cycloheximide, froze at the same level as controls infused with vehicle alone (or that received the drug infusion several hours later) when tested with the light. Hence, within the BLA, second-order fear is not consolidated via a simple recapitulation of the processes required for consolidation of first-order fear. Among the signaling pathways that link receptor activation and gene expression, consolidation of second-order fear requires CaMKCaMKII/IV signaling, but not ERK/MAPK or PKA/PKC signaling; and among the nuclear events that regulate consolidation of first-order conditioned fear, consolidation of second-order fear requires transcriptional activation and DNA methylation, but not *de novo* protein synthesis.

The findings just described raise several questions regarding how second-order fear is consolidated in the BLA. The first concerns the identity and methylation state of genes involved in this consolidation. The second is whether expression of these genes occurs downstream of the signaling cascades involved in first- and/or second-order fear, including ERK/MAPK and CaMKII/IV signaling. The third is how the expression of these genes affects consolidation of second-order fear in the absence of *de novo* protein synthesis. The aim of this thesis is to address these gaps in our knowledge. It will do this by combining the behavioural protocols used in our laboratory (Holmes et al., 2013, 2014; Lay et al., 2018; Parkes & Westbrook, 2010) with techniques developed in molecular biology, including Florescent-Activated Cell Sorting (FACS), rt-qPCR and RNA-sequencing. By combining this methods, we will be able to identify the genes involved in consolidation of second-order fear in the BLA, including their methylation state and time-course of expression (Experiments 1 and 2); the impact of inhibiting ERK/MAPK or CaMKII/IV signaling on this gene expression (Experiments 3 and 4); and finally, the impact of targeted knockdown of these genes on consolidation of second-order fear, and its neural correlates (Experiment 5 and 6).

**Experiment 1**

The aim of this experiment is to examine whether genes that are known to regulate consolidation of first-order fear in the BLA also regulate consolidation of second-order fear. The genes of primary interest are those that code for transcription factors (*Egr1, Crebbp*), kinases (*Mapk2, Map2k7, CaMKII/IV, CaMKIIa, PKC-b1, PKI-a*), signaling molecules *(Mras, Rasa1*) and proteins that service synaptic/structural changes (*Arc*). Briefly, four groups of rats undergo two stages of training. Rats in the group of interest (2nd Order) are exposed to light-shock pairings in stage 1 and tone-light pairings in stage 2. The three remaining groups serve as controls for the assessment of changes in gene expression in Group 2nd Order: the first is exposed to light-shock pairings in stage 1 and the context alone in stage 2 (Group 1st Order); the second is exposed to the context alone in stage 1 and light-tone pairings in stage 2 (Group SPC); and the third is exposed to the context alone in both stages 1 and 2 (Group Context). In order to assess the time-course of genes expressed in relation to 2nd order conditioning, rats in each group are sacrificed either 30, 90 or 270 min after stage 2 training; and in order to assess the specificity of changes in gene expression to the BLA, they are compared to changes observed in a neighboring region of the medial temporal lobe, the perirhinal cortex (PRh).

**Materials and methods**

*Subjects*

Subjects are naïve male Long Evans rats, approximately 90 days old at the start of the experiment. They are obtained from a local breeding facility (Randwick), and upon arrival in our facility, are housed in plastic cages (eight rats per cage) measuring X x Y x Z. The cages are kept in a colony room that is maintained at 20-22˚C, and in which the lights are on during 7am-7pm. Rats have ad libitum access to food and water for the duration of the experiment.

*Procedure*

Context Exposure. All rats will be familiarized with the context on Days 1 and 2. There will be two, 20-minute sessions of context exposure on each of these days. One exposure will occur in the morning and the other three hours later in the afternoon.

Stage 1 training. On Day 3, all rats receive a single exposure to the context for 22 minutes. During this session, rats in two groups (2nd Order and 1st Order) receive four pairings of a flashing light (3.5 Hz) and foot shock (0.8 mA, 0.5 s). Each presentation of the light lasts for 10 seconds and co-terminates with presentation of the foot shock. The first light presentation occurs five minutes after placement in the context. The interval between each light presentation is fixed at five minutes. Rats remain in the context for an additional one minute after the final light presentation. Rats in the remaining two groups (SPC and Context) are exposed to the context alone during this stage of training.

Context Extinction. On Day 4, rats receive two context alone exposures, each 20 minutes in duration, with one in the morning and the other three hours later in the afternoon. These exposures are intended to extinguish any context-elicited freezing that would otherwise obscure the development of second-order fear to the tone in the next stage of training.

Stage 2 training. On Day 5, all rats receive a single 25 minute session of exposure to the context. During this session, rats in two groups (2nd Order and SPC) receive four pairings of a novel tone (1000 Hz, 75 dB) and the already conditioned light. Each presentation of the tone lasts for 30 seconds, and its offset is accompanied by onset of the flashing light for 10 seconds. The first tone presentation occurs five minutes after placement in the context. The interval from light-offset at the end of one trial to the next tone-onset is fixed at five minutes. Rats remain in the context for an additional two minutes after the final light presentation. Rats in the remaining two groups (1st Order and context) are exposed to the context alone during this stage of training.

*Scoring*

Freezing is used to index conditioned fear. It is defined as the absence of all movement except that required for breathing (Fanselow, 1980) and measured using a time sampling procedure in which each rat is scored as either ‘freezing’ or ‘not freezing’ every two seconds. A percentage score is calculated for the proportion of total observations that each rat is freezing. All test data will be scored by the experimenter and then cross-scored by an experienced observer who is blind to the experimental groups. The Pearson product moment correlation will be calculated to assess the reliability between the scores of the two observers. Any discrepancies between the scores will be resolved in favour of those by the naïve observer.

Freezing data across training sessions will be analyzed using a mixed model ANOVA, with between-subject factors of stage 1 training (light-shock pairings or context alone) and stage 2 training (tone-light pairings or context alone), and a within-subject factor of trial. For all statistical analyses, the criterion for rejection of the null hypothesis is set at alpha = .05. Standardized 95% confidence intervals will also be reported for each statistically significant difference, and effect sizes will be reported as Cohen’s d.

*Tissue preparation*

Thirty, 90 and 270 minutes after stage 2 training, rats will be given a lethal dose of sodium pentobarbital (X mg/ml/rat), their brains will be rapidly removed, frozen in liquid nitrogen and stored at -80˚C for further processing. This processing will involve sectioning of each brain into 1 mm-thick coronal slices (using a brain matrix) and tissue collection from four 1 mm punches centered on the BLA (two punches from each of two consecutive sections) and four 2 mm tissue punches centered on the PRh (two punches from each of two consecutive sections).

*Fluorescence-activated cell sorting (FACS)*

The BLA is a heterogeneous structure with multiple cell sub-populations. As such, fluorescence-activated cell sorting (FACS) will be used to isolate cells activated during the formation of second-order conditioned fear, and thereby assess the genes expressed in relation to this fear. Briefly, the BLA (and PRh) tissue punches are first minced, digested and filtered. The cells are then incubated with (i) fluorescent tagged antibodies that label neurons (NeuN+ve) and (ii) a marker of neuronal activation (c-fos +ve). They are then washed and analysed using a BD FACSAria™ IIIu cell sorter (UNSW Mark Wainwright Analytical Centre). Based on their individual fluorescent properties, NeuN+ve cells will be sorted into separate collection tubes as Fos+ve or Fos-ve, and processed for RNA extraction.

*RNA extraction*

RNA will be isolated using the Trizol extraction method (Sigma). Messenger RNA (mRNA) will be DNAse-treated and reverse transcribed in a C1000 Touch Thermal Cycler (Bio-Rad Laboratories, CA, USA) using the QuantiTect Reverse Transcription Kit (Qiagen, CA, USA) according to the manufacturer’s instructions.

*RT-PCR*

Quantitative PCR will be performed in a StepOnePlus system with the use of SYBR Select Master Mix (Applied Biosystems, VIC, Australia). All qPCR primers (Integrated DNA Technologies, IA, USA) will be designed using Primer3 software (Untergasser et al. 2012) and veriﬁed using the BLAST-like alignment tool (BLAT; Kent 2002). Annealing temperatures for each primer will be optimized using temperature-graded PCR, and speciﬁcity for target sequences conﬁrmed with 1.5% agarose gels. Primer efﬁciencies will also be determined using a standard curve. For each target (run-in triplicate for each sample), mRNA levels will be normalized to the housekeeping-gene GAPDH.

*Statistical analysis*

The ΔΔCt method will be used to determine the relative expression of gene-candidates in 2nd Order, 1st Order and SPC relative to Context. The results will be presented as mean fold change value with confidence intervals and standard errors. The distribution of ΔCt values will be assessed using the Shapiro-Wilk test; and equality of group variances assessed using Levene's test. Depending on the outcome of these preliminary assessments, the data will be analysed using standard parametric (contrasts in ANOVA) or non-parametric (Wilcoxon test) methods. In either case, appropriate corrections will be employed to maintain the chance of a Type 1 error at alpha = .05.

*Expected outcomes*

We expect that rats exposed to light-shock pairings in stage 1 (2nd Order and 1st Order) will show myriad changes in gene expression in the BLA (but not the PRh) relative to rats that did not receive these pairings in stage 1 (SPC). We additionally expect that, among those exposed to light-shock pairings in stage 1, those exposed to tone-light pairings in stage 2 (2nd Order) will exhibit a different pattern of gene expression to those exposed to the context alone in stage 2 (1st Order). Specifically, based on findings by Lay and coauthors (2018), we expect that the formation of second-order conditioned fear will involve expression of genes related to CaMKII/IV signalling and DNA methylation.

**Experiment 2**

Recent work in our laboratory has shown that, within the BLA, consolidation of second-order fear requires CaMKII/IV signalling, but not ERK/MAPK or PKA/PKC signalling (Lay et al. 2018). Given that CaMKII/IV is an essential functional link between activated membrane receptors and gene expression alterations, we will examine whether the genes identified in the previous experiment are downstream of CaMKII/IV signalling in the BLA. Immediately after stage 2 pairings of the novel tone and fear-conditioned-light, rats will receive a BLA infusion of the CaMKII/IV inhibitor, KN62, or vehicle. Thirty minutes later, all rats will be sacrificed, their brains rapidly removed, and frozen in liquid nitrogen. Tissue will then be collected and processed for assessment of the genes expressed in relation to second-order conditioning. We expect that pharmacological inhibition of CaMKII/IV signalling will prevent transcriptional regulation of genes that are linked to this signalling.

**Materials and methods**

*Subjects*

Subjects are naïve male Long Evans rats, of the same age and from the same source as those used in Experiment 1. Their housing and maintenance conditions are as described for Experiment 1.

*Surgery and Drug Infusions*

Before behavioural training and testing, rats will be implanted with guide cannulae directed toward the BLA. For this surgery, rats will be anesthetized with an intraperitoneal injection of 1.3 ml/kg of ketamine (Ketapex; Apex Laboratories), at a concentration of 100 mg/ml and 0.3 ml/kg, and xylazine (a muscle relaxant; Rompun, Bayer), at a concentration of 20 mg/ml. Anaesthetized rats will be mounted on a stereotaxic apparatus (David Kopf Instruments) and 26-gauge guide cannulae (Plastics One) will be implanted through holes drilled in both hemispheres of the skull. The tips of the guide cannulae will be aimed bilaterally at the BLA using the following coordinates: 2.3 mm posterior to bregma, 4.9 mm lateral to the midline, and 7.9-8.0 mm ventral to the skull. The guide cannulae will be secured to the skull with four jeweller’s screws and dental cement. A dummy cannula will be kept in each guide at all times except during microinjections. Immediately after the surgical procedure, rats will receive a subcutaneous injection of a prophylactic (0.4 ml) dose of 300 mg/kg solution of procaine penicillin. Rats will be allowed seven days to recover from surgery, during which time they will be handled and weighed daily.

KN-62 or vehicle will be infused bilaterally in the BLA by inserting a 33-gauge internal cannula into the guide cannula. The internal cannula will be connected to a 25 µl glass syringe attached to an infusion pump (Harvard Apparatus) and project an additional 1 mm ventral to the tip of the guide cannula. The internal cannula will remain in place for an additional 2 min after the infusion and was then removed.

*Drugs*

The CaMKII/IV inhibitor, KN-62 (Sigma-Aldrich, Castle Hill, NSW), will be dissolved in 45% w/v 2-hydropropyl-β-cyclodextrin (45% w/v HBC; Sigma-Aldrich, Castle Hill, NSW) to a concentration of 680 ng/µl as described by Rodrigues, Farb, Bauer, LeDoux, and Schafe (2004). A 45% w/v solution of HBC will be used as the vehicle. A total volume of 0.5 µl will be delivered to both sides at a rate of 0.25 µl/min.

*Histology*

Following behavioural testing, rats will receive a lethal dose of sodium pentobarbital (1 ml). The brains will be removed and processed as described above for assessment of the gene changes in the BLA and PRh that are associated with second-order fear conditioning. A subset of animals will be used to verify the cannula placements. For these animals, their brains will be remained, frozen and then sectioned coronally at 40 µm through the BLA and PRh. Every second section will be collected on a glass slide and stained with cresyl violet. The location of the cannula tips will be determined under a microscope using the regional boundaries defined by Paxinos and Watson (2007; see Figures 1, 2, 8b, and 9b). Rats with incorrect placements or damage will be excluded from all statistical analyses.

*Procedure*

The rats will undergo the same behavioural procedures as in Experiment 1. Immediately after tone-light pairings in stage 2, rats will receive bilateral infusions of KN62 or vehicle directly into BLA. Scoring and statistical analyses of freezing data will be the same as described in Experiment 1.

*Expected outcomes*

The experiment will result in determining the genes that are downstream to CaMKII/IV signalling pathway and implicated in second-order fear conditioning.

**Future experiments**

To evaluate the previously identified genes that are critical for second-order conditioning, we will examine if the functional knockout of them will disrupt second-order memory consolidation. This will elucidate the gene expression pattern underlying memory updating at the level of transcriptome.

The next set of experiments will be focused on looking for the epigenetic mechanisms of gene expression regulation in both first- and second-order conditioning. Specifically, the methylation status of previously identified gene promoters will be examined via bisulphite PCR. The main aim of this experiment is to identify differences in patterns of DNA methylation of genes of interest after first- and second-order conditioning. The subsequent experiments will determine if those differences in DNA methylation are linked to CaMKII/IV pathways.

**Timeline**

March – July 2018: Write research proposal; deliver proposal talk; learn FACS

July – December 2018: Experiment 1 and Experiment 2

December 2018 – March 2019: carry out the analysis of Experiment 1&2 results, design future experiments.