Decoding Observational Learning: A Circuit Level Analysis of the Social Brain

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Abstract

The ability to engage in appropriate social interaction is a critical component of daily life that requires integration of multiple neural processes and can be perturbed in numerous psychiatric diseases (Adolphs et al. 2003; Frith et al. 2008). One approach to begin understanding how the brain supports a complex array of social behaviors is to study innate, evolutionarily conserved social behaviors. Observational fear learning is one such social behavior that offers a distinct advantage for survival and is thus highly conserved across various species including rodents (Heyes et al. 1990; Kavaliers et al. 2001), monkeys (Mineka et al. 1984), and humans (Olsson et al. 2007). The data presented in this thesis combines in vivo electrophysiology, optogenetics, and rodent behavior in order to answer a number of questions about the role of the anterior cingulate cortex (ACC) and the basolateral amygdala (BLA) in observational fear learning. We show that both the ACC and the BLA contain neurons that show conditioned responses to the cue and are therefore neural correlates of observational fear learning. We photo-identify neurons within the ACC-BLA network and show that the ACC-BLA network has an enhanced representation of cue information when compared to out of network neurons. In addition, we show that ACC neurons that project to the BLA encode cue information. Next, we inhibit ACC input to the BLA during the cue and show that this impairs observational learning but not classical fear conditioning. Further, inhibition of ACC input to the BLA changes the cue response of a subset of BLA neurons. Lastly, we show that ACC input to the BLA is necessary for normal social interaction. Together, this data provides the first circuit level analysis of observational fear learning. It establishes that the transfer of cue information from the ACC to the BLA plays a causal role in enabling observational learning and that this same input is needed for general social behavior.

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Chapter 1

Introduction

Chapter adapted from Allsop et al, 2014, *Frontiers in Behavioral Neuroscience*

Understanding social cognition

Survival in the world is predicated on an animal's ability to appropriately respond to stimuli in the environment. Thus animals have various mechanisms that allow them to avoid dangerous or aversive stimuli and approach rewarding stimuli or stimuli that increase their ability to survive and pass their genes on to their offspring. Towards this end, one of the most important abilities animals can have is the ability to learn which environmental stimuli are dangerous and which are rewarding, in order to proactively interact with the environment in flexible, beneficial ways.

Many animals also have the ability to assess and appropriately respond to a wide range of social stimuli (Weitekamp and Hofmann 2014). This ability to engage in social behavior also has a survival advantage. Here, we define social behavior as any behavior that is dependent on the processing of social stimuli from a conspecific. Using this definition, social behaviors are seen in a wide range of animals from nematodes to humans (Insel and Young 2000; Adolphs 2009; Sokolowski 2010). Indeed animals with simple nervous systems such as flies, ants, fish, birds, and bees engage in social behaviors seen in more complex animals such as courtship, mating, parenting, and aggression (Sokolowski 2010; Weitekamp and Hofmann 2014). Yet it is clear that with increasing neural complexity comes an increased array of complex social behaviors

such as alliance formation, cooperative hunting, empathy, and altruism (de Waal 1986; Soares et al. 2010; de Waal and Suchak 2010).

Whether simple or complex, it is thought that social behaviors are ubiquitous because they offer distinct evolutionary advantages such as decreased susceptibility to predation, increased success of foraging, efficient parenting, and enhanced reproductive selection and fitness (Alexander 1974; Schülke et al. 2010). However, as advantageous as the ability to engage in social behavior is, the addition of interactions with conspecifics to an animal's environment adds to the unpredictability of that environment. An animal that is solely concerned with its "non-social abiotic or biotic environment" has an often-fixed set of challenges to overcome (Taborsky and Oliveira 2012). This would include problems such as finding appropriate resources for food and shelter or avoiding predators. The addition of a social domain to this environment drastically increases the complexity of the challenges that an animal has to overcome (Taborsky and Oliveira 2012). Not only is a conspecific's own behavior variable and less predictable than other abiotic or biotic components of the environment, but an animal must also take into consideration that conspecific's interaction with the environment as well as other animals in order to engage in adaptive behaviors. Thus, it is not surprising that brain size is correlated with an animal's ability to engage in complex social behaviors (Dunbar and Shultz 2007). In fact, many have posited the idea of the social brain: a set of neural circuits, networks, and signaling molecules uniquely designed to sense and interpret social information and respond appropriately (Newman 1999; Insel and Young 2000; Brothers 2002; Adolphs 2009; Soares et al. 2010; O'Connell and Hofmann 2012; Rushworth, Mars, and Sallet 2013). Much work has been done in order

to try to define the brain regions responsible for various social behaviors (Adolphs 2001; Adolphs 2009; Bicks et al. 2015) **(Table 1).** Still it has become clear that many regions and signaling molecules involved in social behaviors also play roles in other non-social behaviors (Frith and Frith 2012; Allsop et al. 2014). Thus it is important to understand exactly what role various circuits and molecules play in generating social behaviors.

Brain Regions	Function	Citation
Fusiform face area	Recognition of faces	Kanwisher et al. 1997
Medial prefrontal Cortex	Theory of mind	Gallagher et al. 2000
Amygdala	Facial expressions/emotion recognition	Adolphs et al. 1994; Whalen et al. 1998
Anterior Insula/Anterior Cingulate Cortex	Empathy	Singer et al. 2004; de Vignemont and Singer, 2006
Temporo-parietal junction	Theory of Mind	Saxe and Kanwisher, 2003
Left inferior frontal cortex/ right superior parietal lobe	Imitation	Iacoboni et al. 1999
Left medial Orbitofrontal cortex	Cooperation	Decety et al. 2004
Hippocampus	Character Judgement	Croft et al. 2010

Table 1: Select brain regions implicated in various human social behaviors

One approach to begin understanding how the brain supports a complex array of social behaviors is to study innate, evolutionarily conserved social behaviors and the circuits that underlie them, in order to understand how these circuits are used to build more complex social and non-social behaviors. An understanding of the circuits involved in building social behaviors can provide a basic understanding of processes such as empathy (Eisenberg and Miller 1987; Preston and de Waal 2002), altruism, (Trivers 1971; Fehr and Fischbacher 2003) and other social behaviors that play important roles in the generation of a functional society.

A translational motivation for understanding social behaviors

Aside from the basic science motivations, there is also a translational appeal for understanding social behaviors. This appeal is that social deficits have emerged as one of the major symptoms observed in many psychiatric diseases including schizophrenia, depression, anxiety, obsessive compulsive disorder, and Fragile X (Kennedy and Adolphs 2012; American Psychiatric Association, 2013; Derntl and Habel 2013). For example, patients with schizophrenia show deficits in facial expression identification and emotion matching when compared to controls (Mueser et al. 1996; Penn et al. 1997). Likewise, patients with depression show social deficits that dramatically affect their quality of life (Segrin 2000; Steger and Kashdan 2009). Furthermore, in patients with general anxiety, social function is significantly affected and has been found to be an important cause for disability when comparing anxious patients to controls (Schonfeld et al. 1997; Kessler et al. 1999; Kroenke et al. 2007). In young adults with anxiety, these

deficits may be even more detrimental because they occur during a period vital for social development (Wittchen, Nelson, and Lachner 1998).

In addition to these diseases that aren't typically thought of as predominantly driven by social deficits, some diseases, such as autism and social anxiety disorder, are primarily characterized by deficits in the social domain (Stein and Stein 2008; Losh M et al. 2009; Kennedy and Adolphs 2012). Patients with social anxiety disorder suffer from significant distress or impairment during social interaction, which interferes with ordinary routine in social settings, at work or school, or during everyday activities (American Psychiatric Association 2013). Thus, individuals with social anxiety disorder avoid interpersonal interactions whenever possible. If they must endure one, it is with extreme emotional and physical discomfort (Schneier 2006; Stein and Stein 2008). Autism Spectrum Disorders are characterized by deficits in a myriad of social behaviors with dramatic impact on one's quality of life (Lord et al. 2000; American Psychiatric Association, 2013). Symptoms can be seen as early as infancy and include a lack of verbal and non-verbal communication, facial gaze, and emotion sharing (Lord et al. 2000). Studies estimate that there are currently over 3 million people in the United States suffering from autism (Fombonne 2003; Buescher et al. 2014) while social anxiety disorder is the most common anxiety disorder (Stein and Stein 2008). These disorders, coupled with other mental health disorders, represent a significant public health burden. In addition, the lack of specific pharmacological treatments for neuropsychiatric diseases such as social anxiety disorder and autism points to a need for a greater understanding of the neural mechanisms that mediate social behaviors.

Current pharmacological treatment approaches for social anxiety disorder and autism spectrum disorders utilize drugs which are used to treat other psychiatric disorders (e.g., anxiety and depression) (Gordon et al. 1993; Stein et al. 1998; Fedoroff and Taylor 2001; Malone et al. 2002; Rodebaugh, Holaway, and Heimberg 2004). In addition, treatments for autism are often ineffective at treating social pathologies (McDougle et al. 2005; but see Hollander et al. 2007; Andari et al. 2010). Thus, there remains a clinical need for a better understanding of the neural substrates underlying social behavior and how they become aberrant in psychiatric disorders.

Although research in humans has provided significant insights about brain regions involved in social behavior (Adolphs 2003; Lieberman 2007), there are considerable ethical and technological limitations to using humans as experimental subjects (Council for International Organizations of Medical Sciences 2002; Institute of Medicine (US) Forum on Neuroscience and Nervous System 2008). Establishing causal relationships between specific neuropsychiatric symptoms and precise brain mechanisms requires invasive techniques that are not suitable for human subjects. In addition, the expense of drug development for psychiatric disorders dictates that drug targets are validated in more economical systems prior to being tested in humans (Frantz 2004). Animal models are one important means to address the limitations of human neuroscience research (Cryan and Holmes 2005; Nestler and Hyman 2010). Animal models enable more invasive methodologies and the application of new technologies in order to provide information about the basic mechanisms involved in driving behavior (Nestler and Hyman 2010; Aston-Jones and Deisseroth 2013; Cruz et

al. 2013; Kim, Chung, and Deisseroth 2013). One such technology is optogenetics. Optogenetics involves the integration of light-sensitive proteins, called "opsins," into cell membranes allowing for millisecond temporal control of cellular activity by photostimulation (Boyden et al. 2005; Fenno, Yizhar, and Deisseroth 2011). The most commonly used light-sensitive opsins are channelrhodopsins (ChRs), halorhodopsins (NpHRs), and Archaerhodopsins (Archs) (Soliman and Trüper 1982; Mukohata et al. 1988; Nagel et al. 2002; Nagel et al. 2003; Zhang et al. 2007). ChRs are a class of cation channels that, when exposed to blue light, cause the depolarization of neuronal membranes where opsins are expressed and results in neuronal excitability (Nagel et al. 2003; Boyden et al. 2005). In contrast, NpHRs are chloride pumps and Archs are proton pumps that, when exposed to yellow light, cause the hyperpolarization of neuronal membranes and results in subsequent inhibition (Zhang et al. 2007; Chow et al. 2010; Gradinaru et al. 2010). Through various targeting strategies, optogenetics allows a high level of spatial and temporal control of specific, molecularly defined neuronal circuits (Tye and Deisseroth 2012). Importantly, optogenetics has been successfully used to elucidate neuronal circuits involved in many complex behaviors relevant to rodent models of psychiatric disease (Nieh et al. 2013; Deisseroth 2014). However, whether it is possible to model psychiatric disease in animals is controversial. For instance, some diagnostic features of psychiatric diseases include terms such as sadness, guilt, delusions, and disorganized thinking (American Psychiatric Association, 2013). These symptoms are difficult, if not impossible, to ascertain in animal models. In addition, the variability in clinical presentation of psychiatric diseases makes modeling emotional disease states in animals a challenge. Nevertheless, scientists have been

able to successfully create models that recapitulate important features of various psychiatric diseases such as anxiety (Lister 1990; Lang, Davis, and Ohman 2000), depression (Willner 1984; Castagné et al. 2001), and autism (Lewis et al. 2007; Ting and Feng 2011).

Indeed, animal models have been a useful tool for scientific inquiry into the brain regions, connections, and signaling involved in social function (Cacioppo 2002b; Insel and Fernald 2004; Crawley 2007; Adolphs 2009; Silverman et al. 2010). Many animals are known to display a wide array of social behaviors that can be assayed in a laboratory setting (Hau, Schapiro, and Jr 2002). For example, Caenorhabditis elegans and Drosophila melanogaster have been successfully used to study the genetic basis of social behaviors such as aggregation, mating, and aggression (Antony and Jallon 1982; Liu and Sternberg 1995; Lee and Hall 2000; Srinivasan et al. 2008; Macosko et al. 2009). For a synopsis of insights provided by the rich genetic toolkits of these model organisms, refer to the review by Sokolowski (2010). Various studies have utilized the social behaviors found in rodents to find neural substrates of innate behaviors like aggression and mating (Choi et al. 2005; Lin et al. 2011; Anderson 2012). Others have made strides in understanding the basis of behaviors such as emotional contagion, empathic responses, and observational learning in rodents (Jeon et al. 2010; Atsak et al. 2011; Bartal, Decety, and Mason 2011). Social behavior has also been studied extensively in non-human primates (Brown and Schafer 1888; de Waal and Suchak 2010). Primates exhibit a very complex set of social behaviors including the formation of long-term alliances and "friendships" that lead to social interactions and hierarchies that

closely resemble human social structures (Cheney, Seyfarth, and Smuts 1986; Whiten et al. 1999; Adolphs 2009). Another important animal model for studying social behavior is the prairie vole. Prairie voles maintain long-term social attachments after mating, known as a pair bond (Getz, Carter, and Gavish 1981; Carter, Devries, and Getz 1995; Wang and Aragona 2004; Young and Wang 2004) and thus serve as an appropriate analog to the type of social bonds observed in humans (Cacioppo 2002a; Insel and Fernald 2004; Adolphs 2009). To date, anatomical and pharmacological techniques have been used in combination with behavioral assays of pair bonding in prairie voles to reveal the importance of molecules such as oxytocin, vasopressin, dopamine, and opioids in selective social attachment (Insel and Hulihan 1995; Cho et al. 1999; Aragona et al. 2003; Aragona et al. 2006; Resendez et al. 2012; Resendez et al. 2013).

Optogenetics offers a great opportunity to begin elucidating the circuits involved in social behavior. Various optogenetic manipulations have provided recent evidence about the neural basis for a number of different rodent social behaviors (Gunaydin et al. 2014; Anderson 2012; Yizhar 2012) and application of optogenetic approaches to models such as the prairie vole holds great promise for future insight into the neurobiology of social attachments and behavior. Optogenetic studies have already provided evidence that manipulation of a single population of synapses can effectively change social behavior in rodents (Gunaydin et al. 2014; Felix-Ortiz and Tye 2014), although social behavior is likely dependent on multiple circuits acting in concert (Baron-Cohen et al. 2000; File and Seth 2003; Bachevalier and Loveland 2006; Rushworth, Mars, and Sallet 2013). Thus clarifying which circuits governing social behaviors interact

with circuits governing other complex behaviors will likely provide insight about the mechanism by which social function is impaired in a wide array of psychiatric diseases. One way of approaching this problem is to study the neural circuits underlying innate[1], evolutionarily conserved social behaviors, how those circuits might be used to build the networks underlying more complex social behaviors, and how this process goes wrong in psychiatric disease.

Observational fear learning: an innate social behavior

There are various innate social behaviors that are critical to an animal's survival that have been conserved across various species. These include courtship behaviors, mating, maternal behaviors, aggression, and observational learning (Matarić 1997; Sokolowski and Corbin 2012). These simpler forms of social behavior provide a reductionist framework in which we can begin to understand the neural basis of social cognition. Observational learning can take many forms and enables animals to learn about action-outcome relationships, places to approach and avoid, and which objects or agents in the environment are aversive or positive (Frith and Frith 2012). For humans, observational learning represents a critical means by which we learn about the world (Meltzoff and Moore 1977; Baeyens et al. 1996; Hopper et al. 2008; Hopper et al. 2010). However, observational learning has also been validated experimentally in a number of

[1] Konrad Lorenz is credited with conceptualizing a distinction between innate and learned behaviors in the mid-20th century (Lorenz 1950; Lorenz 1965). In this view, innate behaviors are not dependent on an animal's experience and are based in set genetic programs, while learned behavior is variable and is dependent on the interaction of the animal with its environment. This dichotomous view has been met with criticism over the years (Lehrman 1953; Johnston 1987) as it has become apparent that the line of distinction is not always so clear and that most behaviors arise due to a combination of genetics as well as experiences. Here, I use this term "innate" to refer to behaviors that manifest without prior conditioning or trial and error processes.

different animal species as well as paradigms. One early seminal study showed that, through observation, cats learned to jump over a hurdle in order to avoid receiving footshocks and that after observation they performed better on an operant reward-conditioning paradigm (John et al. 1968). A similar ability to perform better on an operant reward task after observation was also seen in dogs (Adler and Adler 1977). Octopi learned to select a specific object after observing conditioned octopi perform the task (Fiorito and Scotto 1992). Even the "non-social", red-footed tortoise was able to learn to navigate around an obstacle after observing a demonstrator tortoise (Wilkinson et al. 2010). Not surprisingly, non-human primates have demonstrated the ability to learn various operant and reward tasks, including tool use, through observation (Darby and Riopelle 1959; Tomasello et al. 1987; Hopper et al. 2008; Whiten, Horner, and de Waal 2005). Lastly, multiple studies have detailed the ability of rats and mice to learn to attain rewards, avoid aversive outcomes, and solve puzzles through observation (Russo 1971; Lore, Blanc, and Suedfeld 1971; Heyes and Dawson 1990; Jurado-Parras, Gruart, and Delgado-García 2012). Still, these more complex forms of observational learning are not the focus of my dissertation.

This thesis focuses on observational fear learning, the process by which an animal learns about which stimuli in the environment are predictive of aversive or dangerous consequences through observation[2] of the experience of another animal. It

[2] Observation in this case does not imply sole reliance on visual information. Although various studies have shown visual information to be an important driver of observational learning in rodents, monkeys, and humans (Mineka et al. 1984; Kavaliers et al. 2001; Olsson et al. 2007; Jeon et al. 2010), here the term "observe" refers to a multi-modal perception of the other animal's behavior. Indeed, it has been shown that olfactory and auditory social cues can also play a role in driving fear responses (Kim et al. 2010a; Bredy and Barad 2009).

is dependent on the detection, processing, and integration of a social signal in order to adaptively change behavior. It is a behavior fundamental to survival as many naturally aversive outcomes such as predation or consuming poisonous food would be life threatening to learn about through direct experience (Neilsen et al 2014; Griffin 2004; Frith and Frith 2012). It has been theorized that at the core of observational fear learning is another process known as emotional contagion (Hatfield, Cacioppo, and Rapson 1994; Panksepp and Lahvis 2011). Emotional contagion is the process by which an emotional state in one animal is able to elicit that same emotion in another animal (Preston and de Waal 2002). Although it isn't possible to directly ascertain specific emotional states from animals, there are behaviors that give us insight into the emotional state of an animal. For instance, in rodents, defensive behaviors such as freezing suggest that animals are in a "fear" state (Davis et al. 1993; Davis et al. 2009). Thus, we can use animal models to study the process by which an aversive "fear" state in one animal elicits the same state in another animal that is not in direct danger.

The idea that animals' behavior can be influenced by the aversive experience of other conspecifics has been experimentally tested in a number of different ways since the 1950s (Church 1959; Rice and Gainer 1962; Greene 1969; Rottman and Snowdown 1972; Langford et al. 2006; Chen, Panksepp, and Lahvis 2009a; Kim et al. 2010; Atsak et al. 2011; Pereira et al. 2012). For instance, an early seminal study showed that rats will decrease responses for a food reward when the response was paired with a footshock delivered to another rat (Church 1959). Yet another study showed that rats observing distressed rats hung up in a harness were motivated to press a bar to lower

them (Rice and Gainer 1962). Additionally, it was found that rats would release trapped cagemates in distress (Bartal, Decety, and Mason 2011; Ben-Ami Bartal et al. 2014). Other recent studies have shown that pain sensitivity in mice can be modulated by observation of a cagemate in pain (Kavaliers, Choleris, and Colwell 2001; Langford et al. 2006) and that freezing behavior in conditioned rats is sufficient to drive freezing in rats that have not been conditioned (Atsak et al. 2011; Pereira et al. 2012). Thus there is a plethora of experimental evidence for not only rapid modulation of behaviors in animals who are observing the aversive experiences of other animals, but also shared defensive behaviors in animals when only one is undergoing an aversive experience. It has been thought that this highly conserved innate ability is the basis for more complex behaviors such as empathy and altruism (Trivers 1971; Preston and de Waal 2002; Bastiaansen, Thioux, and Keysers 2009; Panksepp and Lahvis 2011).

However, observational fear learning does not end with the notion that a fear state in one animal can lead to the same state in another animal. Critically, animals must be able to use this experience to learn new information about the environment. In fact, the idea that the aversive experiences of one animal could be used to drive learning during observation has been tested under experimental conditions in various animal species (Mineka et al. 1984; Jeon et al. 2010; Kim et al. 2010; Atsak et al. 2011; Pereira et al. 2012; Chen, Panksepp, and Lahvis 2009b; Langford et al. 2006). A landmark study showed that when rhesus monkeys watched videos of monkeys behaving fearfully to certain fear-relevant toys, they learned to fear those toys (Mineka et al. 1984). It has also been demonstrated that mice learn to avoid biting flies after

observing those flies biting conspecifics (Kavaliers, Choleris, and Colwell 2001). Additionally, it has been demonstrated that rodents can acquire contextual and cued fear conditioning through observation of other mice receiving aversive foot shocks or expressing fear responses to a conditioned stimulus (Chen, Panksepp, and Lahvis 2009; Jeon et al. 2010; Bruchey, Jones, and Monfils 2010; Kim et al. 2012; Jones et al. 2014) Lastly, human subjects have been shown to acquire conditioned fear responses after observation of another subject undergoing fear conditioning (Hygge and Ohman 1978; Vaughan and Lanzetta 1980; Olsson, Nearing, and Phelps 2007).

Despite the experimental evidence detailing the ability of rodents, monkeys, humans, and other animals to learn through observation, surprisingly little is known about its neural basis. However, some studies have sought to begin unraveling how the brain enables observational learning and have found a number of different regions involved. For instance, in rodents, the anterior cingulate cortex (ACC), amygdala, medial and intralaminar nuclei of the thalamus (MITN), prefrontal cortex, and nucleus accumbens have all been shown to have various roles in different observational learning paradigms (Knapska et al. 2006; Jeon et al. 2010; Kim et al. 2012; Jurado-Parras, Gruart, and Delgado-García 2012). In humans, the ACC, amygdala, dorsolateral and ventromedial prefrontal cortex, ventral striatum, cuneus, and cerebellum have all been shown to play a role in various types of observational learning (Torriero et al. 2007; Shane et al. 2008; Burke et al. 2010; Cooper et al. 2011; Goubert et al. 2011).

In studies that have looked specifically at observational fear learning, the ACC and amygdala are repeatedly implicated. The amygdala is a structure within the medial temporal lobe comprised of approximately 13 nuclei, and their respective sub-nuclei (Brodal 1947; Krettek and Price 1978a; Pitkänen, Savander, and LeDoux 1997; Sah et al. 2003) Among these nuclei, the lateral (LA), basal (BA), basomedial (BM) and central amygdala (CeA) and the connections between them have been well characterized for their role in processing fear and reward (LeDoux et al. 1990; Pitkänen, Savander, and LeDoux 1997; Sah et al. 2003; Namburi et al. 2015; Janak and Tye 2015). The LA, BA, and BM are sometimes grouped together functionally and referred to as the basolateral complex (BLA). The amygdala has been though to be critical for valence assignment to stimuli and emotional processing (LeDoux 2000; Namburi et al. 2015; Janak and Tye 2015).

The ACC is a large region of cortex that lies just dorsal to the corpus callosum (Devinsky, Morrell, and Vogt 1995; Allman et al. 2001). It can be divided into dorsal, ventral, anterior and posterior components and these distinctions seem to underlie functional differences (Vogt, Finch, and Olson 1992; Devinsky, Morrell, and Vogt 1995; Bush, Luu, and Posner 2000; Beckmann, Johansen-Berg, and Rushworth 2009; Etkin, Egner, and Kalisch 2011). Thus, across various species the ACC has been implicated in a number of different functions including attention, autonomic functions, action selection, conflict monitoring, competitive effort, pain processing, learning and memory, social prediction, and empathy (Tow and Whitty 1953; Kennard 1955; Gabriel 1990; Devinsky, Morrell, and Vogt 1995; Bussey et al. 1996; Rainville et al. 1997; Cohen et al.

1999; Hutchison et al. 1999; Botvinick, Cohen, and Carter 2004; Johansen and Fields 2004; Singer et al. 2004; Williams et al. 2004a; Hillman and Bilkey 2012; Sheth et al. 2012; Haroush and Williams 2015). It is thought to have such a wide variety of functions due to its broad efferent and afferent connectivity with regions such as the amygdala, hippocampus, hypothalamus, peri-aqueductal grey, thalamus, sensory cortices, and motor cortex (Bush, Luu, and Posner 2000; Beckmann, Johansen-Berg, and Rushworth 2009; Etkin, Egner, and Kalisch 2011). Overall, a simplified model divides the ACC's function into two major categories: cognitive appraisal and emotional modulation (Vogt, Finch, and Olson 1992; Devinsky, Morrell, and Vogt 1995; Carter et al. 1998; Bush, Luu, and Posner 2000; Quirk and Beer 2006).

In rodents it was found through lesion studies that both the ACC and LA were necessary for observational fear learning (Jeon et al. 2010). Furthermore, that study also found that during observational fear learning there was increased theta frequency synchronization between the amygdala and ACC. A follow-up study showed that electrical stimulation of the ACC was sufficient to enhance observational fear learning (Kim et al. 2012). In another study, they found that observational fear learning increased c-fos in the dorsomedial prefrontal cortex (dmPFC), which includes the ACC, as well as the BLA (Ito, Erisir, and Morozov 2015). In addition, synaptic changes occurred at dmPFC inputs to the BLA as a result of observational fear learning (Ito, Erisir, and Morozov 2015). Yet another study in rodents showed that normal dopamine and serotonin signaling within the ACC was necessary for observational fear learning (Kim et al. 2014a). Lastly, in humans observing demonstrators undergoing fear conditioning,

there was increased BOLD activity in the ACC, anterior insula, and amygdala (Olsson, Nearing, and Phelps 2007). It is clear from all of these studies that the ACC and the amygdala play some important role in observational fear learning. Elucidating the exact nature of that role is the focus of the work presented in this dissertation.

Fear conditioning: how the brain learns from direct experience

In order to properly interpret data from an observational fear conditioning experiment, one must be familiar with the body of literature detailing the manner in which animals learn about what to fear through direct experience (Pavlov 1927; Davis 1992; Fendt and Fanselow 1999; LeDoux 2000; Maren and Quirk 2004; Pape and Pare 2010; Johansen et al. 2011; Janak and Tye 2015). Pavlovian fear conditioning has become well known as a learning paradigm in which a neutral stimulus (conditioned stimulus) such as a light or tone is paired to an innately aversive stimulus (unconditioned stimulus). The unconditioned stimulus (US) is able to elicit defensive behaviors such as freezing (Small 1899; Griffith 1920; Blanchard and Blanchard 1971; Bouton and Bolles 1980), changes in blood pressure and heart rate (Holdstock and Schwartzbaum 1965; Hoffman and Fitzgerald 1978), changes in pain sensitivity (Chance, Krynock, and Rosecrans 1978; Fanselow and Baackes 1982), and heightened reflexes such as the startle reflex (Lang, Bradley, and Cuthbert 1990; Davis et al. 1993). When the conditioned stimulus (CS) is paired in a predictive manner to the US, animals begin to show defensive behaviors to the CS (Rescorla 1988). These behaviors are referred to as conditioned responses (CR). These defensive behaviors have been seen as representative of a fear state in animal models such as rodents due to the fact that

the constellation of behavioral changes that occur during conditioning are similar to that seen in humans when they report being in a fear state (Darwin, Ekman, and Prodger 1998; Davis 1992; Fendt and Fanselow 1999; American Psychiatric Association 2013). However, it is useful to keep in mind that when in danger, animals show varying "innate species-specific defensive reactions", thus the defensive behaviors we see during fear conditioning experiments represent the constellation of behaviors animals likely use to respond to danger in the wild (Bolles 1970). For example, rodents display robust freezing during fear conditioning because in the wild, predators are more likely to attack moving prey than still prey (Fanselow and Lester 1988).

Pavlovian fear conditioning has been a powerful experimental paradigm in behavioral neuroscience for a few reasons. Firstly, the ability to learn about which stimuli in the environment represent a potential threat and respond with appropriate behaviors is critical to survival. Thus, this form of conditioning has been shown to work in a wide range of animals including flies, worms, fish, birds, rodents, cats, dogs, monkeys, and humans (Carew and Sahley 1986; LeDoux 2000). This highlights its fundamental importance to the evolutionary fitness of animals and the wide applicability of experimental findings that use this behavioral model. Secondly, fear conditioning is a very robust form of learning and memory (Squire 1987; Maren 2005). Thus it has served as an important model for detailing the neural and molecular mechanisms underlying associative learning and memory (Thompson 1986; Atkins et al. 1998; Rumpel et al. 2005; Fanselow and Poulos 2005). Thirdly, fear conditioning provides us with a tractable experimental paradigm to begin solving the complex problem of how the brain

enables emotion and how emotions influence behavior (Darwin, Ekman, and Prodger 1998; LeDoux 2003; Delgado, Olsson, and Phelps 2006). Lastly, the prevalence of psychiatric disorders such as general anxiety disorder, post-traumatic stress disorder, and specific phobias provide a translational motivation for understanding how fear is processed in the brain and how that process can become pathological (Lissek et al. 2005; Mahan and Ressler 2012; Calhoon and Tye 2015; American Psychiatric Association 2013).

The anterior cingulate cortex and amygdala and in fear conditioning

Decades of fear conditioning research have established the amygdala as a critical region necessary for fear learning and expression (Davis 1992; Fendt and Fanselow 1999; LeDoux 2000; Maren and Quirk 2004; Pape and Pare 2010; Johansen et al. 2011; Janak and Tye 2015). Early lesion studies in monkeys[3] first provided evidence that damage to the amygdala results in profound alterations in a wide range of emotional responses, most notably, fear (Brown and Schafer 1888; Kluver and Bucy 1939; Weiskrantz 1956). Later studies firmly established a role for the amygdala in fear learning and memory across a range of mammals including rodents, monkeys, and

[3] In the landmark study by Brown and Schafer, they removed the temporal lobe (which includes the amygdala) of monkeys and saw significant deficits in the fear and avoidance behavior of a subset of animals (Brown and Schafer 1888). Later, Kluver and Bucy would also show blunted fear responses in monkeys with temporal lobectomies (Kluver and Bucy, 1939). Finally, Weiskrantz would show that isolated amygdala lesions could reproduce some of the phenotypes seen by Kluver and Bucy and Brown and Schafer. For instance, monkeys with amygdala lesions "permitted petting and handling without visible excitement" and even "approached and reached for observers" while control animals "continued to display their fear of and hostility toward humans by running to the farthest corner of the cage, frequently urinating and defecating, grimacing, and screeching" (Weiskrantz 1956).

humans (Robinson 1963; Horvath 1963; Kellicutt and Schwartzbaum 1963; Fonberg 1965; Blanchard and Blanchard 1972; Adolphs et al. 1994; Bechara et al. 1995; LaBar et al. 1995; Morris et al. 1996; Anderson and Phelps 2001).

Numerous studies have lead to an understanding of how information during classical fear conditioning is processed in the amygdala. Sensory information about the CS is relayed to the BLA from the sensory thalamus as well as sensory cortex(LeDoux, Farb, and Ruggiero 1990; LeDoux et al. 1990; Clugnet and LeDoux 1990; McCabe et al. 1993; Romanski and LeDoux 1993; Campeau and Davis 1995; Quirk, Armony, and LeDoux 1997a; Rogan, Stäubli, and LeDoux 1997; Kwon et al. 2014). Meanwhile, it is thought that information about the US also reaches the BLA from thalamic and cortical regions as well as regions such as the periaqueductal grey known to process nociceptive information (Romanski et al. 1993; Shi and Davis 1999; Lanuza, Moncho-Bogani, and LeDoux 2008; Johansen et al. 2010) while the CeA also receives US information from nociceptive areas (Bernard and Besson 1990; Burstein and Potrebic 1993). Thus the BLA is well suited to integrate CS and US information and neurons within the BLA have been shown to respond to CSs as well as USs and also show plasticity during or as a result of fear learning (Maren, Poremba, and Gabriel 1991; Uwano 1995; Quirk, Repa, and LeDoux 1995a; Quirk, Armony, and LeDoux 1997b; Rogan, Stäubli, and LeDoux 1997; McKernan and Shinnick-Gallagher 1997; Johansen et al. 2010; Nabavi et al. 2014; Namburi et al. 2015). Once the CS-US association is integrated in the BLA it then relays this information to the CeA, which serves as the main behavioral output of the amygdala (LeDoux et al. 1988; Kapp et al. 1992; Killcross, Robbins, and Everitt 1997; Duvarci, Popa, and Paré 2011; Li et al. 2013). The CeA then

projects to a number of regions such as the peri-aqueductal grey, parabrachial nucleus, Bed nucleus of the stria terminalis, and the hypothalamus in order to generate the behavioral and autonomic changes seen during conditioning (Krettek and Price 1978b; LeDoux et al. 1988; Veening, Swanson, and Sawchenko 1984; Rizvi et al. 1991; Petrovich, Canteras, and Swanson 2001; Dong, Petrovich, and Swanson 2001). While this general idea of information flow through the amygdala has been a useful model and seems to be generally accurate, in fact, the picture is much more complicated than this model suggests (Cahill et al. 1999; Paré, Quirk, and Ledoux 2004; Wilensky et al. 2006; Gross and Canteras 2012; Herry and Johansen 2014). For example, recent studies have provided compelling evidence that expands the role of CeA microcircuitry and its direct thalamic input in fear acquisition and expression (Ciocchi et al. 2010; Haubensak et al. 2010; Penzo et al. 2015).

In addition to the amygdala, the prefrontal cortex (PFC), has also been shown to play a role in fear conditioning and expression (Fuster 2001; Herry and Johansen 2014; Etkin, Egner, and Kalisch 2011; Courtin et al. 2013; Courtin et al. 2014). The PFC is a term used to describe a number of separate but interconnected regions in the cortex. In humans and non-human primates, it is divided into orbital, lateral, and medial regions encompassing Brodman's areas 8-13, 24, 25, 32, 46, and 47 (Öngür and Price 2000; Fuster 2001). In rodents, it encompasses the prelimbic (PL), infralimbic (IL), and ACC regions (Öngür and Price 2000; Quirk and Beer 2006). The PFC has been shown to play a role in fear conditioning and expression in humans as well as rodents (Gilmartin and McEchron 2005; Laviolette, Lipski, and Grace 2005; Milad et al. 2007; Corcoran

and Quirk 2007; Mechias, Etkin, and Kalisch 2010; Etkin, Egner, and Kalisch 2011; Burgos-Robles, Vidal-Gonzalez, and Quirk 2009; Julien Courtin et al. 2014). In a seemingly conflicting role, some regions of PFC have also been shown to be important for promoting and maintaining fear extinction (Morgan and LeDoux 1995; Milad and Quirk 2002; Phelps et al. 2004; Gottfried and Dolan 2004; Burgos-Robles et al. 2007; Maroun et al. 2012; Do-Monte et al. 2015). Within the PFC, the ACC has also been shown to have some role in fear conditioning (Frankland et al. 2004; Tang et al. 2005; Milad et al. 2007; Bissiere et al. 2008; Etkin, Egner, and Kalisch 2011). However, It remains unclear, to what extent the ACC's role in fear conditioning overlaps with its role in observational conditioning.

The anterior cingulate cortex and amygdala in social behavior

The ACC has been shown to have various roles in the social behavior of rodents, monkeys, and humans (Devinsky, Morrell, and Vogt 1995; Rudebeck et al. 2006; Chang, Gariépy, and Platt 2013; Rilling et al. 2002; Singer et al. 2004; de Vignemont and Singer 2006; Rushworth, Mars, and Sallet 2013; Haroush and Williams 2015). A combination of approaches across humans and animal models have implicated the ACC to be involved in social behaviors such as social interest (Rudebeck et al. 2006), maternal behavior (Slotnick 1967), vocalizations (Aitken 1981), cooperation (Rilling et al. 2002), and interaction (Hadland et al. 2003), as well as higher order social cognitive functions such as social prediction (Haroush and Williams 2015), social decision making (Chang, Gariépy, and Platt 2013), empathy (Singer et al. 2004; de Vignemont and Singer 2006), and theory of mind (Vogeley et al. 2001; Amodio and Frith 2006). Early

surgical interventions in patients revealed that Cingulate lesions lead to profound changes in personality and affect, most notably affecting their social lives (Jarvie 1954; Tow and Whitty 1953; Saver and Damasio 1991; Devinsky, Morrell, and Vogt 1995). ACC lesions in monkeys have further validated the necessity of the ACC for normal social behavior (Hadland et al. 2003; Rudebeck et al. 2006). Lastly, ACC dysfunction has been implicated as part of the pathology for a number of psychiatric diseases including autism (Beasley et al. 2006; Thakkar et al. 2008; Oblak, Gibbs, and Blatt 2009; Dichter, Felder, and Bodfish 2009). However, as previously mentioned, the ACC has also been shown to be important in non-social domains of cognition and behavior (Vogt, Finch, and Olson 1992; Devinsky, Morrell, and Vogt 1995; Carter et al. 1998; Bush et al. 2002; Quirk and Beer 2006). Thus, it is still uncertain exactly what the primary role of the ACC is and whether or not its social functions are secondary to its other cognitive functions.

The amygdala's role in social behavior has also been well-established (Brown and Schafer 1888; Kluver and Bucy 1939; Jonason and Enloe 1971; Kling and Steklis 1976; Amaral 2003; Adolphs 2010). Diseases such as autism, Urbach-Wiethe disease, Kluver-Bucy syndrome, and Williams syndrome have provided clues regarding the involvement of the amygdala in social behavior as amygdala damage or dysfunction appears to precipitate aberrant sociality in these diseases (Baron-Cohen et al. 2000; Meyer-Lindenberg et al. 2005; Todd and Anderson 2009; Adolphs 2010; Haas et al. 2010) In support of the notion that the amygdala plays a role in social functioning, it has also been found that higher amygdala volume and stronger intrinsic connectivity is

correlated with having a larger, more complex social network (Bickart et al. 2011; Bickart et al. 2012). In animals, many lines of investigation has established a role for the amygdala in social behaviors such as aggression, sex, and social affiliation (Rosvold, Mirsky, and Pribram 1954; Emery et al. 2001; Amaral 2003; Machado and Bachevalier 2006; Machado et al. 2008; Adolphs 2010; Bliss-Moreau et al. 2013; Chang et al. 2015). However, it should be noted that the amygdala's role in anxiety and fear can also affect social behaviors, thus it can be sometimes difficult to dissociate whether social deficits after amygdala perturbation are specific or secondary to changes in anxiety and fear (Felix-Ortiz et al. 2016; Siuda et al. 2016).

Towards understanding observational learning at the circuit level

In this chapter I have outlined the basic science and translational motivations for the pursuit of my thesis work. Social behaviors are a critical part of the behavioral repertoire of humans and other animals. This is highlighted by the break down in quality of life that occurs when humans can no longer engage in appropriate social interaction due to psychiatric disease (Schonfeld et al. 1997; Segrin 2000; Steger and Kashdan 2009; Kennedy and Adolphs 2012). Furthermore, the generation of culture and the structure and function of society are dependent on social norms and behaviors such as empathy and altruism (Sherif 1936; Trivers 1971; Cialdini and Goldstein 2004). Thus, understanding the neural basis for the generation and maintenance of social behaviors and structures has the potential to allow us to understand fundamental aspects of humanity and society. In addition, the public health burden of social deficits seen in psychiatric diseases such as autism, anxiety, and schizophrenia provide motivation to

understand how abnormal neural function gives rise to aberrant social behavior. I made the case that a wide variety of animal models are useful in this pursuit as they allow for a level of detailed experimentation and analysis that isn't currently available in humans. However, because many social behaviors are complex and involve a variety of brain regions, I have suggested that one way to approach this problem is to focus on innate forms of social behaviors in order to delineate the neural circuits that are at the core of these conserved behaviors. These are likely to be the circuits that are involved in generating the more complex behaviors that are a part of an animal's social repertoire.

Overall, we make a case that studying observational fear learning provides an excellent basis to begin understanding how the social brain works on a fundamental level. Firstly, the idea that this is a robust, very early form of social cognition is evident from its widespread occurrence across the animal kingdom, even in solitary animals that don't normally engage in social behaviors (John et al. 1968; Adler and Adler 1977; Fiorito and Scotto 1992; Wilkinson et al. 2010). The fact that social isolation during development impairs an animals ability to learn through observation (Yusufishaq and Rosenkranz 2013) and that the type of social relationship between a demonstrator and observer modulates the efficiency of learning (Jeon et al. 2010a; Golkar, Castro, and Olsson 2015) strongly suggests that observational learning is a social behavior and cannot be thought of as simply another form of associative learning. Secondly, within observational fear learning, there are dissociable processes as discussed above that provide us with the opportunity to use tools such as optogenetics, electrophysiology, and imaging to interrogate the different circuit components necessary for specific

aspects of this behavior. For instance, we can ask at least three distinct questions during an observational learning paradigm:

1) How do animals detect and process social signals?
2) How are those signals incorporated into motivational systems to change behavior?
3) How does this processing enable learning?

We can then interrogate at multiple levels of analysis the circuits involved in each step of the process. Lastly, the rich literature detailing the mechanisms of classical fear conditioning allow us some context and insight for detangling the circuits that govern the social elements of observational learning, and thus are likely specialized for social cognition, as opposed to those circuits that are involved in general learning mechanisms.

It has become clear that the anterior cingulate cortex (ACC) and the amygdala play a role in observational learning. In mice, observational fear learning was impaired by injection of lidocaine into the ACC as well as the basolateral amygdala (BLA) (Jeon et al. 2010). Interestingly, when doing simultaneous recordings from the ACC and amygdala, there was increased theta frequency synchronization between the two regions during observational fear learning (Jeon et al. 2010). Lastly, in humans, it was shown that the ACC and the amygdala are recruited when subjects acquired a fear to a conditioned stimulus through observation with no direct experience of the aversive event (Olsson, Nearing, and Phelps 2007). The notion that these two regions may work together during observational learning is bolstered by studies showing that the ACC and

BLA have reciprocal connections with each other (Cassell and Wright 1986; Gabbott et al. 2005; Bissiere et al. 2008) and that optogenetic stimulation of ACC terminals in the BLA invoked LTP in BLA pyramidal neurons (Morozov, Sukato, and Ito 2011).

While there is evidence that the ACC and amygdala are necessary for observational fear learning, there are several outstanding, important questions and the combination of optogenetics and electrophysiology has not been utilized in order to provide a circuit level analysis of the temporal dynamics of neural responses in either region. Thus, *how* the ACC or amygdala encodes information necessary for observational learning is unknown. Furthermore, it is unknown if neurons in the ACC or BLA encode the learned meaning of a CS during observational learning and if so whether that information is directly transmitted between these two regions. In addition, the directionality of information flow during observational learning is also unknown. Along those lines, it has not been determined if neurons in the ACC that provide input to the amygdala encode specific information critical to observational learning. Additionally, whether ACC projections to the amygdala are necessary for observational learning has not been determined. Lastly, how neural activity in one region affects the neural encoding of information in the other region during observational learning has not been determined. Lastly, the concept that circuits involved in observational fear learning are also used for normal social interaction has not been directly tested.

Through combining optogenetics, electrophysiology, and rodent behavioral paradigms, we provide the first circuit level analysis of observational fear learning and in the following chapters introduce data that provides insight to all of the questions above.

This thesis work provides the basis for an increasingly advanced understanding of how neural circuits contribute to the generation of social behaviors.

Chapter 2

Neural dynamics of the Anterior Cingulate Cortex and Amygdala during observational fear learning

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Abstract

Observational fear learning is a powerful survival tool, allowing an individual to learn about environmental stimuli that predict specific threats without direct experience. This ability has been conserved from rodents to humans, and has been linked to the anterior cingulate cortex (ACC) and the basolateral amygdala (BLA). Here we record from the ACC as well as the BLA in an observer mouse during observational fear learning and show that both regions contain neurons that respond to the conditioned stimulus. In addition, we show that in an observer, both regions contain neurons that encode the learned predictive meaning of a cue during observational learning. Lastly, we provide evidence that on a population level, neurons in the ACC learn about the cue prior to those in the amygdala. Together this work demonstrates that the ACC and BLA contain neural correlates of observational learning at the neural level and that the ACC leads the BLA in acquiring the learned meaning of the cue.

Background

It is well-known that animals use direct sensory experiences to learn about aversive stimuli and their predictors (Pavlov 1927; Davis 1992; Fendt and Fanselow 1999; LeDoux 2000; Maren and Quirk 2004; Pape and Pare 2010; Johansen et al. 2011; Janak and Tye 2015). However, learning about aversive stimuli through direct experience often puts an animal in life-threatening situations (Griffin 2004; Nielsen et al. 2012). Thus, the ability to use the experiences of others to extract information about events that are predictive of aversive outcomes is critical to evolutionary fitness. This kind of observational learning is a fundamental social behavior that is highly conserved

across various species (Mineka et al. 1984; Heyes and Dawson 1990; Kavaliers, Choleris, and Colwell 2001; Olsson, Nearing, and Phelps 2007; Barber and Kimbrough 2015). For example, it has been shown that rodents display defensive behaviors when in the presence of conspecifics undergoing aversive or stressful experiences (Church 1959; Rice and Gainer 1962; Greene 1969; Chen, Panksepp, and Lahvis 2009b; Kim et al. 2010b; Atsak et al. 2011; Pereira et al. 2012). In addition, mice observing demonstrators undergo negative experiences showed increases in depression-like and anxiety-like behaviors (Warren et al. 2013) as well as fear learning (Guzmán et al. 2009; Chen, Panksepp, and Lahvis 2009b; Bruchey, Jones, and Monfils 2010; Jeon et al. 2010; Kim et al. 2012; Yusufishaq and Rosenkranz 2013). However, despite the fundamental importance of observational learning to the survival of humans and other animal species, little is known about its neural basis.

In both rodents and humans, the ACC and amygdala have been implicated in observational fear learning and social cognition (Jeon et al. 2010; Olsson, Nearing, and Phelps 2007; Kim et al. 2012; Adolphs et al. 1994; Kim et al. 2014). In humans, both brain regions were recruited when subjects acquired fear responses to a conditioned stimulus through observation (Olsson, Nearing, and Phelps 2007). Likewise, in mice engaged in observational fear learning there was increased theta frequency synchronization between the ACC and the lateral amygdala (Jeon et al. 2010). Additionally, pharmacological lesions of either region inhibited learning (Jeon et al. 2010; Kim et al. 2012; Kim et al. 2014). The notion that these two regions may work together during observational learning is bolstered by studies showing that the ACC and

the basolateral complex of the amygdala (BLA), which includes the lateral amygdala (Janak and Tye 2015) have reciprocal connections with each other (Cassell and Wright 1986; Gabbott et al. 2005; Bissiere et al. 2008) and optogenetic stimulation of ACC terminals in the BLA invokes a persistent strengthening of synapses in putative pyramidal neurons (Morozov, Sukato, and Ito 2011). Despite this evidence, how these regions encode and transmit information necessary for observational learning remains unknown. Furthermore, the function, directionality, and necessity of the ACC-BLA circuit in observational learning have heretofore not been determined.

Results

In order to address the role of the ACC and BLA in observational fear learning, we designed a behavioral paradigm in which mice observe a demonstrator mouse undergo cued fear conditioning through a transparent divider with holes allowing for the transmission of auditory, visual, and olfactory information from one mouse to the other **(Fig. 1a)**. In this study, observation refers to the multimodal perception of the behavior of the demonstrator. Mice termed "Experienced observers" (EO) first briefly explore the shock floor side of the chamber, receive a single shock, and are immediately placed on the plastic "safe" floor side of the chamber where they will no longer be shocked. A "demonstrator" familiar with the observer is then placed on the shock floor side of the chamber and 30 cue-shock pairings are delivered in which a cue is predictive of shock delivery to the demonstrator. 24 hrs later, observers are tested for cue learning by being placed back on the shock floor without a demonstrator and cues are played in the absence of shock (**Fig. 1b**). EO mice demonstrated increased freezing to the cue during

test day as well as during observational conditioning (**Fig. 1c, Fig. 2a)** To verify that this learning was driven by the fear responses of the demonstrator we included experienced solo (ES) mice that received the initial shock experience but then observed cues and shocks delivered to an empty chamber (**Fig. 1b**). ES mice did not demonstrate conditioned responses to the cue during training or test day (**Fig. 1c, Fig. 2a**). To confirm that conditioned responses to the cue observed in the EO group were due to the fact that the cue predicted delivery of shock, we also had unpaired observers (UO).

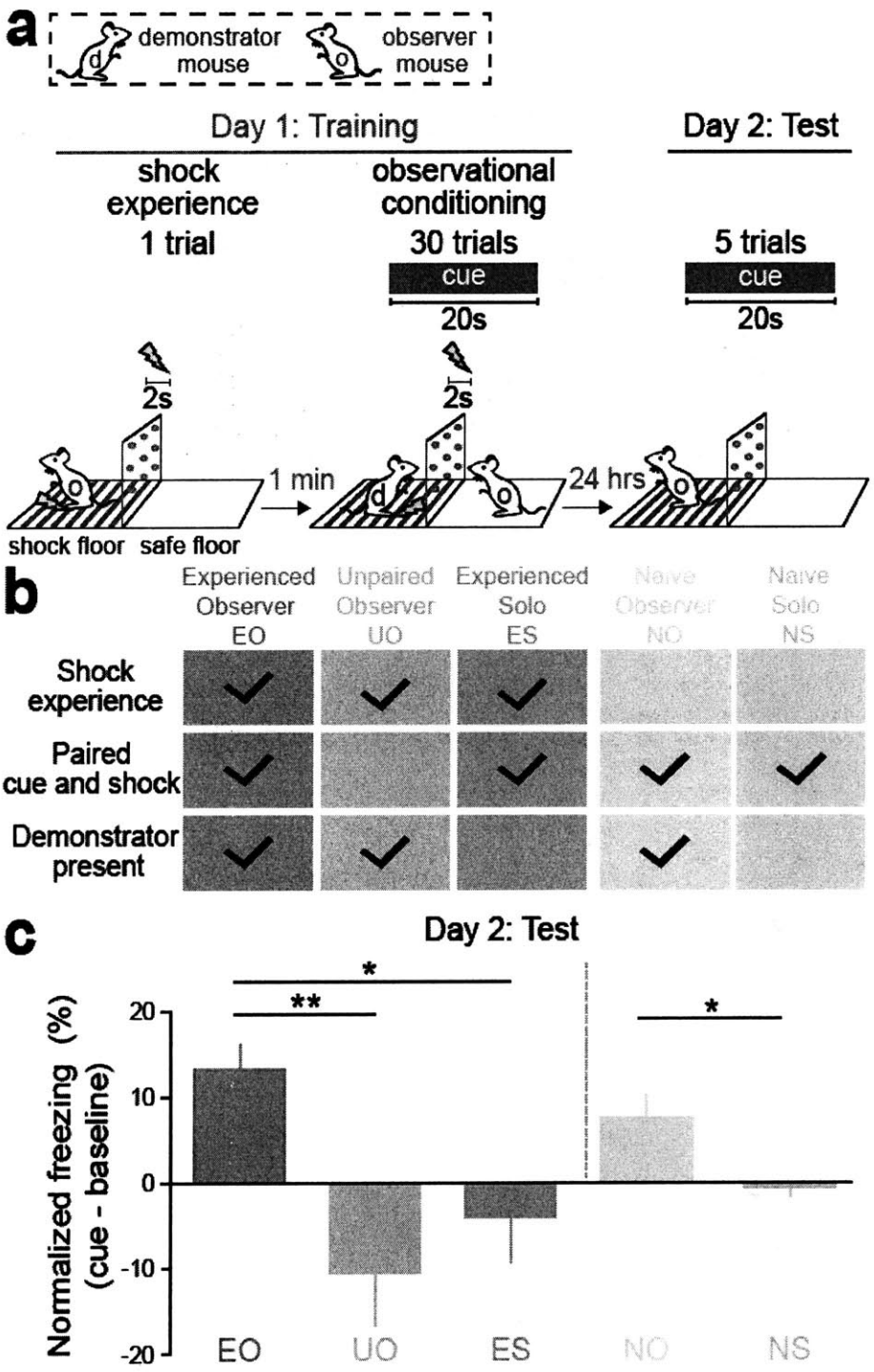

a
demonstrator mouse
observer mouse
Day 1: Training
Day 2: Test
shock experience
1 trial
observational conditioning
30 trials
cue
20s
5 trials
cue
20s
2s
2s
1 min
24 hrs
shock floor
safe floor
b
Experienced Observer EO
Unpaired Observer UO
Experienced Solo ES
Naive Observer NO
Naive Solo NS
Shock experience
Paired cue and shock
Demonstrator present
c
Day 2: Test
Normalized freezing (%) (cue - baseline)
20
10
0
-10
-20
*
**
*
EO
UO
ES
NO
NS

Figure 1 Mice demonstrate cue learning 24 hours after observational conditioning

a. Outline of observational conditioning paradigm for experienced observers (EO). On Day 1, Training Day, an observer mouse receives one foot shock before being transferred to a safe plastic floor. It then observes a demonstrator receive cue-shock pairings through a transparent divider containing holes. 24 hrs later (Day 2, Test Day) the observer is placed back on the shock side of the chamber and cues are delivered in the absence of shock.

b. Outline of conditioning paradigms for all behavioral groups. Experienced Observer conditions are described above (EO, n=7). Mice in the unpaired observer (UO, n=5) group receive an initial shock experience and then observe unpaired cues and shocks delivered to demonstrators. Experienced solo (ES, n=6) mice received an initial shock experience and then observe paired cues and shocks delivered to an empty chamber. Naïve observer (NO, n=7) mice did not receive an initial shock experience but observed demonstrators receive paired cues and shocks. Naïve solo (NS, n=4) mice did not receive an initial shock experience and observed paired cues and shocks delivered to an empty chamber.

c. On Day 2, Test Day, EO mice showed increasing freezing to the cue when compared to UO and ES mice (one-way ANOVA, $F_{2,15}=6.95$, $P=0.0073$, Bonferroni post-hoc analysis, EO vs UO,$^{**}P<0.01$, EO vs ES, $^{*}p<0.05$). NO mice showed increased freezing to the cue when compared to control mice (NS) (two-tailed, unpaired student's t-test, $^{*}P= 0.0422$).

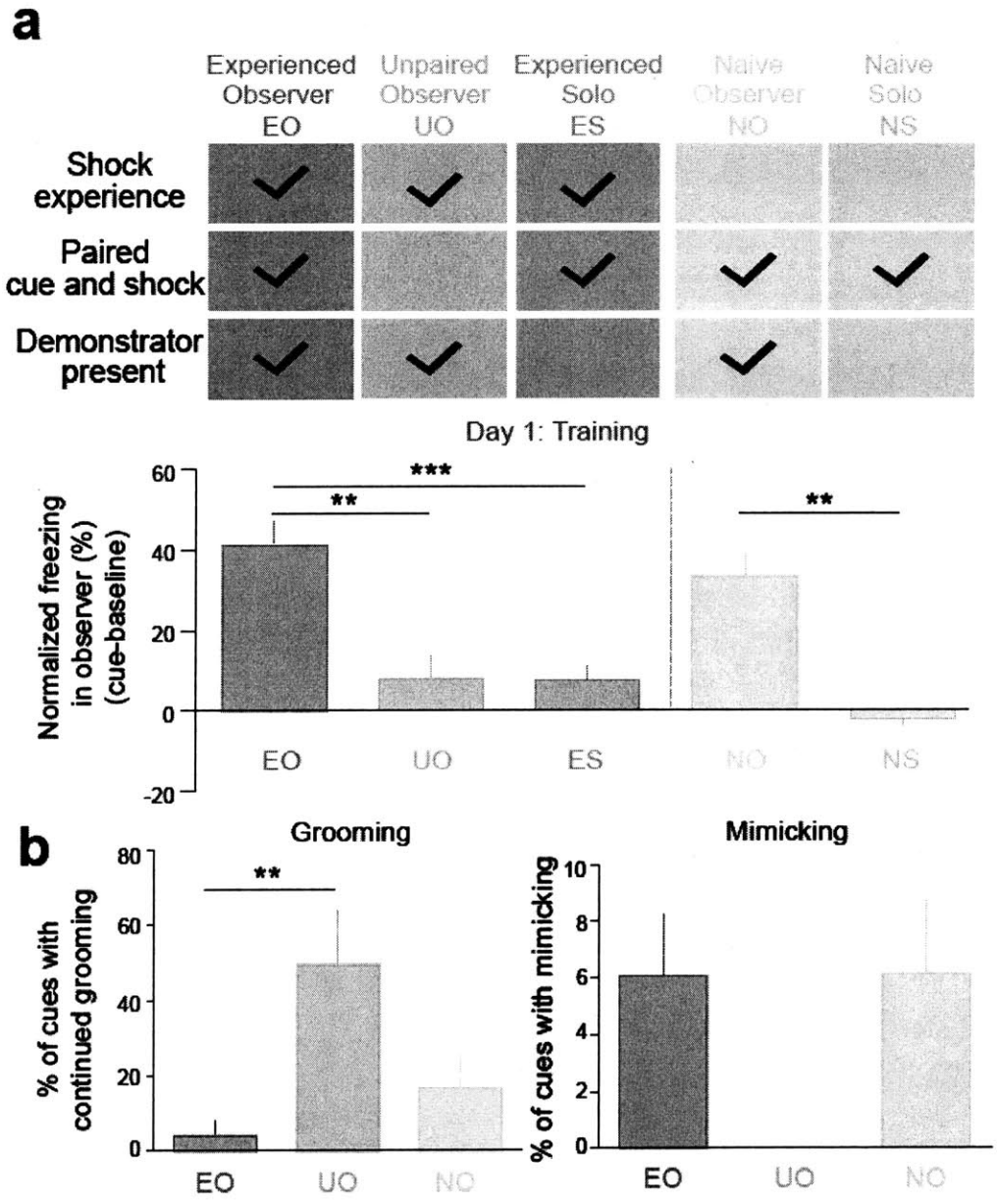

a
Experienced Observer EO
Unpaired Observer UO
Experienced Solo ES
Naive Observer NO
Naive Solo NS
Shock experience
Paired cue and shock
Demonstrator present
Day 1: Training
Normalized freezing in observer (%) (cue-baseline)
60
40
20
0
-20
***
**
**
EO
UO
ES
NO
NS
b
Grooming
% of cues with continued grooming
80
60
40
20
0
**
EO
UO
NO
Mimicking
% of cues with mimicking
10
8
6
4
2
0
EO
UO
NO

Figure 2 Mice demonstrate cue learning during observational conditioning

a. On the observational conditioning day, EO mice showed increasing freezing to the cue when compared to UO and ES mice (one-way ANOVA, $F_{2,15}$=6.95, P=0.0073, Bonferroni post-hoc analysis, EO vs UO,**P<0.01, EO vs ES, *P<0.05). NO mice showed increased freezing to the cue when compared to control mice (NS) (two-tailed, unpaired student's t-test, **P= 0.0012).

b. Additional behaviors observed during observational conditioning. UO mice show continued grooming after cue onset compared to EO (one-way ANOVA, $F_{2,14}$=6.273, P=0.0113, Bonferroni post-hoc analysis, EO vs UO,**P<0.01). Both EO and NO mimicked behavior of demonstrator.

In this group, experienced mice observed demonstrators receiving foot shocks explicitly unpaired to the cue (**Fig. 1b**). UO mice did not display conditioned freezing to the cue during the conditioning epoch and on the test day showed decreased freezing to the cue (**Fig. 1c, Fig. 2a**). This was consistent with the observation that demonstrator mice never received foot shocks when the cue was being played. Thus, in the UO animals the cue likely became a signal of safety. Lastly, we probed the role of the initial shock experience with a naïve observer (NO) group. These mice did not experience an initial shock but did observe demonstrators receiving paired cues and shocks (**Fig. 1b**). NO mice also showed increased freezing to the cue during training as well as test day, comparable to the EO mice (**Fig. 1c, Fig. 2a).** Naïve solo (NS) mice that never experienced foot shock and observed an empty chamber where cues and shocks were delivered showed no freezing (**Fig. 1c, Fig. 2a).** In addition to freezing, EO and NO mice also demonstrated other behaviors consistent with learning the aversive nature of the cue such as interruption of grooming and mimicking of the demonstrator (**Fig. 2b).**

Lastly, we also assessed whether observational conditioning lead to increased affiliative interaction of the observer with the demonstrator by analyzing social interaction in the home cage prior to and immediately following observational conditioning **(Fig. 3a)**. We observed that EO mice showed increased affiliative social interaction with demonstrators after observational conditioning **(Fig. 3b).** This was not seen in the ES group, suggesting that this increase in interaction was driven by the observation of distress in the demonstrator. This increase in interaction may be indicative of empathic processes (Burkett et al. 2016) or the social buffering of stress previously observed in rodents (Taylor 1981; Beery and Kaufer 2015).

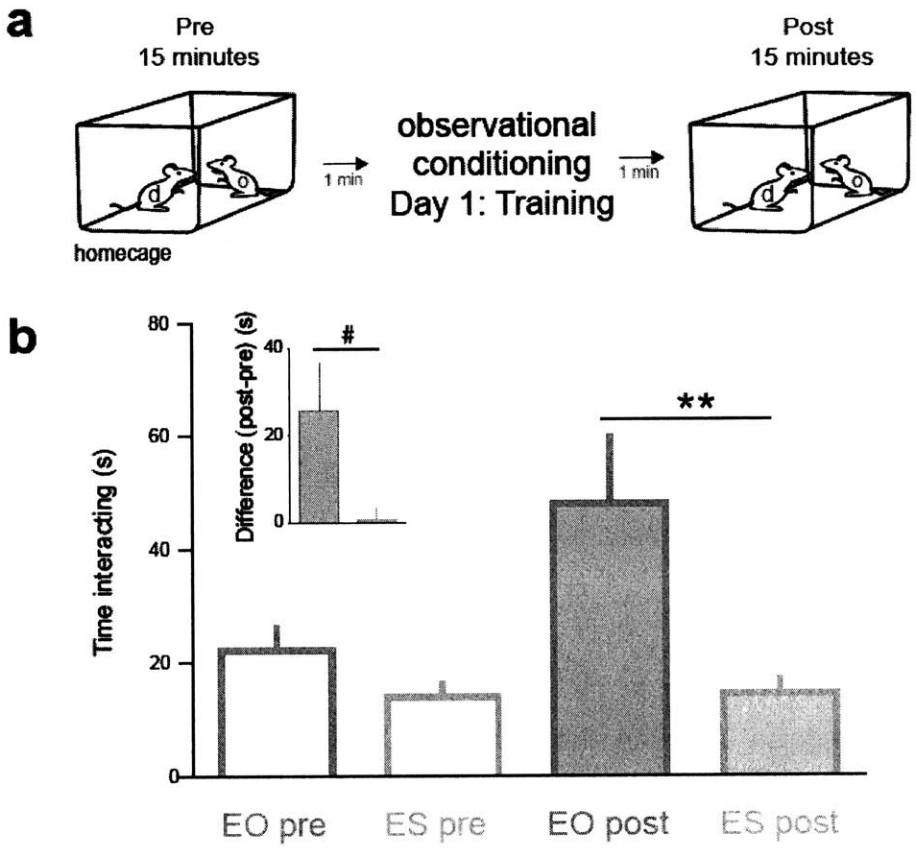


Figure 3 Experienced Observers show increased social interaction after observational conditioning

a. The social interaction of observers with demonstrators was scored in their homecage prior to and directly following observational conditioning.

b. Experienced Observers (EO) spent more time interacting following observational conditioning in contrast Experienced Solo (ES) animals which did not have an increase in interaction (two-way ANOVA, Bonferroni post hoc analysis, **p <.01) (two-tailed, unpaired student's t-test, #*P* = 0.0548).

The observed cue-induced freezing in EO and NO demonstrates that mice learn to associate an aversive outcome with a previously neutral cue through observation of a distressed cage mate. However, from this experiment it is unclear whether mice learned that the shock floor was the source of the aversive outcome or if they only learned about the predictive conditioned stimulus through the demonstrator's distress. If a mouse acquired knowledge of the shock floor as an aversive stimulus, we would expect this mouse to avoid the shock grid. In contrast, if a mouse acquired an association between the cue and distress of a conspecific without learning that the shock floor was aversive, this mouse would not necessarily avoid the shock grid and might even avoid the location from which the distress was observed.

To distinguish between these two possibilities, we performed an additional experiment wherein we placed EO and NO mice in a chamber with a shock floor and safe floor without a divider and allowed them to explore both floors. 24 hrs later they underwent observational conditioning. They were then tested 24 hrs later in the same chamber as on day 1 and allowed to freely explore again (**Fig. 4a**). We quantified the amount of time spent on the shock floor relative to their initial preference on Day 1 and found that NO mice spent more time on the shock floor, while EO spent less time on the shock floor (**Fig. 4b**). This is consistent with the notion that EO mice learned to avoid the shock floor, while NO mice learned to avoid the location they were in when observational conditioning occurred. Importantly, avoidance of the shock floor in the ES group demonstrates that in this paradigm a single direct experience of shock drives

shock avoidance, while animals that have not experienced shock (NO) do not avoid the shock floor. Taken together, EO mice learn that the cue is aversive because it predicts shock delivery, while NO mice only learn that the cue is aversive, but not about the shock-predictive significance of the cue (**Fig. 4c).** Thus, for the remainder of the study, we focused our investigation on mice in the EO group that underwent the direct shock experience.

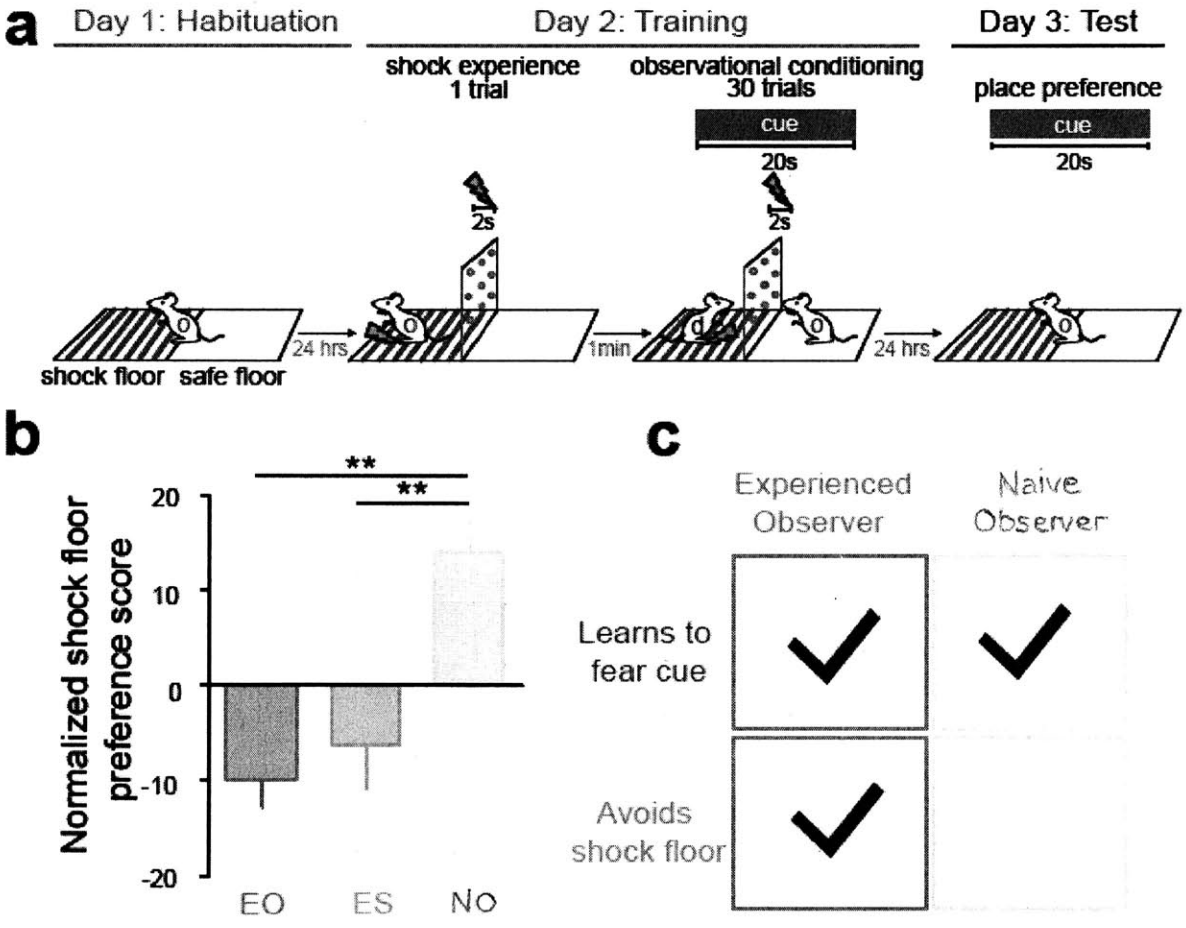


Figure 4 Experience of shock is necessary for shock floor avoidance

a. Outline of adapted conditioning paradigm to test for place preference after conditioning. On Day 1, mice were allowed to explore a chamber with a shock floor side and a safe floor side without a barrier. On Day 2, EO, ES, NO, and NS mice underwent observational conditioning as outlined above. On Day 3 mice were once again placed into the chamber without the barrier and allowed to explore.

b. On the Test Day, EO and ES mice showed a normalized negative preference score (see methods for calculation) indicating avoidance of the shock floor, while NO mice showed a positive preference score indicating a lack of shock floor avoidance. EO and ES mice were significantly different from NO mice in there avoidance of the shock floor (one- way ANOVA, $F_{3,25}=5.663$, $P=0.0042$, Bonferroni post-hoc analysis, $^{**}P<0.01$).

c. Summary of learning experience in adapted observational conditioning paradigm. EO and NO mice both show conditioned freezing to the cue on test day after observational conditioning. However, EO mice also avoid the shock floor.

To confirm that EO mice learned the predictive meaning of the cue in a manner independent of contextual cues, we ran a group of mice that underwent observational conditioning and were then tested 24 hrs later in a novel context (**Fig. 5a**). These animals showed significantly greater freezing during the cue relative to baseline (**Fig. 5b)** demonstrating that EO animals learn about cues through observation in a context independent manner.

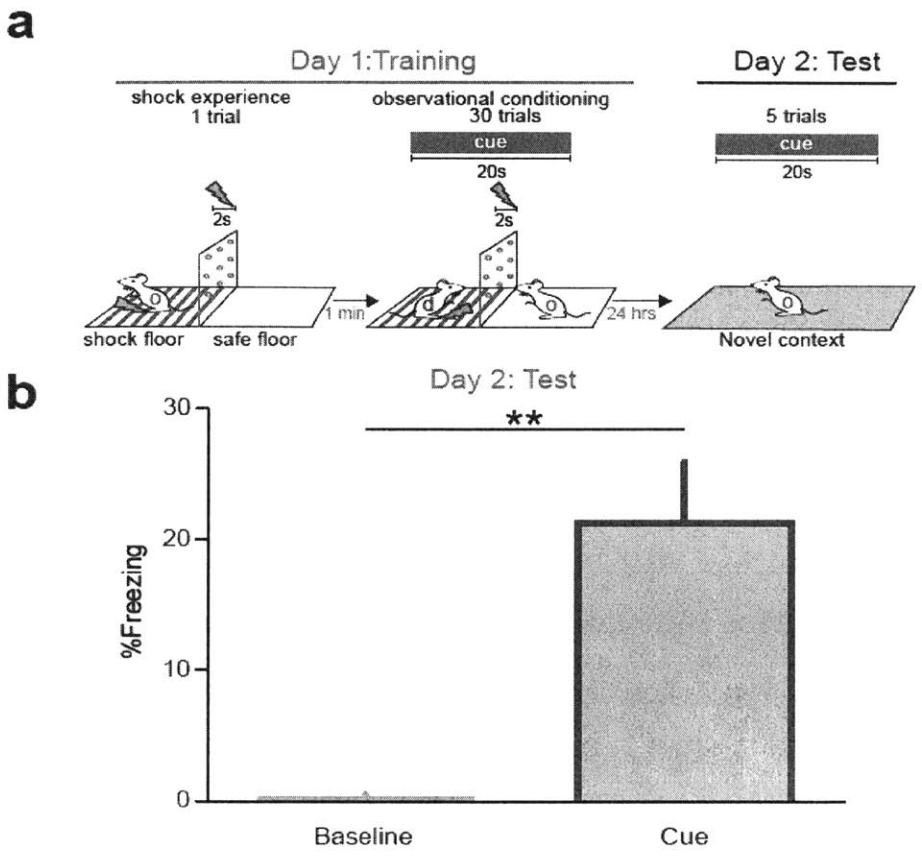


Figure 5 Experienced Observers learn about the cue in a context independent manner

a. Modified observational conditioning paradigm for experienced observers (EO) to test for context independent cue learning. EO mice received one foot shock before being transferred to a safe plastic floor. Mice then observed a demonstrator receive cue-shock pairings through a transparent, divider that contains holes. 24 hrs later the observer was placed into a novel context and cues were delivered in the absence of shock.

b. EO mice showed significantly increased freezing responses to the cue compared to baseline freezing (two-tailed, unpaired student's t-test, $^{**}P=0.0011$)

In order to elucidate the manner in which neurons in the ACC and BLA encode information during observational learning, we performed single unit recordings in EO mice during our observational conditioning paradigm **(Fig. 6a, Fig. 7a-d, Fig. 8a-d).** To assess whether neurons in either region would demonstrate conditioned responses, a subset of observers were run on a modified version in which after initial shock experience, they observed demonstrators receiving cues in the absence of shock (cue only) prior to receiving cue-shock pairings (during the observational conditioning epoch) (**Fig. 6a**). We found that during observational conditioning, 32% (n=36/114) of ACC neurons and 19% (n=27/139) of BLA neurons showed phasic excitation to the cue while 6% (n=7/114) of ACC and 22% (n=31/139) of BLA neurons showed phasic inhibitions to the cue (**Fig. 6b-d, Fig. 7a-c**). We found that 21% (n=9/43) of cue-responsive neurons in the ACC and 27% (n=9/34) of cue-responsive neurons in the BLA had a significant response to the cue only during observational conditioning, but not during the cue only habituation epoch (conditioning selective response) **(Fig. 6e, Fig. 9a-b).** We also found that 21% (n=9/43) of ACC and 15% (n=5/34) of BLA cue-responsive neurons showed significantly different responses to the cue during the cue-only habituation epoch and during the observational conditioning epoch during which the cues were paired with shock to another mouse (conditioned cue response) (**Fig. 6e, Fig. 9a-b**). Neurons that fit into both of these categories were also categorized as having a conditioned cue response. In the ACC, 9% (n=4/43) of cue responsive neurons met both criteria, while in the BLA, 8% (n=3/34) of cue responsive neurons met both criteria.

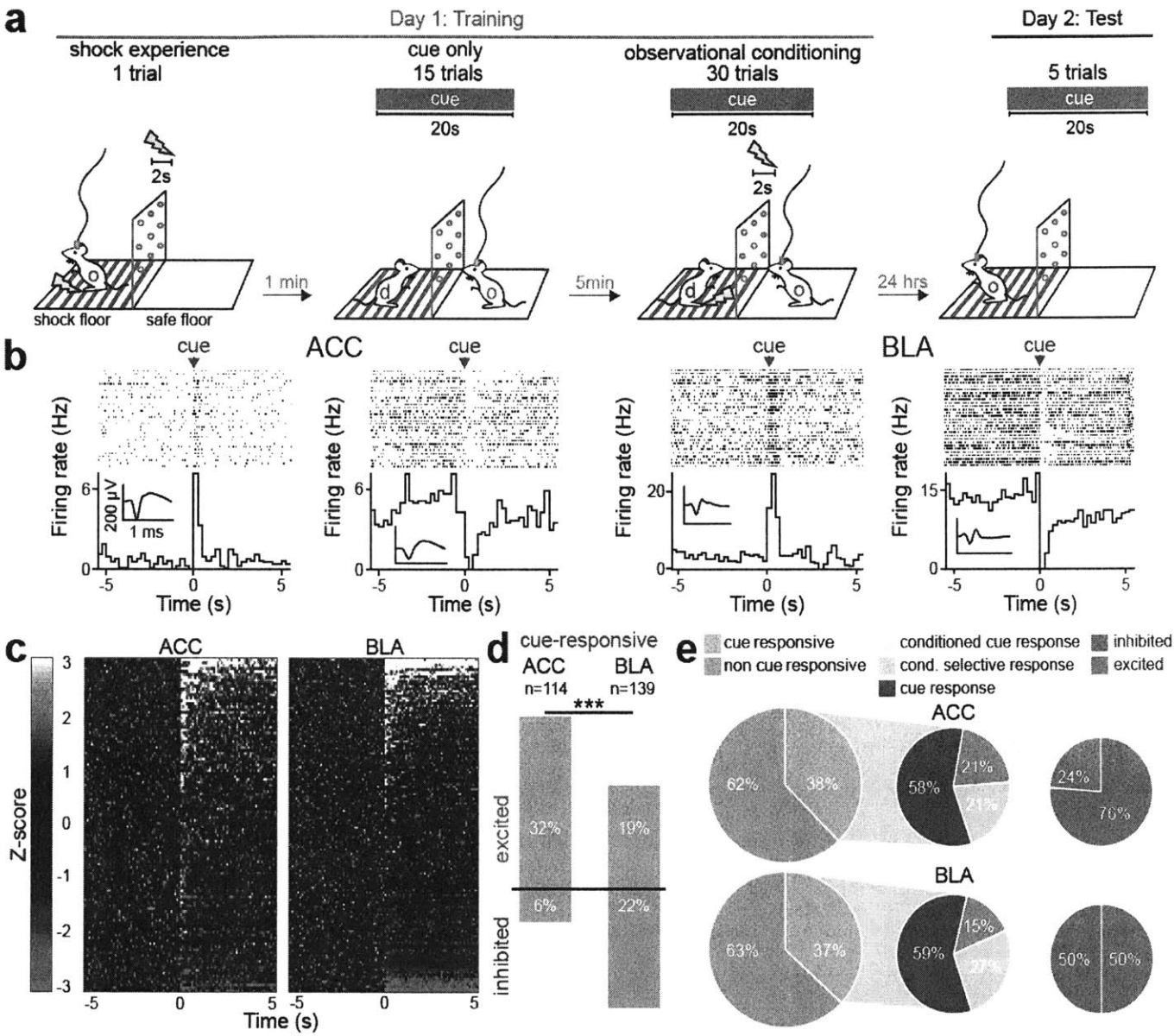

a
Day 1: Training
Day 2: Test
shock experience
1 trial
cue only
15 trials
observational conditioning
30 trials
5 trials
cue
20s
2s
1 min
5min
24 hrs
shock floor
safe floor
b
ACC
BLA
cue
Firing rate (Hz)
200 µV
1 ms
Time (s)
c
ACC
BLA
Z-score
Time (s)
d
cue-responsive
ACC
BLA
n=114
n=139
***
excited
inhibited
32%
19%
6%
22%
e
cue responsive
non cue responsive
conditioned cue response
cond. selective response
cue response
inhibited
excited
ACC
BLA
62%
38%
58%
21%
21%
24%
76%
63%
37%
59%
15%
26%
50%
50%

Figure 6 ACC and BLA neurons contain neural correlates of observational learning

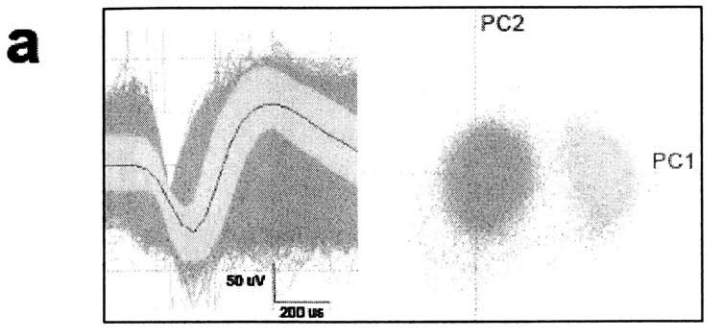
a. Outline of observational fear learning behavioral task. EO mice received one foot shock and were moved to the plastic floor to observe the demonstrator. Cues were then delivered to the chamber in the absence of shock delivery (cue only). Following this period, paired cues and shocks were delivered to the demonstrator (observational conditioning). 24 hrs later mice underwent a conditioning test.

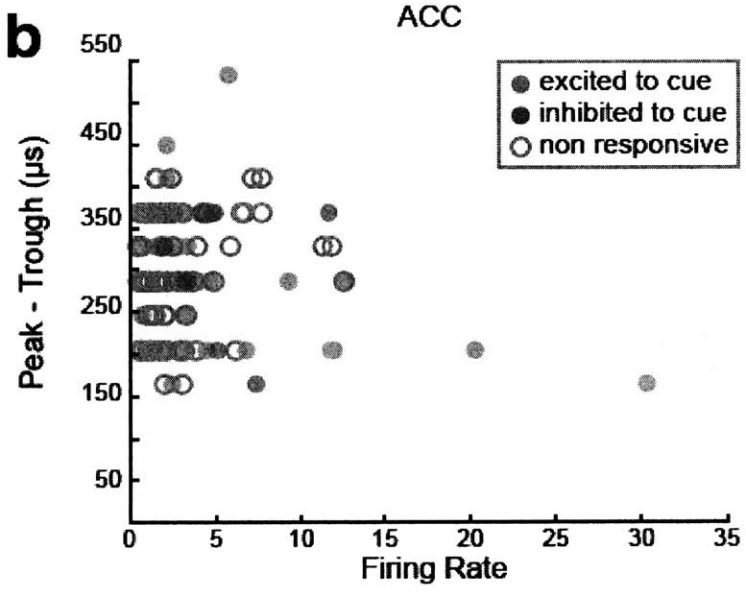
b. Examples of neurons in the ACC and BLA that showed excitation or inhibition to the cue (PSTH (200 ms bins)).

c. Heat maps of all neurons recorded in the ACC (7 mice, n=114) and BLA (8 mice, n=139) (100 ms bins).

d. Percentage of all neurons recorded in the ACC and BLA that showed excitation or inhibition to the cue. The ACC and BLA had significantly different population representations of excitatory and inhibitory cue responses (Chi-square test, X^2 = 15. 28, *df*=2, ***P= 0.0005).

e. 38% (n=43/114, 7 mice) of ACC and 37% (n=34/92, 5 mice) of BLA neurons were cue responsive neurons. Of cue responsive neurons, 58% (n=25/43) of ACC and 59% (n=20/34) of BLA neurons maintained cue responses across the entire session, while 21% (n=9/43) of ACC and 27% (n=9/34) of BLA neurons only showed significant responses to the cue during observational conditioning and not the cue only period (selective cue response). Lastly, 21% (n=9/43) of ACC and 15% (n=5/34) of BLA neurons changed their response to the cue during conditioning when compared to the cue only response (conditioned cue response).


a
PC2
PC1
50 uV
200 us


b
ACC
excited to cue
inhibited to cue
non responsive
Peak - Trough (µs)
550
450
350
250
150
50
0
5
10
15
20
25
30
35
Firing Rate

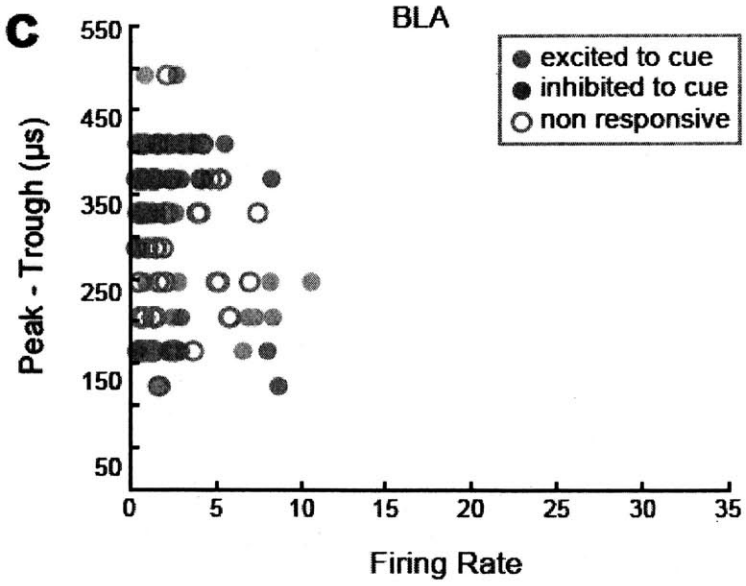

c
BLA
excited to cue
inhibited to cue
non responsive
Peak - Trough (µs)
550
450
350
250
150
50
0
5
10
15
20
25
30
35
Firing Rate

Figure 7 In vivo electrophysiological properties of ACC and BLA neurons

a. Representative waveform and PCA analysis for unit identification from unit recorded in the BLA.

b-c. Scatterplots depicting all neurons recorded in the ACC (b) and the BLA (c) plotted by firing rate and time between the peak and trough. Neurons are color coded to indicate their response to the cue.

d. Scatter plot of all ACC-BLA network neurons based on their photo-responses.

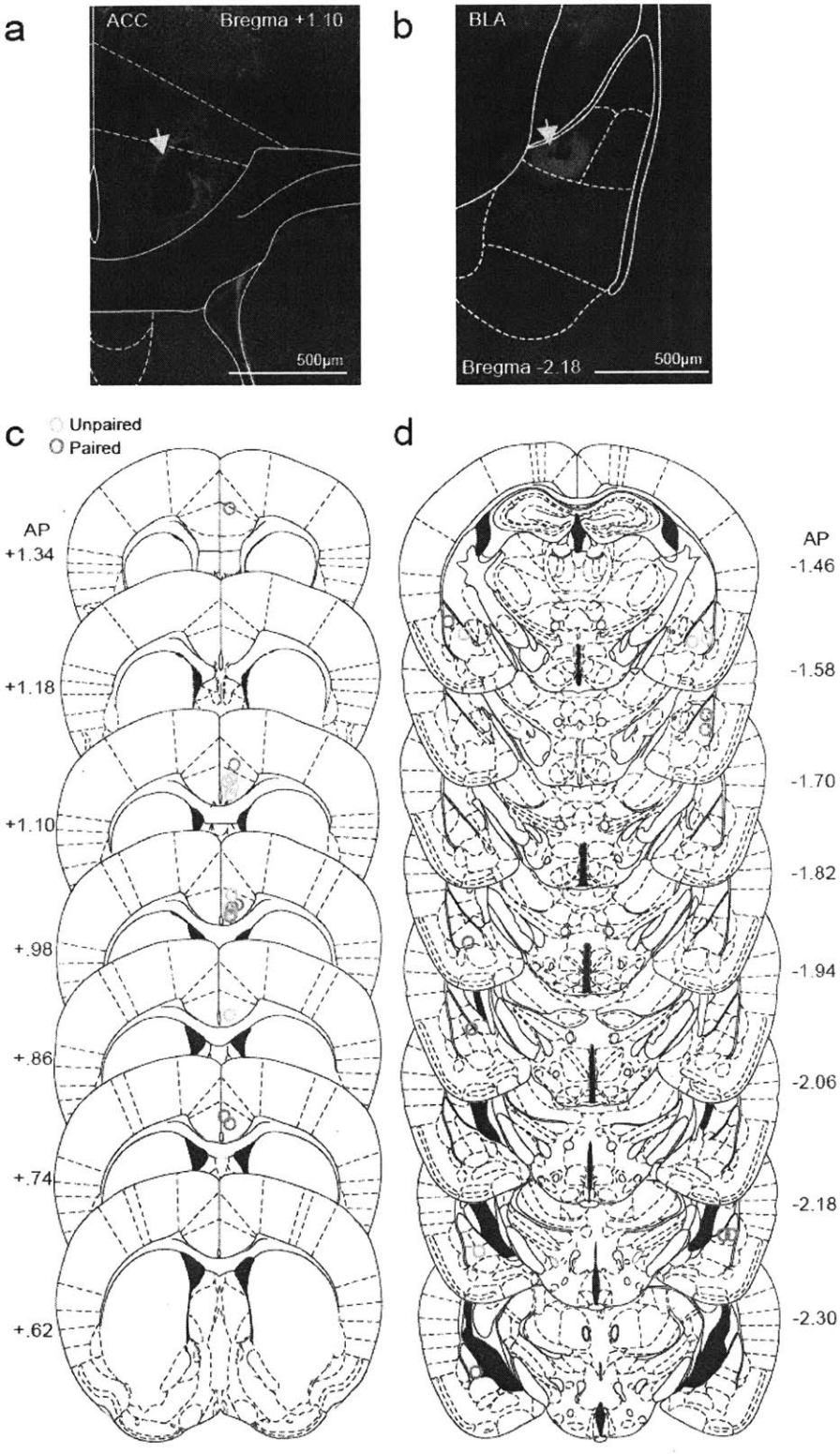

a
ACC
Bregma +1.10
500μm
b
BLA
Bregma -2.18
500μm
c
Unpaired
Paired
AP
+1.34
+1.18
+1.10
+.98
+.86
+.74
+.62
d
AP
-1.46
-1.58
-1.70
-1.82
-1.94
-2.06
-2.18
-2.30

Figure 8 Histological placements of ACC and BLA electrodes

a-b. Representative confocal images of electrolytic lesions in ACC (a) and BLA (b). **c-d.** Histologically verified electrode placements in ACC (c) and BLA (d) for all subjects included in the study. Symbols represent electrolytic lesion for unpaired (yellow) and paired (red) mice.

On a population level, the z-score of phasic responses to the cue in all ACC neurons recorded were enhanced during the observational conditioning epoch (**Fig. 10a**). This enhancement of z-score response was not seen in the ACC when cues and shocks were unpaired, demonstrating that the enhancement of the population response to the cue only occurred when cues had predictive value, and thus was not a result of increased salience or contextual fear conditioning **(Fig. 10a).** Thus, during observational learning, the ACC and BLA show conditioned responses to the predictive cue. This is indicative that both regions contain neural correlates of observational learning.

In order to further characterize the changes in firing rate that occurred due to conditioning, we applied state-space analyses to the neurons in both regions that showed conditioned cue responses or conditioning selective responses **(Fig 9a-b).** The state-space model provides a means by which one can accurately track changes in neural dynamics both within and across trials in a statistically robust manner that includes confidence intervals for comparative statements (Smith et al. 2010). In addition, using this approach, one can estimate the neuronal firing rate at an individual trial and assess whether or not it is different from any other trial.

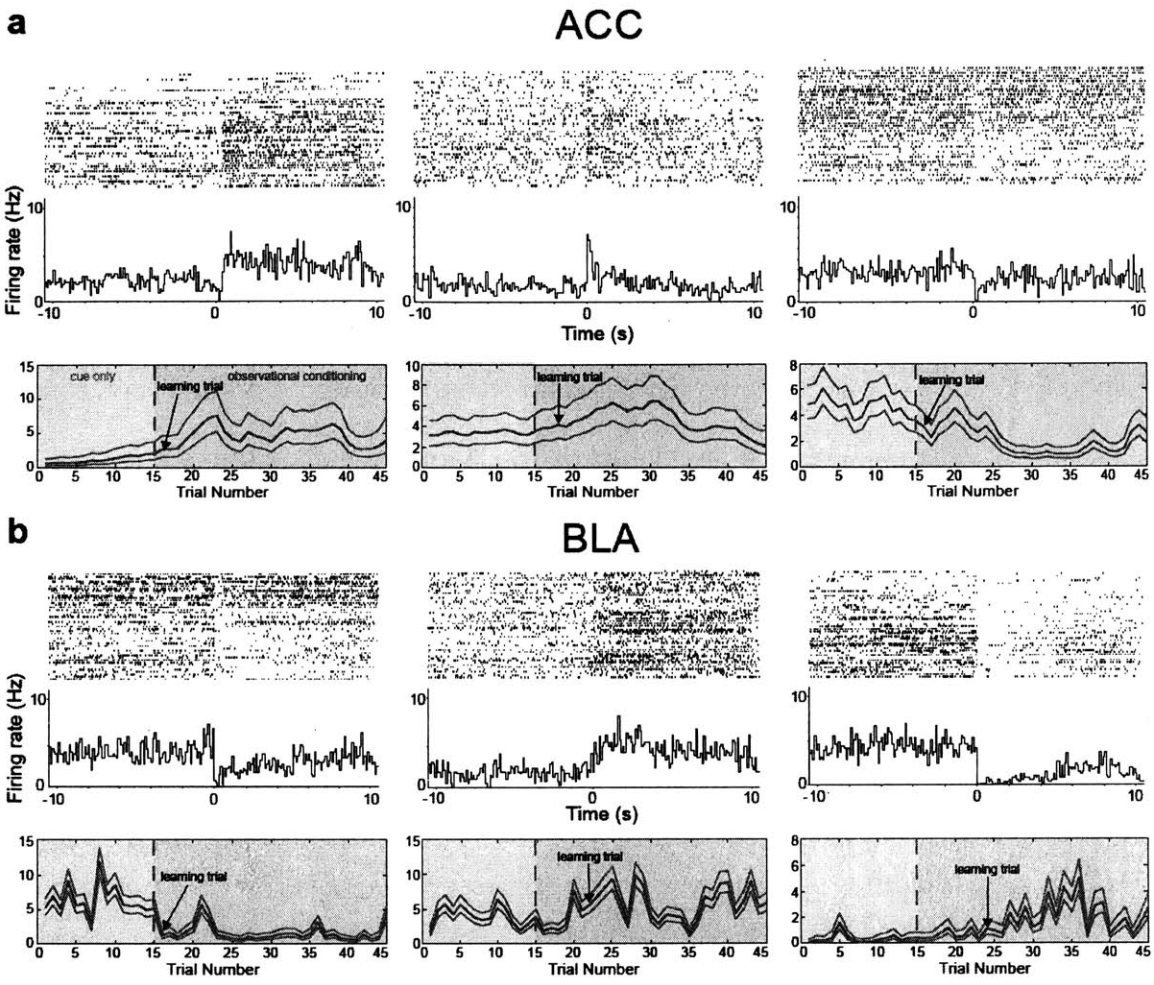
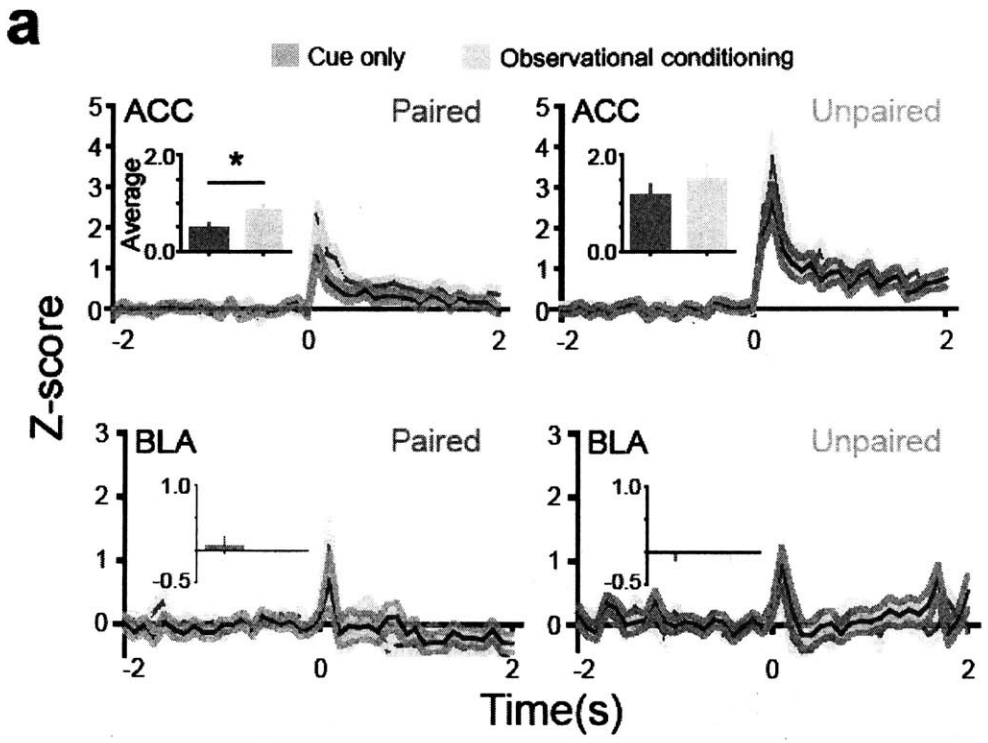
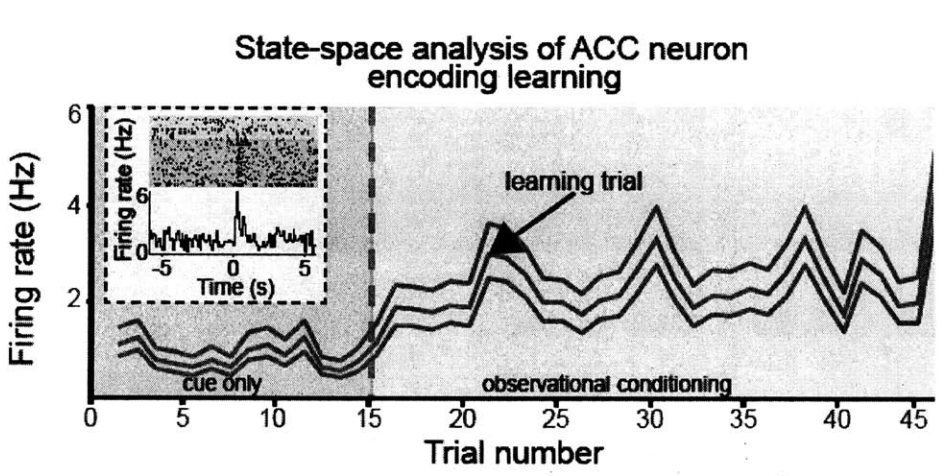


Figure 9 State-space analysis of learning neurons within the ACC and BLA

a-b Representative peri-stimulus time histogram (PSTH)(100ms bins) of neurons in ACC (a) and BLA (b) responding to the cue and the state space analysis of these neurons showing their learning trial.

The state-space approach is a statistical approach that consists of both a state equation and observation equation. The state equation defines the "state process" which is the unobservable process of learning that governs the spike rate function. The observation equation relates the electrophysiological data we collect to the state process of learning. Thus, one can estimate the spike rate function based on the observed data. State-space analysis of these neurons, showed the change in firing rate response to the cue that occurred across learning and provided a probabilistic estimate of the trial at which neurons began to encode the learned meaning of the cue **(Fig. 10b, Fig. 9a**). We used this analysis to find the learning trials of neurons in both regions and showed that the ACC learning population had a significantly earlier distribution of learning trials than the BLA **(Fig. 10c).** Thus, if information about the cue is passed between both regions, it is likely that the direction of that information flow would be from the ACC to the amygdala.


a
Cue only
Observational conditioning
ACC
Paired
Unpaired
BLA
Average
Z-score
Time(s)


b
State-space analysis of ACC neuron encoding learning
Firing rate (Hz)
learning trial
cue only
observational conditioning
Trial number

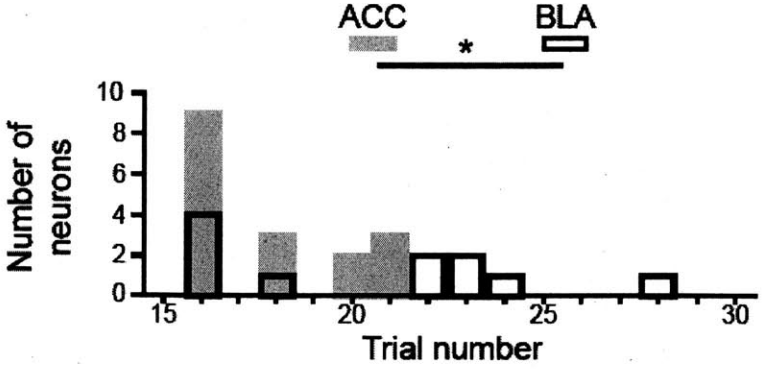

c
ACC
BLA
Number of neurons
Trial number

Figure 10 The ACC leads the BLA in the acquisition of learning

a. Z-score responses of the population of neurons recorded in ACC (paired, n=114, 7mice; unpaired, n=95, 8 mice) and BLA (paired, n=92, 5 mice; unpaired n=75, 4 mice). Inset shows the average Z-score of the respective population during the first second after the cue (0-1) in the cue only and observational conditioning epochs. In the ACC, the average Z-score response is significantly greater when the cue is predictive of shock (observational conditioning) than during cue only presentations (Paired, two tailed t-test, $*P=0.0013$).

b. State-space analysis of a learning neuron (shown in inset) showing a conditioned response to the cue. This analysis provides a probabilistic estimate of the trial at which the neuron "learns" about the cue.

c. State-space analysis was applied to neurons that showed selective cue responses and conditioned cue responses in the ACC and BLA in order to track changes over time and provide an estimate of each neuron's learning trial. The ACC had an earlier trial distribution of learning trials than the BLA, suggesting that learning occurs in the ACC prior to the amygdala (Kolmogorov-Smirnov test, $*P<0.05$).

Discussion

Altogether, we show that mice can learn from other mice about discrete cues that predict aversive outcomes without directly experiencing pairing of the cue and outcome. This is consistent with previous studies (Chen, Panksepp, and Lahvis 2009b; Jeon et al. 2010; Kim et al. 2012; Jeon and Shin 2011) and is likely ethologically relevant as natural aversive outcomes such as predation might be too costly to experience and thus the ability to learn predictors of predation without prior experience would be advantageous. Still, prior experience equips animals with the added advantage of not only being able to identify predictive cues, but to also avoid the source of the aversive outcome.

Next, we show that neurons within the ACC as well as the BLA show conditioned responses to the cue during observational fear learning. Rapid plasticity during learning has previously been seen in the amygdala, the ACC, and auditory cortex during classical fear conditioning (Quirk, Repa, and LeDoux 1995a; Quirk, Armony, and LeDoux 1997; Maren 2000; Steenland, Li, and Zhuo 2012). For example, Quirk and colleagues recorded from the LA of rats during classical fear conditioning and showed that BLA neurons show conditioned responses to the cue (Quirk, Repa, and LeDoux 1995) . Here we also see rapid plasticity in the form of conditioned neuronal responses in the ACC and BLA during observational learning underscoring that this plasticity may underlie observational learning at the neuronal level.

Lastly, it was known that both the ACC and BLA are necessary for observational fear learning (Jeon et al. 2010). It was also known that the two regions have reciprocal

connections. However, it was unclear whether information flow between the two regions is important for observational learning and if so what the directionality of that information flow might be. Here we utilized a state-space analysis in order to determine the learning trial of each neuron. This allowed us to assess how long it took neurons in both regions to significantly change their response to the cue during learning. Comparing both regions to each other provides direct evidence that there might be a specific direction of information flow from the ACC to the BLA during observational fear learning. Our data suggests that the ACC sends information about the learned cue to the amygdala in order to drive learned responses to the cue.

Methods

Subjects

Adult (8-12 weeks) male C57BL/6J were used for all experiments. All mice were pair-housed on a reverse 12 hour light-dark cycle with food and water *ad libitum*. All experiments were conducted in accordance with NIH guidelines and with approval of the MIT Institutional Animal Care and Use Committee and the MIT Department of Comparative Medicine.

Stereotactic surgery procedures

All surgeries were conducted under aseptic conditions using a digital small animal stereotaxic instrument (David Kopf Instruments, Tujunga, CA, USA). Mice were anaesthetized with isoflurane (5% for induction, 1%–2.5% for maintenance). Injections were performed using a beveled 33-gauge microinjection needle. A 10-µl microsyringe (nanofil; WPI, Sarasotam FL, USA) was used to deliver virus at a rate of 0.1 µl per min using a microsyringe pump (UMP3; WPI) and controller (Micro4; WPI). Mice were given a post-surgical recovery time of at least 7 days prior to start of any experimental procedures.

Surgical procedures for *in vivo* multi-unit electrophysiological recordings (Figure 2 and 3):

To target the basolateral amygdala (BLA) for in vivo recordings, a craniotomy was made in the right hemisphere at anteroposterior (AP) -1.6 mm and mediolateral (ML) +3.35 mm. Two to three skull screws were implanted around the site of the craniotomy. One

layer of adhesive cement (C&B Metabond; Parkell, Edgewood, NY, USA) followed by cranioplastic cement (Dental cement; Ortho-Jet, Lang Dental, Wheeling, IL, USA) was used to stabilize screws to the skull. A 16-channel multi array electrode (Innovative Neurophysiology) was then lowered at approximately 0.01 mm/s to -4.75 dorsoventral (DV) as measured from bregma. A ground wire was placed in the contralateral posterior hemisphere at an approximate depth of 1mm. An additional layer of cranioplastic cement (Ortho-Jet, Lang Dental, Wheeling, IL, USA) was applied to the skull as well as around the wires. The electrode was then lowered to - 4.9 DV and stabilized with additional layers of cement.

In order to record from ACC neurons, one craniotomy was drilled over the ACC (AP: −0.6 mm, ML: 1.0 mm). The electrode was lowered at approximately 0.01 mm/s u to -1.9 mm DV. The ground wire was implanted at a depth of approximately 1 mm into the posterior ipsilateral hemisphere. Cranioplastic cement was placed around the electrode and ground wire and the eletrode was then lowered to −2.1 mm DV. Sham surgery was performed on the observer's cage mate. A craniotomy was made and mice were left under anesthesia for an equivalent amount of time as their observer but no electrode was implanted. A small amount of cranioplastic cement was placed on the skull to cover up the craniotomy. Cage mates were reunited directly after surgery.

Behavioral Tasks

Observational conditioning task

Mice were placed in a soundproof conditioning chamber (Med Associates, St Albans, VT, USA) with a shock floor side and a plastic floor side separated by a transparent plastic divider containing holes. Mice in the Experienced Observers (EO) group received a "shock experience" by being placed on the shock floor side of the chamber and allowed to freely explore. After 5 minutes they received 1 un-predicted, un-cued, foot shock (all mice greater than 30 g were shocked with 1.5 mA, while mice less than 30 g were shocked with 1mA) and were immediately transferred to the plastic floor side of the chamber. The cage mate of the observer was then placed into the shock side of the chamber as the demonstrator for "observational conditioning". After 5 minutes of habituation, demonstrators underwent 30 trials that occurred at random intervals (60, 90, 120, 150, 180 seconds) in which a 20 second compound cue (light and 10 kHz tone) predicted the delivery of a 2 second shock (1 mA - 1.5 mA) 10 seconds after the onset of the cue. Directly after, mice were placed back into their home cages. 24 hrs later, observer mice were placed back into the shock side of the chamber and 30 cues were delivered to the chamber in the absence of shock. Mice in the Unpaired Observers (UO) group received a "shock experience", however during observational conditioning cues and shocks delivered to the demonstrator were explicitly unpaired. Experienced Solo animals (ES) also received a "shock experience" but then no demonstrator was placed into the shock side of the chamber. Paired cues and shocks were delivered to the empty side of the chamber. Naïve Observers (NO) did not receive a "shock experience" but instead were placed on the plastic side of the chamber where they observed

demonstrators in the same way as EO. Lastly, Naïve Solo animals (NS) also did not receive a "shock experience" but instead were placed on the plastic side of the chamber. Paired cues and shocks were delivered to the shock floor side of the chamber in the absence of a demonstrator.

Observational Place Preference Task

On Day 1 mice were placed in the same soundproof conditioning chamber mentioned above except that there was no barrier between the shock floor and plastic floor so mice could freely explore both. After 30 minutes mice were removed. On Day 2, a barrier was placed in the chamber separating the two floors and mice underwent observational conditioning according to their behavioral groups as described above. On Day 3, the barrier was removed and mice were again allowed to freely explore for 30 minutes. After 5 minutes of exploration, cues were played.

Observational test in novel context

On Day 1, EO mice were observationally conditioned as described above. To test for cue learning in a novel context, on Day 2 observer mice were placed in a soundproof conditioning chamber that had a plastic floor covered with fresh bedding. 20 second cues (10 KHz tone and house light) were delivered to the box and time spent freezing during the baseline (20 seconds prior to the cue) and cue was measured.

Social interaction after observational conditioning

For the social interaction assay described, the time spent engaging in social interaction by EO or ES mice was scored in the homecage for the 15 minutes prior to observational

conditioning. The overall score of social interaction was defined as any period of time in which the observer mouse was actively investigating demonstrator, including behaviors such as face or body sniffing, anogenital sniffing, direct contact, and close following (<1 cm). Directly following observational conditioning, the demonstrator and observer mice were placed back into their homecage and time spent interacting was again scored for 15 minutes.

Analysis

Behavioral Analysis of Observational conditioning

Behavioral performance was recorded by digital video cameras. All videos were manually analyzed offline by an experimenter blind to experimental conditions. Freezing behavior of the observer was scored on both conditioning and test day as the amount of freezing during the cue minus the amount of freezing in the 20 seconds directly preceding the cue. Freezing was defined as absence of movement, with the exception of respiration. Based on preliminary behvavioral data, for the training day, analysis of freezing behavior was performed on trials 5-20. Analysis of freezing behavior on test day was performed on the first 5 trials. Additional other stereotyped behaviors shown by the observer such as grooming and escaping were quantified by manual scoring. Observer animals were said to be mimicking when they displayed escape behaviors in direct response to demonstrators exhibiting escaping behaviors during the cue.

Grooming and mimicking behaviors were quantified during the 20s of the cue and compared to the baseline taken as the 20s prior to the cue onset. The number of trials

in which animals that were engaged in grooming stopped at cue onset was quantified as a percentage of all trials. This was averaged across all animals in the respective groups. The percentage of trials in which the Observer showed mimicking behaviors was also quantified and averaged across animals in a given group.

Behavioral analysis of Observational Place Preference Task

For behavioral analysis of observational Place Preference video of all animals was recorded then viewed and scored by a blind experimenter. The percentage of time mice spent with all four limbs on the shock floor side of the chamber on Day 1 was scored and calculated. The percentage of time spent on the shock floor on Day 3 was also scored and calculated. A preference score (%time on shock floor on Day3-%time on shock floor on Day1) was calculated for each animal. This score was then divided by the average of the naïve solo group to give a normalized preference score (%time on shock floor Day3- %time on shock floor Day 1/ average NS preference score). The normalized preference score for the group was calculated as an average of all of the normalized preference scores for animals in that group.

Electrophysiology during Observational learning

To enable us to record from neurons during observational learning, mice were connected to a head stage that plugged into a commutator and pre-amplifier (Tucker-Davis Technologies). EO and UO mice were conditioned as described above while connected to the TDT system for multichannel spike acquisition. In all ACC animals and a subset of BLA animals, immediately after the initial shock experience mice were

placed on the plastic floor side of the chamber. A demonstrator was placed on the shock floor and cues were delivered at random intervals in the absence of shock delivery. This is referred to as the "cue only" period. Following this period, animals observed cues and shocks being delivered to the box in a paired (EO) or unpaired (UO) manner. In animals expressing ChR2 in ACC-BLA projecting neurons and an optrode in the ACC, photo Neural activity was recorded continuously during the entire behavioral paradigm.

Electrophysiology Analysis of cue responses

Neural activity was recorded, and unit discrimination was performed with multichannel spike acquisition (TDT) and sorting software (Plexon Inc.). Responses of single units to the cue onset was deemed statistically significant if the firing rate within one or more 100-ms bins in the response window (0–0.5 s after cue onset or offset) was significantly different ($P < 0.01$) from a 1 s baseline epoch using a Wilcoxon signed-rank test. In some neurons that showed sustained responses a baseline of 20 seconds was used and a response window of 9 seconds. P value was adjusted for multiple comparisons.

Electrophysiology Analysis of learned cue responses

To compute if neurons encoded the learned value of the cue, neuronal cue responses were analyzed for the cue only and observational conditioning periods. Because there were more trials in the observational conditioning period, we used trials 20-35 for comparison with the cue only (trials 1-15) using the baseline and response windows described above for cue responses. Using the Wilcoxon signed-rank test, A neuron was

classified as a learning neuron if it either had a significantly different response window ($P < .01$) during conditioning when compared to the cue only period or if the response when comparing baseline to response window was significant ($P < .01$) during observational conditioning but not significant during the cue only period. P value was adjusted for multiple comparisons.

To compute the estimated learning trial of individual neurons we employed the state-space approach outlined in Smith et al. 2010. We performed analysis on all neurons that were classified as learning after application of the statistical tests described above. The learning trial was defined as the first trial during observational conditioning in which that trial and the subsequent trial had a >95% probability of having a greater or lesser firing rate (depending on whether the neuron was excited or inhibited to the cue, respectively) than the average cue only firing rate. We performed the analysis with 1 second and 9 second response windows and used the earliest trial estimate. The algorithm successfully provided estimates for 79% (n=11/14) BLA and 76% (n=16/21) of ACC neurons. These estimates were used to generate the histogram shown in Figure 10.

Statistical Analysis

Statistical analyses were performed using commercial software (GraphPad Prism; GraphPad Software, Inc, La Jolla, CA, USA; MATLAB, Mathworks, Natick, MA, USA). Group comparisons were made using one-way or two-way ANOVA followed by Bonferroni post-hoc tests. Single variable comparisons were made with two-tailed unpaired Student's t tests while chi-square analyses were used to compare populations.

Non-parametric Wilcoxon rank-sum tests were used to determine whether changes in firing rates were statistically significant in in vivo electrophysiological recordings (MATLAB)). Multiple comparisons were corrected for by adjusting p values. Differences between the experimental groups were assessed using analysis of variance (ANOVA) followed by post-hoc tests when applicable using an $\alpha = 0.05$. All state space analyses were performed according to Smith et al. 2010.

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Author Contributions

SAA and ACFO performed recordings for in vivo electrophysiology experiments and SAA, EHN, DB, ACS, EB analyzed in vivo electrophysiological data. SAA, RW, AV, ACFO, AE, and AM performed behavioral experiments and analysis. SAA, AV, ACFO performed surgeries for all animals included in the study. ACFO, RW, AV, SAA, AE, and AM performed histological confirmation. SAA and KMT designed experiments. SAA and KMT wrote the paper with edits by ACFO and RW. All authors provided feedback on manuscript.

Chapter 3

A cortico-amygdala circuit encodes observational learning

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Abstract

Observational fear learning is an evolutionarily conserved social behavior that has been linked to activity in the anterior cingulate cortex (ACC) and the basolateral amygdala (BLA). However, little is known about the processes that occur within this circuit, and the degree of specificity it has for observational learning. To investigate how information is encoded and transmitted through this network, we performed electrophysiological recordings from neurons identified as part of the ACC-BLA network by optogenetic-mediated phototagging to reveal that this network encodes information obtained through observational learning. We also demonstrate that selective inhibition of the ACC-BLA projection impairs observational fear conditioning and other social behaviors, but not classical fear conditioning. Finally, inhibition of ACC input to the BLA alters the manner in which the BLA represents a cue that predicts shock to another mouse. Together, we show that information sourced from observing the experience of another mouse is transmitted from the ACC to the BLA and that this routing of information is necessary for observational fear learning.

Background

Clarifying which circuits in the brain give rise to the array of social behaviors seen in an animal's behavioral repertoire remains an important problem in neuroscience (Adolphs 2009; Yizhar 2012). One approach is to study the neural circuits that underlie innate evolutionarily conserved social behaviors in order to understand how those circuits might be used to build the networks underlying more complex social behaviors. Observational fear learning is a social behavior that is fundamental for survival. Thus, it

is evolutionarily conserved and can be seen in a number of different animals including rodents, monkeys, and humans (Mineka et al. 1984; Heyes and Dawson 1990; Kavaliers, Choleris, and Colwell 2001; Olsson, Nearing, and Phelps 2007; Barber and Kimbrough 2015). Previous studies have established a role for the ACC and BLA in observational fear learning (Olsson, Nearing, and Phelps 2007; Jeon et al. 2010). For instance, in rodents undergoing observational fear conditioning there was increased theta frequency synchronization between the two regions suggesting that they may communicate with each other during observational learning and lesions in either region impaired learning (Jeon et al. 2010). Likewise, in humans, both the ACC and amygdala showed increased BOLD activity during an observational conditioning task (Olsson, Nearing, and Phelps 2007). Lastly, in Chapter 2, we provided data that neurons in both regions show conditioned responses to a predictive CS during observational fear learning.

However, although both regions contain neural correlates of observational learning, it is unclear whether communication between the two regions is necessary for learning. Additionally, it remains unclear how neural activity in one region might impact neural activity in the other region during learning. Lastly, whether or not the ACC-BLA circuit plays a role in social behavior remains unknown. Thus, here we combine optogenetics, electrophysiology, and rodent behavior to identify and characterize the function and necessity of the ACC-BLA circuit during observational fear learning.

Results

Our finding that neurons in the ACC learn about the cue prior to neurons in the BLA suggests that cue information might be transmitted from the ACC to the BLA during observational learning. To test this hypothesis, we used an intersectional viral strategy to express Channelrhodopsin-2 (ChR2) in ACC neurons that monosynaptically project to the BLA (**Fig. 11a-b**). Ex vivo recordings demonstrated that ChR2-expressing neurons fired action potentials to blue light while non-expressing neighboring cells showed no responses to light (**Fig. 11c**). Analysis of ChR2 photoresponse latencies during patch-clamp recordings allowed us to set a photo-identification latency threshold for ACC-BLA projectors of 8 ms (**Fig. 11c**).

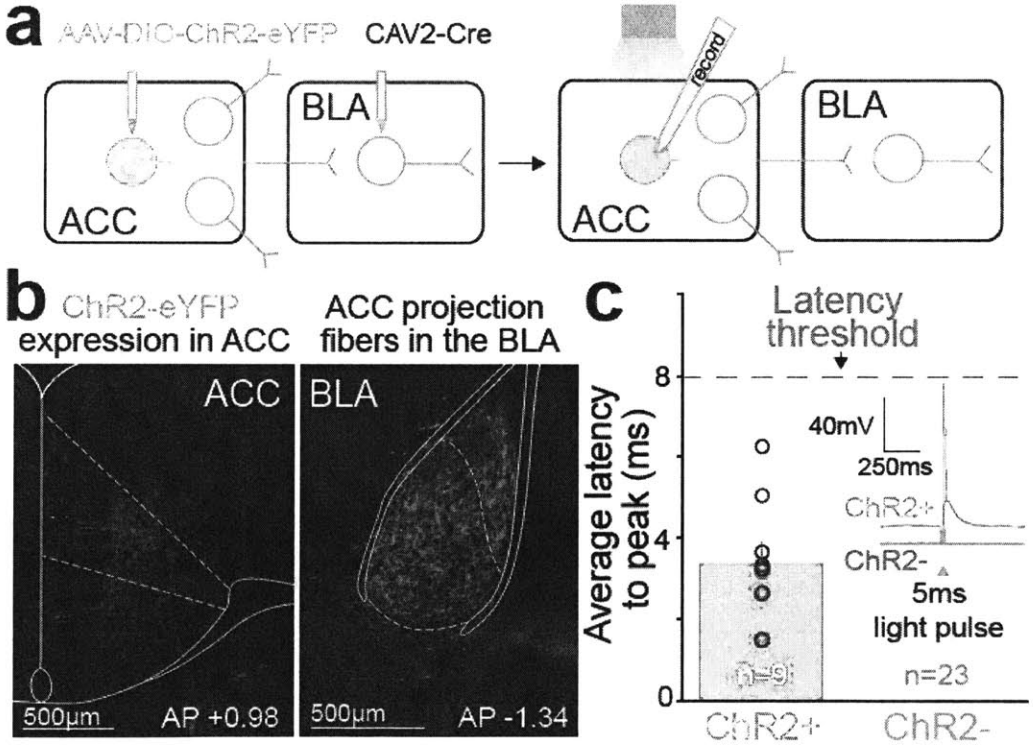


Figure 11 Ex-vivo recording from ChR2 expressing neurons and non-expressing neighbors

a. Schematic of intersectional viral approach. Retrogradely traveling virus CAV2-Cre was stereotaxically injected into the BLA and AAV-DIO-ChR2-eYFP into the ACC. This allowed us to express ChR2 only in ACC neurons that monosynaptically project to the BLA.

b. Representative confocal image of ChR2 expression in the ACC and projection fibers in the BLA.

c. In vitro electrophysiological recordings were performed in coronal slices taken from the ACC. Response of ACC neurons to blue light stimulation was recorded. ChR2+ neurons showed a range of latency responses to light stimulation, while all ChR2- cells showed no action potentials in response to light stimulation. Inset shows representative voltage traces in response to light stimulation from a ChR2+ and ChR2- cell. Latency threshold was then defined at 8ms for subsequent analysis of in vivo electrophysiological recordings.

We performed *in vivo* recordings from ACC neurons in observers during conditioning and immediately phototagged neurons in order to identify projectors. We grouped all photoresponsive neurons as being part of the "ACC-BLA network", while neurons without responses were termed "non-network neurons" (**Fig. 12a, Fig. 13**). Within the ACC-BLA network, a number of neurons showed short latency responses (<8ms). These neurons were termed "direct ACC-BLA projectors" as they express ChR2 and thus provide monosynaptic input to the BLA. We also photo-identified network neurons with long latency responses (20-100ms) as well as inhibitions **(Fig 12a, Fig. 13).** These network neurons were connected to ACC-BLA projectors through either collaterals from projectors or polysynaptic feedback from BLA neurons that receive input from projectors **(Fig. 12a)**. We found that 68% (n=23/34) of network neurons encoded

cue information, while only 27% (n=22/80) of non-network neurons responded to the cue (**Fig. 12b**). We also found that the majority of the direct ACC-BLA projectors encoded the cue (67%, n=4/6) (**Fig. 12b).** Furthermore, 74% (n=14/19) of network neurons that were excited by light stimulation were excited to the cue while only 20% (n=3/15) of network neurons that were inhibited by light were excited to the cue. In fact, the majority of photo-inhibited neurons recorded (53%, n=8/15) were unresponsive to the cue (**Fig. 12c-d).**

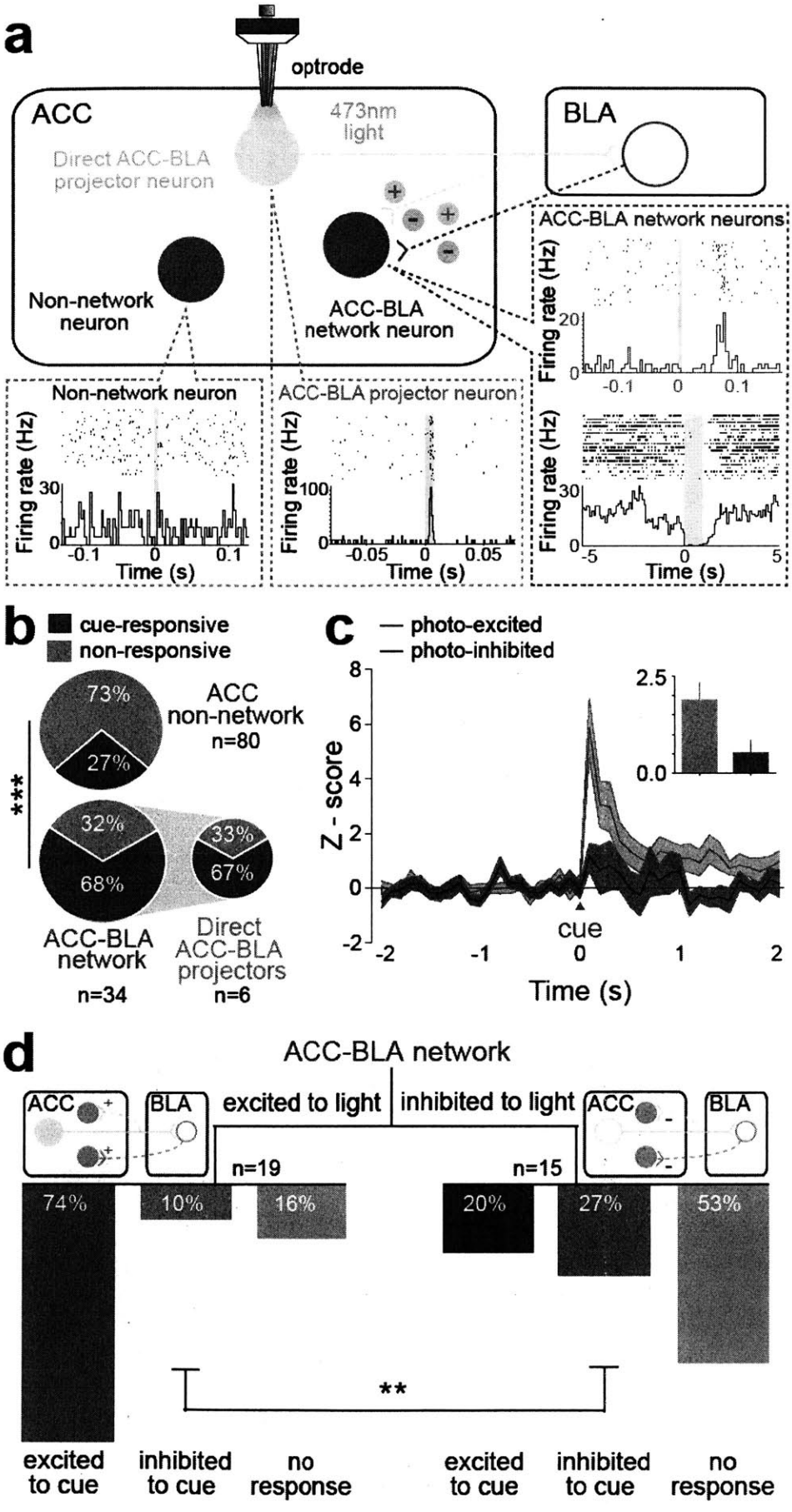

a
optrode
ACC
473nm light
Direct ACC-BLA projector neuron
BLA
Non-network neuron
ACC-BLA network neuron
ACC-BLA network neurons
Firing rate (Hz)
Non-network neuron
ACC-BLA projector neuron
Time (s)
b
cue-responsive
non-responsive
73%
27%
ACC non-network
n=80
***
32%
68%
33%
67%
ACC-BLA network
n=34
Direct ACC-BLA projectors
n=6
c
photo-excited
photo-inhibited
Z - score
cue
Time (s)
d
ACC-BLA network
excited to light
inhibited to light
n=19
n=15
74%
10%
16%
20%
27%
53%
**
excited to cue
inhibited to cue
no response

Figure 12 The ACC-BLA network is enhanced for cue representation when compared to the ACC

a. An optrode was placed into the ACC of mice (n=7) expressing ChR2 in ACC neurons projecting to the BLA. In vivo recordings during observational conditioning and phototagging were performed. Circuit model shows proposed ACC-BLA network connectivity of ACC neurons based on in vivo phototagging. Call outs show example photo-responses from each type of neuron.

b. 68%(n=23/34) of neurons within the ACC-BLA network encoded the cue and 67%(n=4/6) of direct ACC-BLA projectors (a subset of network neurons) encoded the cue during observational conditioning, while only 27% (n=22/80) of non-network neurons encode the cue. The proportion of cue responsive network neurons was significantly greater than non-network neurons (Chi-square, X^2=14.55,*df*=1 ***P=0.0001).

c. Z-score response of the population of neurons that were excited or inhibited to light stimulation. Inset shows the average Z-score of the respective populations during the first second after the cue (0-1).

d. Response tree of ACC-BLA network neurons. Network neurons were categorized based on whether they showed an excitatory or inhibitory response to photostimulation. The population of neurons excited to light had a greater percentage of neurons that were excited by the cue during observational conditioning than the population that was inhibited to light (Chi-square test, X^2=9.721, *df*=2, **P=0.0077). Schematic depicts potential circuitry of photo-excited neurons. Neurons excited to light with long latencies (20 ms- 100 ms) could receive direct or indirect glutamatergic input from collaterals of "direct ACC-BLA projectors" or could receive direct or indirect glutamatergic feedback from BLA neurons excited by "direct ACC-BLA projectors". Schematics depict potential circuitry of photo-excited and photo-inhibited neurons. Neurons inhibited to light could receive feed-forward inhibition, or direct inhibition from long-range

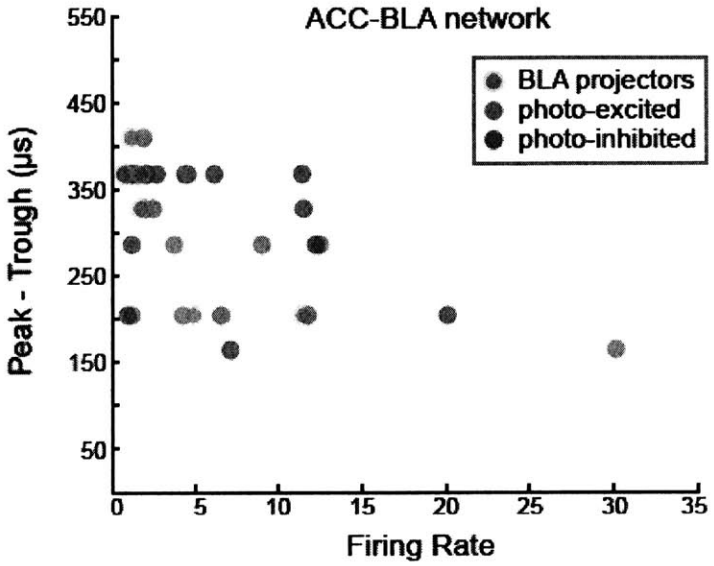


Figure 13 In vivo electrophysiological characterization of ACC-BLA network neurons
Scatter plot of all ACC-BLA network neurons based on their photo-responses

Given the preferential cue-encoding within the ACC-BLA network, and that most direct projectors encoded cue information we hypothesized that transfer of cue information from the ACC to the BLA might be necessary for observational learning about the cue. To test this hypothesis, we expressed Halorhodopsin (NpHR) bilaterally into the ACC of observer animals and placed optical fibers over the BLA in both hemispheres (**Fig. 14a-f, Fig. 16a**). This allowed us to photoinhibit ACC input to the BLA only during cue presentation (**Fig. 15a**). Mice receiving this manipulation showed no differences in freezing or grooming to the cue during training but did show impaired mimicking behavior (**Fig. 15a-b**).

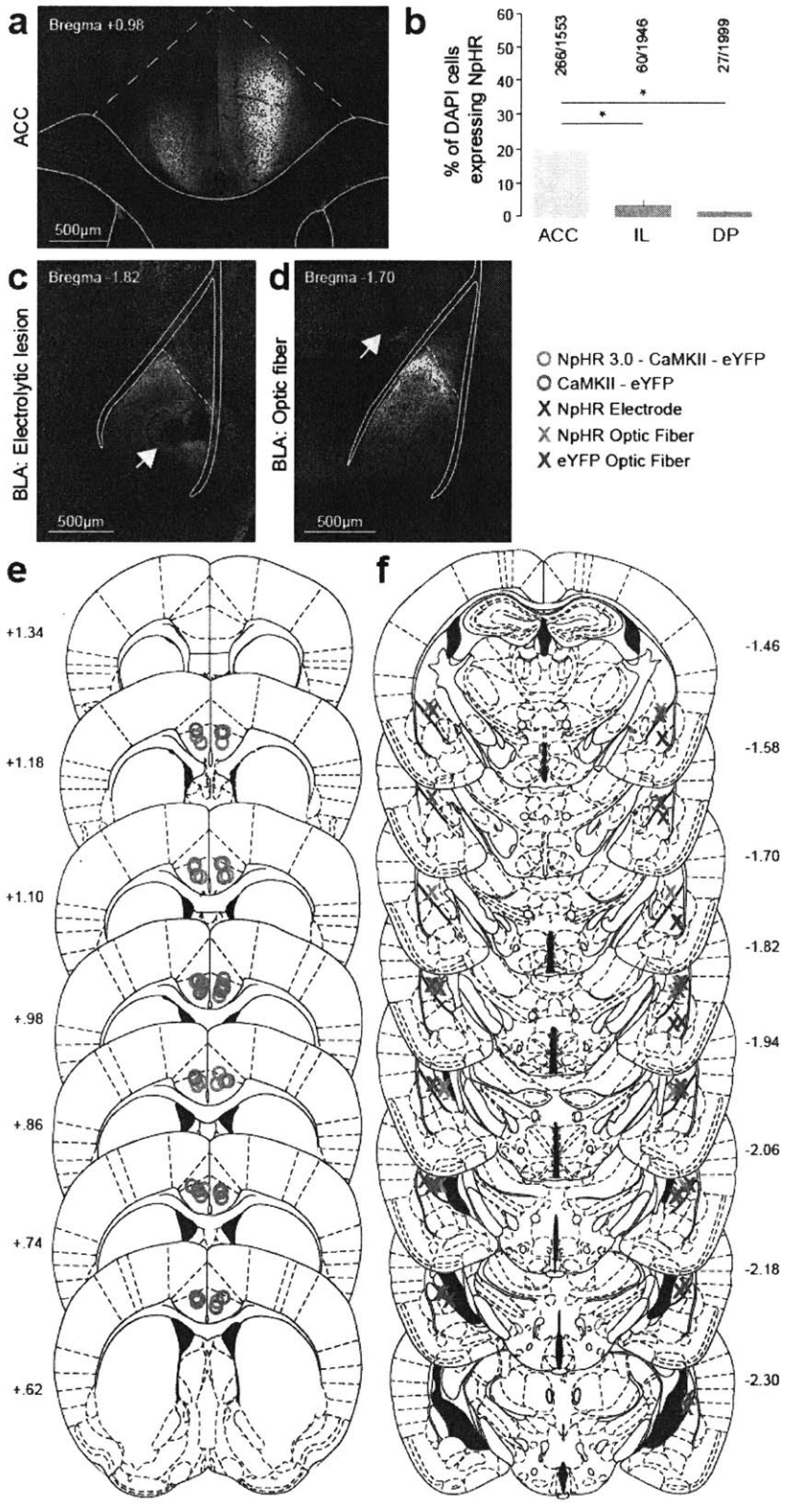

a
Bregma +0.98
ACC
500µm
b
% of DAPI cells expressing NpHR
60
50
40
30
20
10
0
266/1553
60/1946
27/1999
*
*
ACC
IL
DP
c
Bregma -1.82
BLA: Electrolytic lesion
500µm
d
Bregma -1.70
BLA: Optic fiber
500µm
NpHR 3.0 - CaMKII - eYFP
CaMKII - eYFP
NpHR Electrode
NpHR Optic Fiber
eYFP Optic Fiber
e
+1.34
+1.18
+1.10
+.98
+.86
+.74
+.62
f
-1.46
-1.58
-1.70
-1.82
-1.94
-2.06
-2.18
-2.30

Figure 14 Confocal and Histological placements of all Halorhodopsin mice

a, Representative confocal image of the ACC of a mouse in which AAV-camkii-NphR3.0-eYFP (NpHR) was injected into the ACC (blue=DAPI, eYFP = green)

b, Percentage of DAPI-positive (+) cells expressing NpHR in ACC, infralimbic cortex (IL), and dorsal peduncular (DP) cortex. Significantly more cells were expressed in the ACC region compared to the other 2 regions (one-way ANOVA, $F_{2,6}=9.745$, $P=0.0130$, Bonferroni post-hoc analysis, $^{*}P<0.05$).

c-d Representative confocal image of electrolytic lesion (c) and optical fiber placement (d) in the BLA of mice injected with NpHR in the ACC (blue=DAPI, green=eYFP, arrow points to lesion of optrode (c) and optical fiber (d).

e-f Schematic of ACC viral injection sites (e) and electrode and fiber placements in BLA (f) for all mice included in this study.

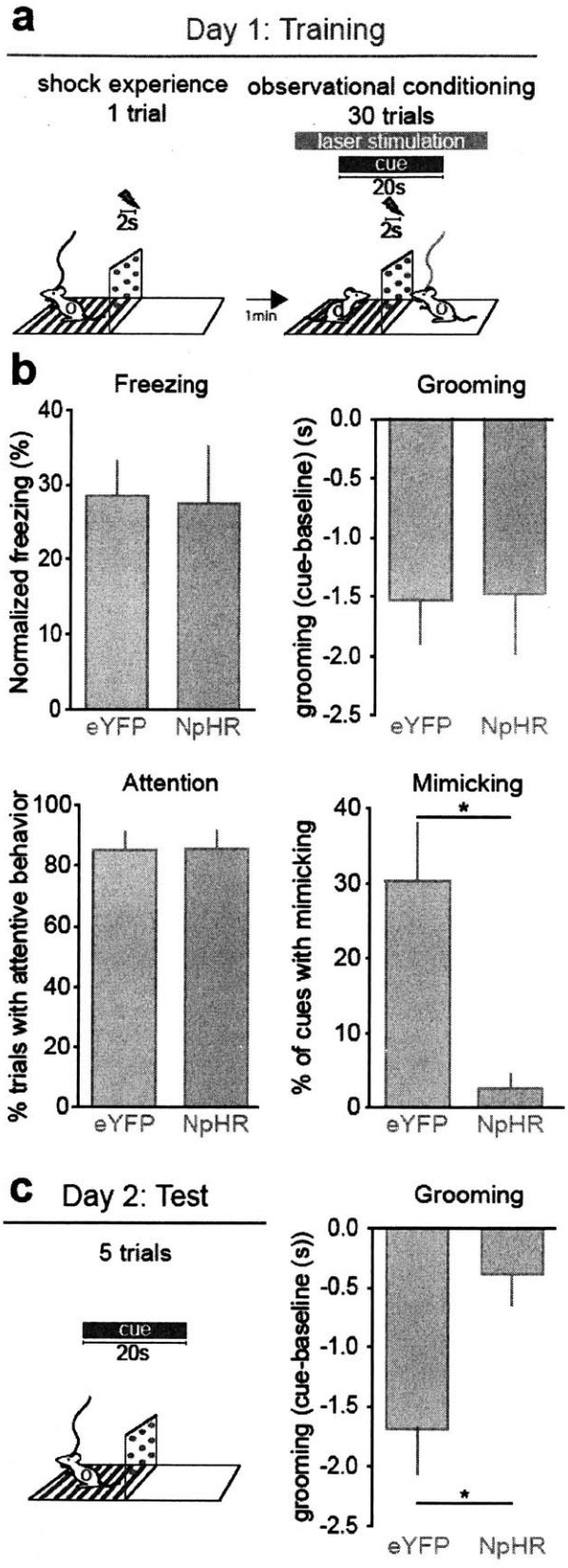

a
Day 1: Training
shock experience
1 trial
observational conditioning
30 trials
laser stimulation
cue
20s
2s
2s
1min
b
Freezing
Normalized freezing (%)
40
30
20
10
0
eYFP
NpHR
Grooming
grooming (cue-baseline) (s)
0.0
-0.5
-1.0
-1.5
-2.0
-2.5
eYFP
NpHR
Attention
% trials with attentive behavior
100
80
60
40
20
0
eYFP
NpHR
Mimicking
% of cues with mimicking
40
30
20
10
0
*
eYFP
NpHR
c
Day 2: Test
5 trials
cue
20s
Grooming
grooming (cue-baseline (s))
0.0
-0.5
-1.0
-1.5
-2.0
-2.5
*
eYFP
NpHR

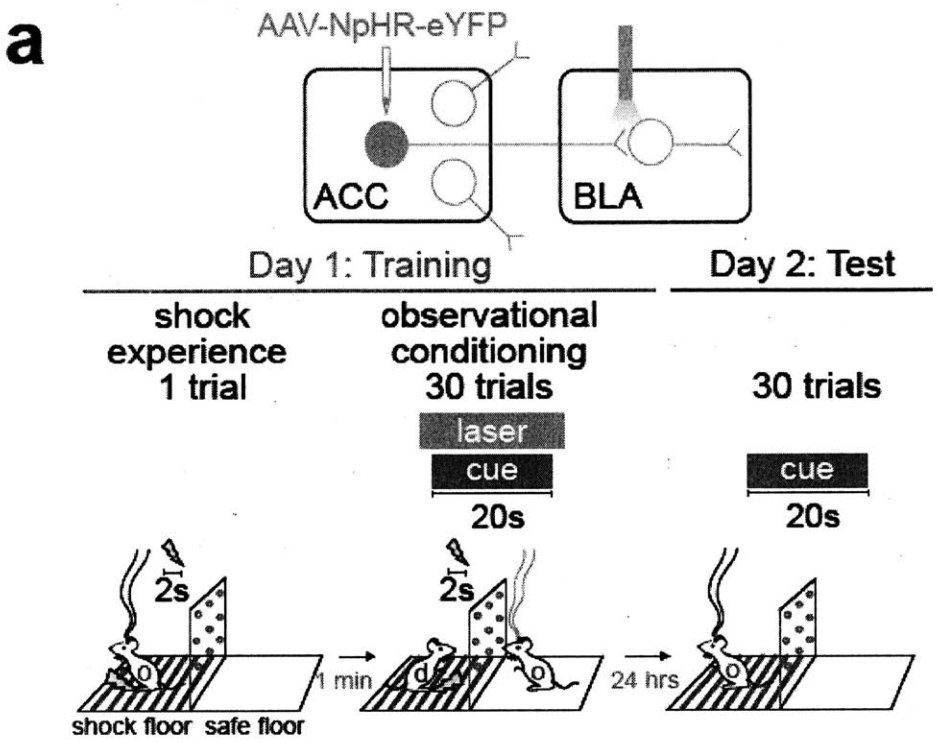
Figure 15 Behavioral Characterization of mice with ACC-BLA inhibition during observational learning

a, Schematic of behavioral paradigm used in NpHR experiments. EO mice receive one foot shock before being transferred to a safe plastic floor. The mouse then observed a demonstrator receive cue-shock pairings through a transparent, divider that contains holes while tethered to a patch cable allowing for bilateral inhibition of ACC input to the BLA during cue presentation.

b, Behavioral responses of EO during observational conditioning session. Neither freezing nor grooming was affected by inhibition of ACC to BLA input. Orienting and startle responses of the observer were used as markers for attentiveness to the cue and shock delivery to the demonstrator. These behaviors were not significantly different between NpHR and eYFP expressing mice during the first 5 cues of conditioning. However, mimicking behavior was significantly reduced in NpHR expressing mice (two-tailed, unpaired student's t-test, $*P=0.0167$).

c, Schematic of behavioral paradigm during Test day. EO mice were re-exposed to the shock floor of the chamber and cues were played without the delivery of footshock or light stimulation. Grooming behavior was significantly different between eYFP and NpHR mice (two-tailed, unpaired student's t-test, $*P=0.0294$)

However, when tested 24 hrs later, in the absence of photostimulation, they showed significantly less freezing to the cue, as well as impaired differential grooming, when compared to control mice expressing only a fluorophore (**Fig. 16a-b, Fig. 15c**). Importantly, when ACC input to the BLA was inhibited during cue presentation in a classical fear conditioning paradigm, the same mice showed no differences in freezing to the cue when compared to control mice (**Fig. 16a-b**). To assess whether the differences seen in observational conditioning were due to deficits in attention during conditioning, we analyzed orientation and startle responses and found no detectable differences between NpHR and eYFP control mice (**Fig. 15b**). This result suggests that there is a causal relationship between the transfer of cue information from the ACC to the BLA and the ability of animals to learn about a predictive cue through observation.


a
AAV-NpHR-eYFP
ACC
BLA
Day 1: Training
Day 2: Test
shock experience 1 trial
observational conditioning 30 trials
30 trials
laser
cue
cue
20s
20s
2s
2s
1 min
24 hrs
shock floor
safe floor

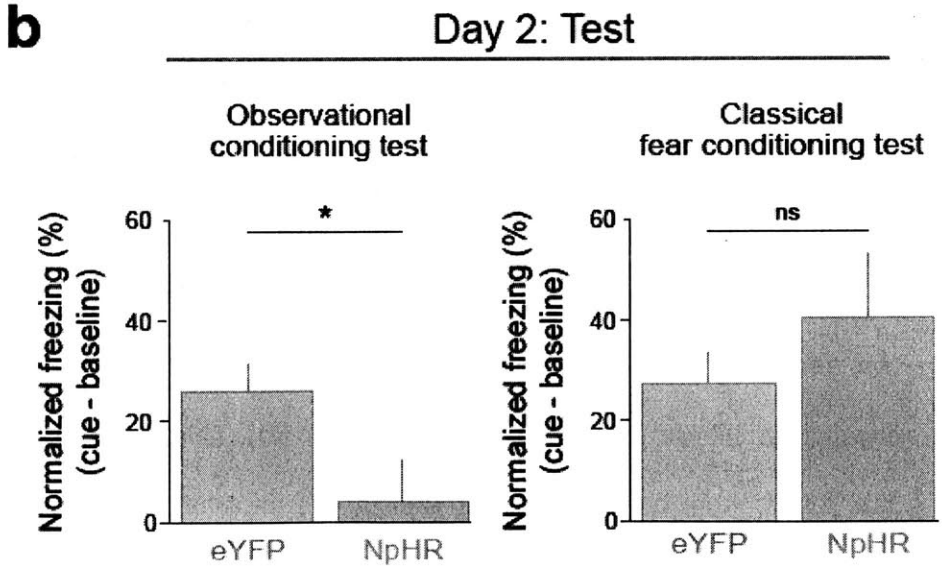

b
Day 2: Test
Observational conditioning test
Classical fear conditioning test
Normalized freezing (%) (cue - baseline)
60
40
20
0
*
ns
eYFP
NpHR

Figure 16 ACC-BLA inhibition during training impairs observational learning

a. Schematic of viral injection and optical fiber placement (top) and behavioral paradigm (bottom). EO mice expressing NpHR bilaterally in the ACC and with bilateral optical fibers over BLA were tethered to a laser to enable optical inhibition of ACC inputs to the BLA. During observational conditioning, yellow light (593 nm) was delivered 1 second prior to cue onset and stayed on until 1 second after cue onset. On Day 2, animals were re-exposed to the shock floor and cues were played while no shocks or light were delivered.

b. On Test Day, freezing to the cue was impaired in NpHR mice (n=7) when compared to control eYFP mice (n=12) (unpaired, two-tailed, t-test, $*P=0.0294$).

No significant difference was detected between NpHR animals (n=7) and eYFP (n=10) in cue specific freezing on test day after a classical fear-conditioning paradigm.

We hypothesized that the deficits in observational learning about the predictive value of the cue seen after inhibition of ACC inputs to BLA arose due to changes in the dynamics of how BLA neurons encoded the cue. To test this directly, we expressed NpHR in ACC neurons of observer mice and placed an optrode in the BLA (**Fig. 14a-c, Fig. 17a**). This enabled us to record from BLA neurons and assess how the dynamics of cue responses might change in the absence of ACC input to the BLA. In this paradigm, EO mice observed demonstrators receive cue-shock pairings, however in a subset of cue presentations, yellow light was delivered in order to inhibit ACC input to the BLA (**Fig. 17a**). We then compared BLA neuronal responses to the cue in the presence and absence of ACC input. We found that BLA neurons showed differences in the significance of their response to the cue when ACC inputs were inhibited (**Fig. 17b-e, Fig. 18c**). Notably, a subset of BLA neurons (22%, n=22/98) only showed significant cue responses when ACC input was available and upon inhibition of ACC input, these

cue responses were significantly different or undetectable **(Fig. 17e).** In addition, across all neurons recorded, significantly fewer neurons were cue-responsive during the trials with inhibition of ACC input (29%, n=28/98) when compared to the non-inhibition trials (44%, n=43/98) **(Fig. 18c).**

Upon examination of the population z-score response of all neurons that were excited (27%) or inhibited (16%) to the cue, we found that the cue excited population response was significantly impaired upon photoinhibition of ACC input when compared to the cue response without photoinhibition **(Fig. 17c-d).** To control for non-specific effects of light stimulation, we also delivered light stimulation during a baseline period where no cues were given (**Fig. 18a-b**). We found that a significantly smaller proportion of BLA neurons (7%, n=7/98) showed changes in firing rate when photoinhibition was delivered during baseline (in the absence of any task-related event) when compared to the proportion of cells (28%, n=27/98) that showed differences in significant cue responses (**Fig. 18b,d**) suggesting a cue-specific mechanism of ACC modulation. This finding that removal of ACC input changes the dynamics of BLA firing to the cue provides a circuit mechanism that could underlie the deficits in cue learning that we observed.

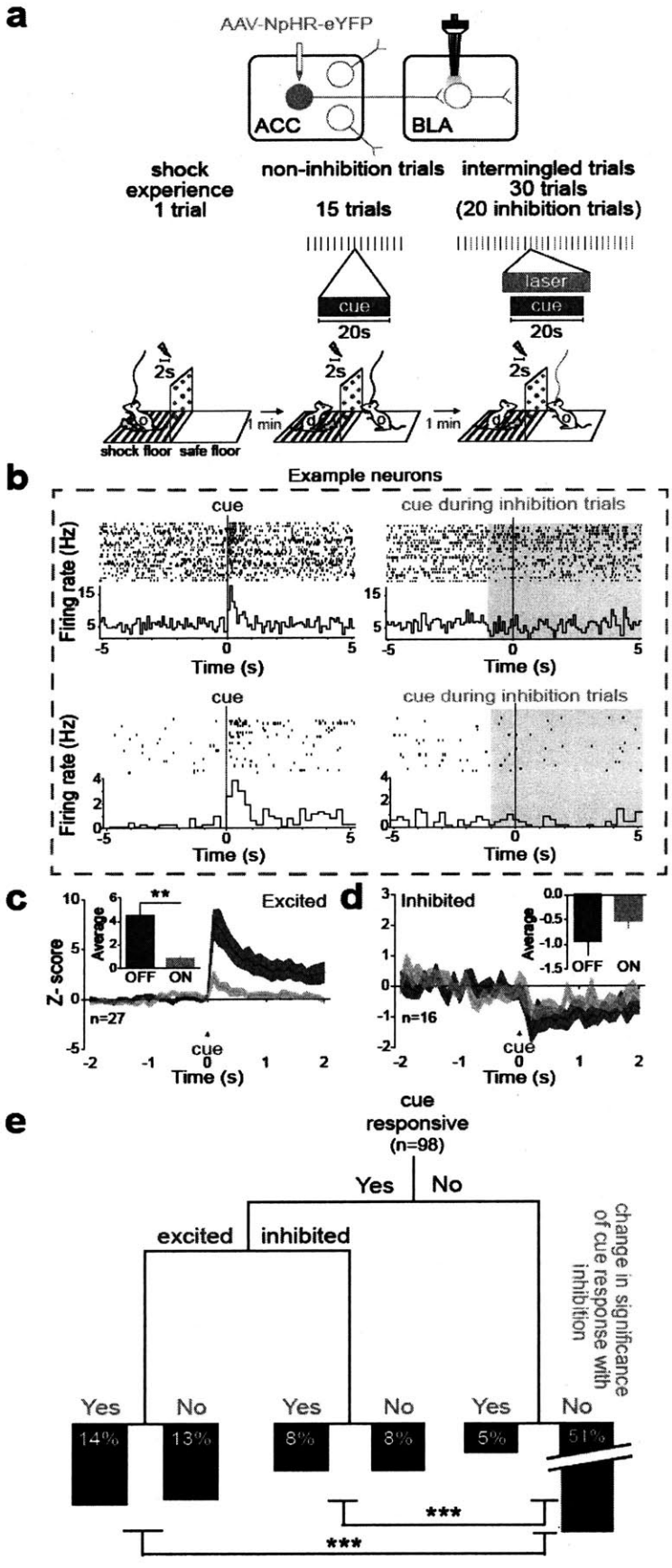

a
AAV-NpHR-eYFP
ACC
BLA
shock experience 1 trial
non-inhibition trials 15 trials
intermingled trials 30 trials (20 inhibition trials)
cue
20s
laser
cue
20s
2s
1 min
2s
1 min
2s
shock floor
safe floor
b
Example neurons
cue
cue during inhibition trials
Firing rate (Hz)
Time (s)
cue
cue during inhibition trials
Firing rate (Hz)
Time (s)
c
Excited
Z- score
Average
OFF
ON
n=27
cue
Time (s)
d
Inhibited
Average
OFF
ON
n=16
cue
Time (s)
e
cue responsive (n=98)
Yes
No
excited
inhibited
change in significance of cue response with inhibition
Yes
No
Yes
No
Yes
No
14%
13%
8%
8%
5%
51%
***
***

Figure 17 ACC input instructs BLA firing to the cue

a. Schematic of viral injection and optrode placement (top) and behavioral paradigm (bottom). Mice (n=5) had NpHR bilaterally injected into the ACC and an optrode implanted in the right BLA in order to record from BLA neurons while inhibiting ACC input. They were tethered to a head stage and laser, received an initial shock experience, and were then placed on the plastic floor to observe demonstrator. Paired cues and shocks were delivered to the demonstrator for 15 cues. The following 30 trials were intermingled with inhibition trials in which yellow light (593nm) from a laser was delivered to the optrode 1 second prior to cue onset and lasted until 1 second after cue offset.

b. Examples of BLA neurons and their response to the cue with and without optogenetic inhibition of ACC input to the BLA.

c. Z-score responses to the cue with and without laser stimulation for population of BLA neurons inhibited to the cue. Inset shows average z-score responses for the first 1 second after the cue.

d. Z-score responses to the cue with and without laser stimulation for population of BLA neurons excited to the cue. Inset shows average z-score responses for the first 2 seconds after the cue. The z-score response of the population of neurons excited to the cue was reduced during laser stimulation (paired, two-tailed t-test, $^{**}P=0.0007$).

e. Response tree representing all BLA neurons. Percentages of BLA neurons that were responsive to the cue and whether ACC inhibition lead to differences in the significance of the cue response. Neurons that were cue responsive had a significantly greater proportion of neurons that were modulated by the ACC (Chi-square test, excited, $X^2=18.60$, $df=1$; inhibited $X^2=13.87$, $df=1$, $^{***}P<0.001$).

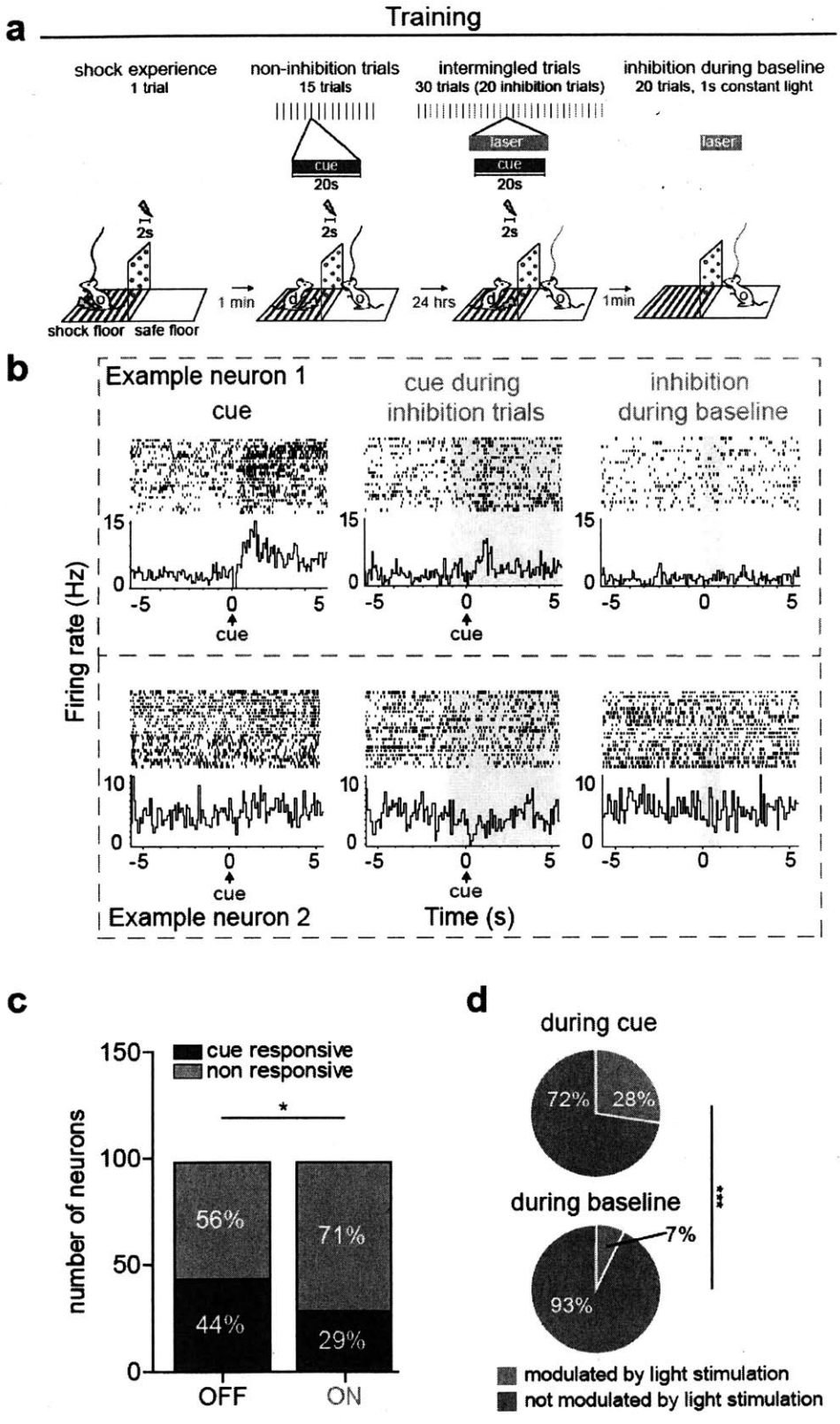

a
Training
shock experience
1 trial
non-inhibition trials
15 trials
intermingled trials
30 trials (20 inhibition trials)
inhibition during baseline
20 trials, 1s constant light
cue
20s
laser
2s
1 min
24 hrs
1min
shock floor
safe floor
b
Example neuron 1
cue
cue during inhibition trials
inhibition during baseline
Firing rate (Hz)
15
0
10
-5
0
5
cue
Example neuron 2
Time (s)
c
cue responsive
non responsive
number of neurons
150
100
50
0
56%
44%
71%
29%
OFF
ON
*
d
during cue
72%
28%
during baseline
7%
93%
***
modulated by light stimulation
not modulated by light stimulation

Figure 18 ACC modulation of BLA firing occurs during the cue

a, Schematic of behavioral paradigm during in vivo optrode recordings. EO mice received one footshock before being transferred to a safe plastic floor. They then observed a demonstrator receive 15 cue-shock pairings through a transparent, divider that contained holes. During 20 of the next 30 trials, laser stimulation (593nm) was delivered 1s prior to cue presentation and remained on until 1s after the cue offset. Immediately following this, mice received 20 trials with laser stimulation during a baseline condition where no cues were presented. Neuronal activity was continuously recorded throughout the experiment.

b, Responses of example neuron in Figure 4 (example neuron 1) and an additional example neuron (example neuron 2) throughout the different phases of the behavioral paradigm.

c, A significantly smaller proportion of cells were cue responsive during the light on trials where ACC input to the BLA was inhibited vs the light off trials.(Chi-square test, X^2=4.969,*df*=1, **P*= 0.0258

d, Significantly more cells were modulated by light stimulation during cue presentation compared to baseline where no task-related events occured (Chi-square test, X^2=12.10,*df*=1, ****P*= 0.0005).

Lastly, based on our result that the ACC-BLA projection was necessary for observational conditioning, but not classical fear conditioning, we hypothesized this might be due to its involvement in processing and utilizing social cues to help drive adaptive behavior. To test this, we performed a juvenile intruder task, in which a juvenile male mouse was introduced into the home cage of a resident observer and the time spent interacting was analyzed (**Fig. 19a-d**). Inhibition of ACC inputs to the BLA led to a significant impairment in social interaction (**Fig. 19a-d**), but did not alter non-social behaviors such as grooming or digging **(Fig. 19b).** In addition, we found no significant results in a novel object exploration task designed to control for the possible confound

that this manipulation generally affected exploration of novel stimuli and not social stimuli specifically **(Fig. 19e-h)**. Lastly, anxiety-related behaviors and locomotion were unaffected by ACC-BLA inhibition **(Fig. 20a-c).** Thus, given that inhibition of this circuit specifically impaired social interaction, this circuit may play a necessary role in observational learning because it is specialized for processing social cues to drive behavior.

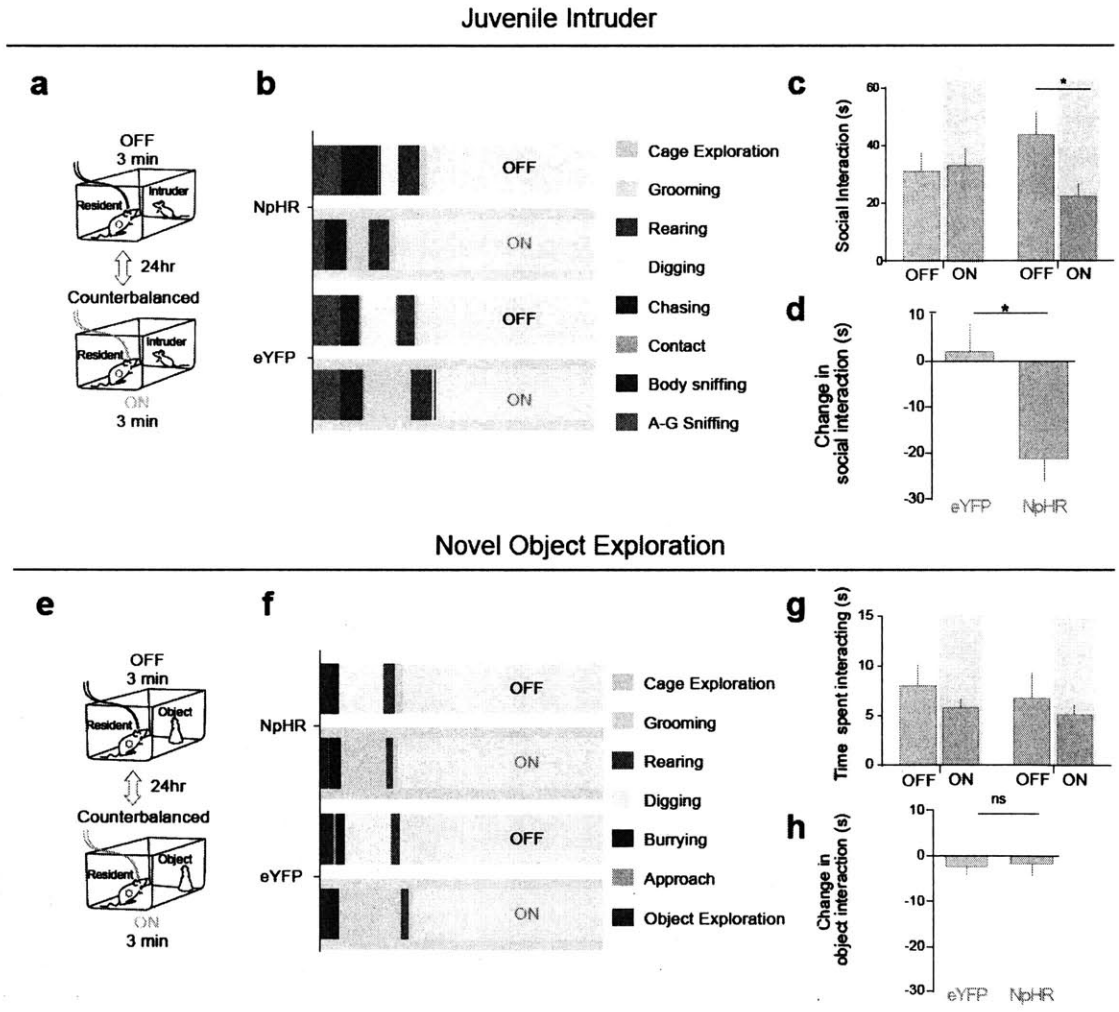


Figure 19

a. Schematic of Juvenile Intruder task.

b. Amount of total time (3min) spent engaging in social interaction (A-G sniffing, body sniffing, contact or chasing), digging, grooming, rearing and cage exploration during the juvenile intruder task in the NpHR and eYFP mice.

c. Inhibition of ACC to BLA input during juvenile intruder task decreases time engaged in social interaction behavior in NpHR but not eYFP mice (2-way ANOVA, $F_{1,13}$=5.209, P=0.04, Bonferroni post-hoc analysis *P=0.0126).

d. Summary of light-evoked changes in juvenile-intruder paradigm. Inhibition of ACC input to the BLA decreased social interaction time in NpHR mice (n=7) compared to eYFP (n=9), (unpaired, two-tailed T-test, *P=0.0163)..

e. schematic of Novel Object Exploration task.

f. Amount of total time (3min) spent engaging in object exploration (exploration, approach), burying, digging, rearing, grooming and cage exploration during the novel object task in NpHR and eYFP mice.

g. Inhibition of ACC to BLA input during novel object exploration task did not significantly change the time engaged in object exploration behavior in NpHR or eYFP mice.

h. Novel object exploration was not altered by light-evoked inhibition of ACC input to the BLA

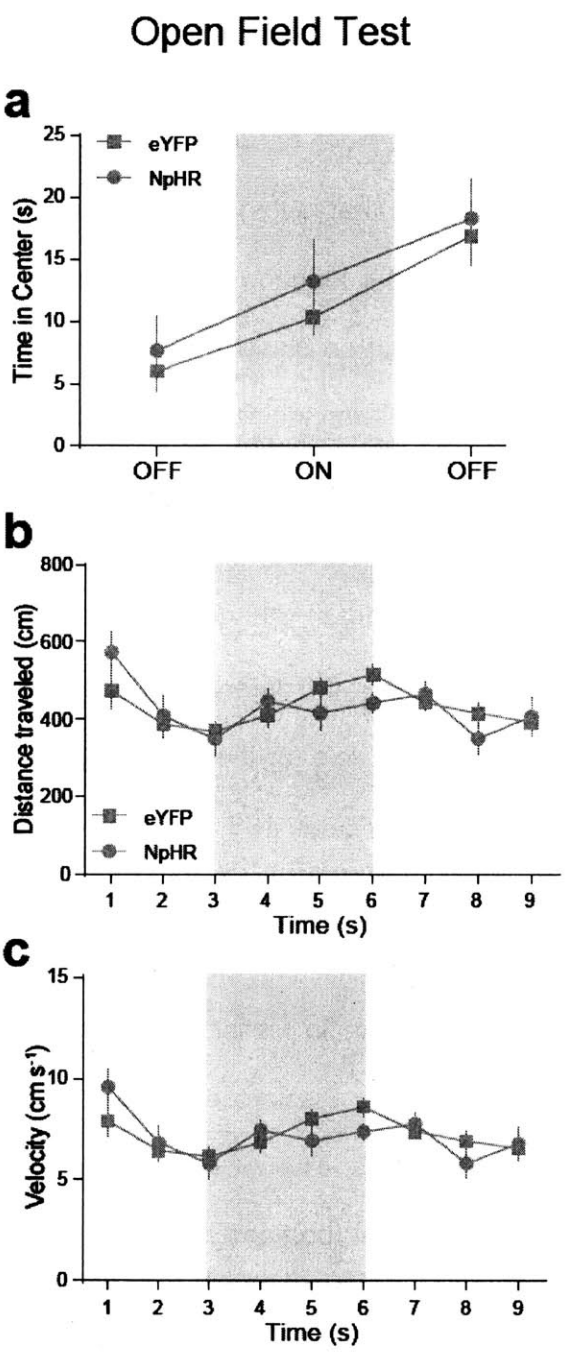


Figure 20 ACC-BLA inhibition during the open field test

a-c. Inhibition of ACC to BLA inputs did not significantly increase center exploration (a), distance traveled (b) or velocity (c) in an open-field.

Discussion

In this study, we show that ACC neurons providing monosynaptic input to the BLA encode cue information and that optogenetic inhibition of this input during the predictive cue impairs observational learning but not classical fear conditioning. The finding that this circuit had no effect on classical fear conditioning suggests that when animals have direct access to an unconditioned stimulus, the computation needed to learn about the predictive value of a cue may circumvent the ACC-BLA projection. However, when the predictive value needs to be learned through observation of others, the associative computation is routed through the ACC before being sent to the amygdala in order to drive appropriate defensive responses to the cue. This is in contrast to previous work showing a role for the ACC in fear conditioning (Peretz 1960; Buchanan and Powell 1982; Gabriel et al. 1991; Morgan and LeDoux 1995; Farr et al. 2000; Kung et al. 2003; C. J. Han et al. 2003; Johansen and Fields 2004; Steenland, Li, and Zhuo 2012) . Additionally, it has been previously hypothesized that ACC interaction with the BLA plays an important role in classical fear conditioning (Gao et al. 2004; Tang et al. 2005; Malin et al. 2007; Bissiere et al. 2008). There are a few potential reasons for this difference in results. The ACC is a large structure and it has been suggested that different anatomical portions of the ACC have different functions (Devinsky, Morrell, and Vogt 1995; Bussey et al. 1996; Allman et al. 2001; B. F. Jones, Groenewegen, and Witter 2005). Thus, differences in the targeted regions of the ACC could lead to different behavioral effects. In agreement with this notion, experiments targeting the segment of ACC that we targeted also showed no effect on classical fear

conditioning (Jeon et al. 2010; Kim et al. 2012). Secondly, we target a specific projection from the ACC in a temporally precise manner. It is likely that manipulations of specific ACC projections may lead to different behavioral results than non-specific lesion studies as has been seen in other brain regions such as the amygdala and VTA (Tye et al. 2011; Lammel et al. 2012).

In addition, it has been previously suggested that the ACC could influence activity in the amygdala (Etkin et al. 2006; Olsson, Nearing, and Phelps 2007; Bissiere et al. 2008). For example, Bissiere and colleagues showed that ACC neurons provided primarily glutamatergic input to the BLA. Here we provide the first direct evidence that BLA neural activity can be dramatically altered by the removal of ACC input. This implies that at least a subset of BLA neurons depend on ACC input in order to encode cue information during observational learning.

Altogether, the present results demonstrate a fundamental role for information sent from the ACC to the BLA in the modulation of observational fear conditioning and social behaviors. This study also provides evidence that circuits underlying very basic, evolutionarily conserved social behaviors such as observational learning are likely to be multi-purposed to allow for the more complex interactions seen in the general repertoire of animal's social behaviors. Understanding how these circuits underlie fundamental aspects of social cognition can give us insight into how these circuits malfunction in psychiatric conditions such as autism spectrum disorders, social anxiety disorder, and schizophrenia.

Methods

Subjects

Adult (8-12 weeks) male C57BL/6J were used for all experiments. All mice were pair-housed on a reverse 12 hour light-dark cycle with food and water *ad libitum*. All experiments were conducted in accordance with NIH guidelines and with approval of the MIT Institutional Animal Care and Use Committee and the MIT Department of Comparative Medicine.

Stereotactic surgery procedures

All surgeries were conducted under aseptic conditions using a digital small animal stereotaxic instrument (David Kopf Instruments, Tujunga, CA, USA). Mice were anaesthetized with isoflurane (5% for induction, 1%–2.5% for maintenance). Injections were performed using a beveled 33-gauge microinjection needle. A 10-µl microsyringe (nanofil; WPI, Sarasotam FL, USA) was used to deliver virus at a rate of 0.1 µl per min using a microsyringe pump (UMP3; WPI) and controller (Micro4; WPI). Mice were given a post-surgical recovery time of at least 7 days prior to start of any experimental procedures.

Surgical procedures for *in vivo* multi-unit electrophysiological recordings (Figure 2 and 3):

In order to record from anterior cingulate cortex (ACC) neurons in a circuit-specific manner an adeno-associated virus serotype 5 carrying a construct for expression of channelrhodopsin-2 fused to a enhanced yellow fluorescent protein, under the control of

a double-inverted open reading frame (AAV5-DIO-ChR2-eYFP) (1 µl) was injected into the ACC (AP: +1.0 mm, ML: -0.3 mm, DV: -2.1 mm) and 1 µl of the retrogradely traveling canine adenovirus carrying cre-recombinase CAV2-EF1α-Cre was injected into the BLA (AP: -1.6 mm, ML: 0.35 mm, DV: −4.9 mm). 5-8 weeks later, a second surgery was performed to implant an optrode. one craniotomy was drilled over the ACC (AP: −0.6 mm, ML: 1.0 mm). Two to three skull screws were implanted around the site of the craniotomy. One layer of adhesive cement (C&B Metabond; Parkell, Edgewood, NY, USA) followed by cranioplastic cement (Dental cement; Ortho-Jet, Lang Dental, Wheeling, IL, USA) was used to stabilize screws to the skull. A 16-channel multi array optrode (Innovative Neurophysiology) was lowered at approximately 0.01 mm/s u to -1.9 mm DV. The ground wire was implanted at a depth of approximately 1 mm into the posterior ipsilateral hemisphere. Cranioplastic cement was placed around the optrode and ground wire and the optrode was then lowered to −2.1 mm DV. Sham surgery was performed on the observer's cage mate. A craniotomy was made and mice were left under anesthesia for an equivalent amount of time as their observer but no electrode was implanted. A small amount of cranioplastic cement was placed on the skull to cover up the craniotomy. Cage mates were reunited directly after surgery.

Surgical procedures for halorhodopsin behavioral and electrophysiological experiments:

In order to inhibit ACC input to the BLA, an Adeno-associated virus carrying the gene for a fusion protein comprised of enhanced Halorhodopsin and enhanced yellow fluorescent protein under the calmodulin kinase II promoter (AAV_5-CaMKIIα-eNpHR3.0-eYFP) or eYFP alone was bilaterally injected into the ACC (AP: +1 mm, ML: ±0.25 mm,

DV: -2.1 mm). After waiting for 5 minutes the needle was raised to -2.0 mm and another 300 µl of virus was injected at the same rate. After waiting 5 additional minutes the needle was raised to -1.9 mm for 10 minutes before being slowly withdrawn. After 4-8 weeks mice underwent a second surgery in which two optical fibers were implanted bilaterally over the BLA (AP: -1.6 mm, ML: ±3.35 mm, DV: -4.5 mm). Fibers were lowered at approximately 0.01 mm/s and were secured using a thin layer of adhesive cement followed by dental cement. In a subset of animals instead of optical fibers an optrode was implanted in the BLA following the same surgical procedure as described above.

Behavioral Tasks

Observational conditioning task:

Mice were placed in a soundproof conditioning chamber (Med Associates, St Albans, VT, USA) with a shock floor side and a plastic floor side separated by a transparent plastic divider containing holes. Mice in the Experienced Observers (EO) group received a "shock experience" by being placed on the shock floor side of the chamber and allowed to freely explore. After 5 minutes they received 1 un-predicted, un-cued, foot shock (all mice greater than 30 g were shocked with 1.5 mA, while mice less than 30 g were shocked with 1mA) and were immediately transferred to the plastic floor side of the chamber. The cage mate of the observer was then placed into the shock side of the

chamber as the demonstrator for "observational conditioning". After 5 minutes of habituation, demonstrators underwent 30 trials that occurred at random intervals (60, 90, 120, 150, 180 seconds) in which a 20 second compound cue (light and 10 kHz tone) predicted the delivery of a 2 second shock (1 mA - 1.5 mA) 10 seconds after the onset of the cue. Directly after, mice were placed back into their home cages. 24 hrs later, observer mice were placed back into the shock side of the chamber and 30 cues were delivered to the chamber in the absence of shock. Mice in the Unpaired Observers (UO) group received a "shock experience", however during observational conditioning cues and shocks delivered to the demonstrator were explicitly unpaired. Experienced Solo animals (ES) also received a "shock experience" but then no demonstrator was placed into the shock side of the chamber. Paired cues and shocks were delivered to the empty side of the chamber. Naïve Observers (NO) did not receive a "shock experience" but instead were placed on the plastic side of the chamber where they observed demonstrators in the same way as EO. Lastly, Naïve Solo animals (NS) also did not receive a "shock experience" but instead were placed on the plastic side of the chamber. Paired cues and shocks were delivered to the shock floor side of the chamber in the absence of a demonstrator.

Optogenetic inhibition during observational learning

Mice that expressed halorhodopsin or eYFP bilaterally in the ACC and had bilateral optical fibers over BLA were tethered to a cable attached to a laser and placed in the same observational conditioning chamber described above. Mice received one foot shock and were then placed on the plastic side of the chamber to observe their demonstrator. 30 cue-shock pairings were then delivered to the box in the same manner

described for EO. Yellow light (593 nm) was delivered one second before every cue and the laser stayed on for one second after the cue offset. 24 hrs later mice were again tethered to the laser and placed back on the shock floor and 30 cues were delivered to the chamber in the absence of shock delivery and laser stimulation. During testing no laser stimulation was delivered. All behavioral scoring for this experiment was performed in the manner described below.

Optogenetic inhibition during classical fear conditioning

Mice that expressed halorhodopsin or eYFP bilaterally in the ACC and had bilateral optical fibers over BLA were tethered to a cable attached to a laser and placed in a behavioral chamber with a shock floor. Mice received 12 trials in which a 20 second cue (10 kHz tone and houselight) was followed by delivery of a .5 ms footshock. The footshock occurred 10 seconds into the cue just as in observational conditioning. 1 second before every cue, yellow light (593 nm) was emitted from the laser and stayed on until 1 second after the cue. 24 hrs later, mice were placed back in the chamber and 12 cues were played in the absence of shock delivery and laser stimulation. All behavioral scoring for this experiment was performed in the manner described below.

Social Interaction assays

Social Interaction in the homecage (Figure 4 and S9) was examined as follows. The cagemate temporarily moved to a holding cage and the experimental mouse was allowed to explore its homecage freely for 1 min (habituation). A novel juvenile (3-4 weeks old) male C57BL/6 mouse was then introduced into the cage and allowed to

interact freely for 3 min (test session). Each experimental mouse underwent two social interaction tests separated by 24 hrs, with one intruder paired with optical stimulation and one with no stimulation. Groups were counterbalanced for order of light stimulation. All behaviors were video recorded and analyzed by an experimenter blind to the testing condition using ODLog software (Macropod software). The overall score of social interaction was defined as any period of time in which the experimental mouse was actively investigating the juvenile intruder, including behaviors such as face or body sniffing, anogenital sniffing, direct contact, and close following (<1 cm). Nonsocial behaviors were also represented in an overall exploration score, which included walking, rearing, digging, and self-grooming.

Novel object exploration

The novel object test was executed exactly like the social interaction assay. Instead of a juvenile intruder, a plastic object was introduced to the animals home cage and total time spent investigating the object over 3 min was quantified. Objects were thoroughly cleaned with acidic acid in between tests. Each experimental mouse underwent two novel object investigation tests separated by 24 hrs, with one trial paired with optical stimulation and one with no stimulation, counterbalanced for order of light stimulation and object.

Open field test

The open field chamber was made of transparent plastic (53 x 53 cm) and divided into a central and a peripheral field. Individual mice were connected to the patch cables and placed in the center of the open field at the start of the session. The open field test consisted of a 9 min session with three 3 min epochs (OFF-ON-OFF) in which the mouse was permitted to freely investigate the chamber.

All behavioral tests were recorded by a video camera. The EthoVision XT video tracking system (Noldus, Wageningen, Netherlands) was used to track mouse location, velocity, and movement of head, body, and tail. All measurements displayed are relative to the center of the mouse body.

Analysis

Behavioral Analysis of Observational conditioning

Behavioral performance was recorded by digital video cameras. All videos were manually analyzed offline by an experimenter blind to experimental conditions. Freezing behavior of the observer was scored on both conditioning and test day as the amount of freezing during the cue minus the amount of freezing in the 20 seconds directly preceding the cue. Freezing was defined as absence of movement, with the exception of respiration. Based on preliminary behavioral data, analysis of freezing behavior for the training day was performed on trials 5-20. Analysis of freezing behavior on test day

was performed on the first 5 trials. Additional other stereotyped behaviors shown by the observer such as grooming and escaping, were quantified by manual scoring. Observer animals were said to be mimicking when they displayed escape behaviors in direct response to demonstrators exhibiting escaping behaviors during the cue.

Grooming and mimicking behaviors were quantified during the 20 s of the cue and compared to the baseline taken as the 20 s prior to the cue onset. The number of trials in which animals that were engaged in grooming stopped at cue onset was quantified as a percentage of all trials. This was averaged across all animals in the respective groups. The percentage of trials in which the Observers showed mimicking behaviors was also quantified and averaged across animals in a given group.

In vivo and Ex-vivo electrophysiological recordings

Ex vivo recordings and analysis

Six to eight weeks after surgery for ChR2 expression in ACC-BLA projectors, 4 mice were anesthetized with 90 mg/kg pentobarbital and perfused transcardially with 10 mL of modified artificial cerebrospinal fluid (ACSF, at ~4°C) containing (in mM): 75 sucrose, 87 NaCl, 2.5 KCl, 1.3 NaH_2PO_4, 7 $MgCl_2$, 0.5 $CaCl_2$, 25 $NaHCO_3$ and 5 ascorbic acid. The brain was then extracted and glued (Roti coll 1; Carh Roth GmbH, Karlsruhe, Germany) on the platform of a semiautomatic vibrating blade microtome (VT1200;

Leica, Buffalo Grove, IL). The platform was then placed in the slicing chamber containing modified ACSF at 4°C. Coronal sections of 300 µm containing the ACC and BLA were collected in a holding chamber filled with ACSF saturated with 95% O2 and 5% CO2, containing (in mM): 126 NaCl, 2.5 KCl, 1.25 NaH2PO4, 1.0 MgCl2, 2.4 CaCl2, 26.0 NaHCO3, 10 glucose. Recordings were started 1 h after slicing and the temperature was maintained at approximately 31°C both in the holding chamber and during the recordings.

The viral injection sites were checked and imaged with a camera (Hamatsu Photonics K.K., Japan) attached to the microscope (BX51; Olympus, Center Valley, PA). The slice images were registered to the mouse brain atlas (Paxinos and Watson) and the center of the injection was taken at the brightest point of the fluorescence. If the injection site was outside the ACC, data was not collected from that animal.

Recordings were made from visually identified neurons expressing ChR2-eYFP and non-expressing neighboring cells. Patched cells were filled with Alexa Fluor (AF) 350 and biocytin. Voltage and current-clamp recordings of BLA projectors were made using glass microelectrodes (5-7 MΩ) shaped with a horizontal puller (P-1000, Sutter, CA) and filled with a solution containing (in mM): 125 potassium gluconate, 20 HEPES, 10 NaCl, 3 MgATP, 8 biocytin and 2 Alexa Fluor 350 (pH 7.25-7.4; 280-290 milliosmol). Recorded signals were amplified using Multiclamp 700B amplifier (Molecular Devices, Sunnyvale, CA). Analog signals were digitized at 10 kHz using a Digidata 1440 and recorded using the pClamp10 software (Molecular Devices, Sunnyvale, CA). Oxygenated ACSF was perfused onto the slice via a peristaltic pump (Minipuls3; Gilson, Middleton, WI) at ~3

mL/min. Cells were confirmed to be expressing ChR2 based on the constant inward current response to a 1 s constant blue light pulse in voltage clamp.

Off-line analysis was performed using Clampfit software (Molecular Devices, Sunnyvale, CA). Light evoked latencies of action potentials (AP) and excitatory postsynaptic potentials (EPSP) were measured for each cell for 20 pulses of a 1 Hz train with 5 ms pulses. Latencies were measured from the onset of the light pulse to the peak of the AP or EPSP.

Electrophysiology during Observational learning

To enable us to record from neurons during observational learning, mice were connected to a head stage that plugged into a commutator and pre-amplifier (Tucker-Davis Technologies, Alachua, FL (TDT)). EO and UO mice were conditioned while connected to the TDT system for multichannel spike acquisition. Immediately after the initial shock experience mice were placed on the plastic floor side of the chamber. A demonstrator was placed on the shock floor and cues were delivered at random intervals in the absence of shock delivery. This is referred to as the "cue only" period. Following this period, animals observed cues and shocks being delivered to the box in a paired (EO) or unpaired (UO) manner. In animals expressing ChR2 in ACC-BLA projecting neurons and an optrode in the ACC, photo neural activity was recorded continuously during the entire behavioral paradigm.

Electrophysiology Analysis of cue responses

Neural activity was recorded, and unit discrimination was performed with multichannel spike acquisition (TDT) and sorting software (Plexon Inc., Dallas, TX). Responses of single units to the cue onset was deemed statistically significant if the firing rate within one or more 100-ms bins in the response window (0–0.5 s after cue onset or offset) was significantly different ($P < 0.01$) from a 1 s baseline epoch using a Wilcoxon signed-rank test. In some neurons that showed sustained responses a baseline window of 20 seconds before cue onset and a response window of 9 seconds was used. *P* value was adjusted for multiple comparisons.

Electrophysiology Analysis of learned cue responses

To compute if neurons encoded the learned value of the cue, neuronal cue responses were analyzed for the cue only and observational conditioning periods. Because there were more trials in the observational conditioning period, we used trials 20-35 for comparison with the cue only (trials 1-15) using the baseline and response windows described above for cue responses. We chose these trials as they corresponded to the trials that we used to measure learning behaviorally. Using the Wilcoxon signed-rank test, a neuron was classified as having a conditioning selective response if it had a significant response to the cue during observational conditioning ($P < 0.01$), but not during the cue only habituation epoch. A neuron was classified as having a conditioned response to the cue if it had a significantly different response window ($P < 0.01$) during conditioning when compared to the cue only period. *P* value was adjusted for multiple comparisons.

Electrophysiological identification of ACC network vs non- network neurons

In animals expressing ChR2 in ACC-BLA projector neurons an optrode was implanted into the ACC as described above. EO animals underwent observational conditioning while we recorded neural activity using the TDT acquisition system. Immediately after conditioning the demonstrator mouse was removed and blue light (473nm, 15-20 MW) from a laser was delivered through the optrode (phototagging). Electrophysiological recording was not stopped between observational conditioning and phototagging. During phototagging different stimulation parameters were used: 1s pulse, 5ms 1Hz pulses, 5ms 10Hz pulses, and 5ms 20Hz pulses. We then analyzed neural responses to photostimulation. Due to the wide range of latencies in response to light stimulation in recorded units, we first used custom-written MATLAB scripts to calculate the latency from photostimulation onset to the first 10 ms bin (within 500 ms) with a 4 standard deviation (SD) increase over the baseline firing rate (−0.5 to 0 s) using the data from the 1Hz stimulation. We then used the Wilcoxon rank-sum test to determine if the firing rate within an experimental window was significantly different than the baseline firing rate. For neurons with latencies lower than 10 ms, a baseline window of 50 ms and response window of 20 ms was used. For neurons with latencies above 10 ms, a baseline of 500 ms was used and a response window of 50 ms that started 20 ms before the calculated latency. Lastly neurons that showed inhibitory responses were analyzed using the data from the 1 second constant pulse of light with a baseline window of 1 s and a response window of 500 ms. Neurons were categorized as ACC network neurons if they showed a significant excitatory or inhibitory response to photostimulation. Neurons were

classified as ACC-BLA projectors if they had latencies under 8 ms and a significant difference in the rank-sum analysis ($P < 0.01$). Excitatory network neurons were defined as those that were excited to light with a latency of 20-100ms and significant difference in the rank-sum analysis ($P < 0.01$). Inhibitory network neurons were defined as those that were inhibited to light within a 500 ms window using the rank-sum analysis ($P < 0.01$).

Electrophysiology during halorhodopsin manipulation of Observational learning

Mice that expressed halorhodopsin bilaterally in the ACC and had an optrode in the right BLA were plugged into the TDT acquisition system and a laser. They were placed into the observational conditioning behavioral chamber described above, received an initial shock experience, and were then transferred to the plastic side of the chamber. The demonstrator was placed on the shock floor and 15 cue-shock pairings were delivered to the chamber. The next 30 trials had a subset of trials where the laser delivered yellow light (593 nm) 1 second before the onset of the cue and stayed on until 1 second after the cue. This was done in a pseudorandom order. Overall, there were 25 trials in which no laser stimulation was delivered and 20 trials where laser stimulation was delivered. Neural activity was recorded continuously through the experiment.

Electrophysiology Analysis of halorhodopsin optrode experiments

Neural activity was recorded, and unit discrimination was performed with multichannel spike acquisition (TDT) and sorting software (Plexon Inc.). All of the non-laser stimulation trials and the laser stimulation trials were grouped together for statistical

analysis. Responses of single units to the cue onset were deemed statistically significant as described above. A neuron's response to the cue was categorized as being modulated by laser stimulation if the Wilcoxon rank-sum test for cue response was significant ($P< 0.01$) in either the laser stimulation or non-laser stimulation condition but not the other condition, or if the response windows were significantly different from each other. Multiple comparisons were corrected for by adjusting p values.

Statistical Analysis

Statistical analyses were performed using commercial software (GraphPad Prism; GraphPad Software, Inc, La Jolla, CA, USA; MATLAB, Mathworks, Natick, MA, USA). Group comparisons were made using one-way or two-way ANOVA followed by Bonferroni post-hoc tests. Single variable comparisons were made with two-tailed unpaired Student's t tests while chi-square analyses were used to compare populations. Non-parametric Wilcoxon rank-sum tests were used to determine whether changes in firing rates were statistically significant in *in vivo* electrophysiological recordings (MATLAB) using an $\alpha = 0.01$. Comparisons of z-score responses were also analyzed using an $\alpha = 0.01$. Multiple comparisons were corrected for by adjusting P values. Differences between experimental behavioral groups were assessed using analysis of variance (ANOVA) followed by post-hoc tests when applicable using an $\alpha = 0.05$. All state space analyses were performed according to Smith et al. 2010.

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Author Contributions

SAA and ACFO performed recordings for in vivo electrophysiology experiments and SAA, EHN, DB, ACS, EB analyzed in vivo electrophysiological data. SAA, RW, AV, and ACFO performed optogenetic experiments and analysis. SAA, RW, AV, ACFO, AE, and AM performed behavioral experiments and analysis. AB and SA performed ex vivo electrophysiological recordings and analysis. SAA, AV, ACFO performed surgeries for all animals included in the study. ACFO, RW, AV, SAA, AE, and AM performed histological confirmation. SAA and KMT designed experiments. SAA and KMT wrote the paper with edits by ACFO and RW. All authors provided feedback on manuscript.

Chapter 4

Conclusions and a way forward

Summary

In this thesis, we sought to address a number of outstanding questions with regards to the role of the ACC and amygdala in observational fear learning. Through combining optogenetics, in vivo and ex vivo electrophysiology, and rodent behavioral approaches, we were able to provide the following novel insights:

1. The ACC and BLA contain neurons that are excited and inhibited to the cue during observational learning.
2. A subset of neurons in the ACC and BLA show conditioned responses to the cue during observational fear learning.
3. The ACC-BLA network has an enhanced representation of cue information when compared to out of network neurons. ACC neurons that project to the BLA encode the cue.
4. Inhibition of ACC input to the BLA during the cue impairs observational learning but not classical fear conditioning
5. Inhibition of ACC input to the BLA changes the cue response of a subset of BLA neurons.
6. ACC input to the BLA is necessary for normal social interaction.

Together, these results suggest a model in which an association between the cue and the social US is made in the ACC during observational fear learning. The ACC is able to learn the predictive meaning of the cue and transfer this information to the amygdala in order to instruct amygdala firing to the cue. The amygdala can then drive the appropriate defensive behaviors in response to the cue. The predictive value of the cue might be learned in the ACC because of its ability to process and utilize social cues in combination with its ability to perform computations necessary for prediction. It is possible that when animals receive a direct shock experience, as in classical fear conditioning, this ability of the ACC is not necessary as the amygdala is able to make the predictive association without ACC input. Alternatively, it is also possible that the ACC sends information about the social US as well as the cue and that this facilitates the amygdala's ability to make an association between the cue and the social US. We don't have data that deal with this possibility directly, but, the fact that we did see less mimicking during conditioning in animals where ACC input was inhibited supports this alternative model. Nevertheless, the fact that observer animals still show reactions to the shock of the demonstrator as well as contextual freezing during the halorhodopsin manipulation implies that even in the absence of ACC input, animals are still able to process the social US. This makes it more likely that the signal being sent from the ACC is about the predictive value of the cue. This interpretation is bolstered by our data showing that cue-specific freezing is the deficit seen in animals where ACC input to the BLA is inhibited during the cue.

Our finding that the ACC-BLA circuit is necessary for observational fear learning as well as normal social interaction is direct evidence for the principle that the brain utilizes circuits involved in basic, evolutionary conserved forms of social cognition in order to build the more complex array of social behaviors seen during general social interaction. Finding, for the first time, direct evidence that a circuit involved in observational learning is also necessary for social interaction provides validation that studying innate social behaviors can provide insight about the way the brain generates complex social behaviors. Still, in the data presented here we have not characterized the role this circuit plays in social interaction. This provides a rich area for future research and a utilization of optogenetics, electrophysiology, and imaging during behavior will provide insight as to how the brain utilizes this circuit in order to enable normal social interaction.

Here we show that the ACC is involved in one type of observational learning. It is still an outstanding question whether this same circuit is involved in other forms of observational learning and if so, what role it plays. For example, it is possible that the ACC-BLA circuit may also be involved in observational reward learning. The ACC has been implicated in reward processing in humans and other animals (Bush et al. 2002; Williams et al. 2004b; Amiez, Joseph, and Procyk 2005). The involvement of the BLA in reward learning has also been established (Everitt et al. 1999; Tye et al. 2008; Stuber et al. 2011; Namburi et al. 2015; Beyeler et al. 2016). Additionally, the ACC and BLA were shown to respond when monkeys give rewards to another monkey (Chang, Gariépy,

and Platt 2013; Chang et al. 2015). Combined, these findings provide evidence that this circuit may also underlie observational reward learning.

To summarize, we demonstrate that both the ACC and amygdala encode observational fear learning, that BLA encoding of the predictive cue is dependent on ACC input and that this input is necessary for observational fear learning. Lastly, we demonstrate that this same input is also necessary for normal social interaction. Thus, we demonstrate that the ACC-BLA circuit is a critical node for both observational learning as well as social interaction. Future studies will further define this circuit's inputs and outputs and the specific roles they play in enabling observational fear learning and other social behaviors. Lastly, we now have a baseline understanding of this circuit's function that can serve as a springboard for asking questions about how pathology in this circuit can contribute to aberrant social behaviors and deficits in observational learning seen in mouse models of psychiatric disease.

Observational versus classical fear conditioning

In this study we demonstrate a role for the ACC-BLA circuit in observational fear learning but not classical fear learning. This is seemingly at odds with various studies that have suggested that the ACC plays a role in various types of fear conditioning through direct experience (Peretz 1960; Buchanan and Powell 1982; Gabriel et al. 1991; Morgan and LeDoux 1995; Farr et al. 2000; Kung et al. 2003; Han et al. 2003; Gao et al. 2004; Tang et al. 2005; Johansen and Fields 2004; Malin et al. 2007; Bissiere et al. 2008; Steenland, Li, and Zhuo 2012). There are two major considerations that can

help explain this seeming conflict. Firstly, the ACC is a large structure and it is known that different parts of the ACC are involved in different functions (Devinsky, Morrell, and Vogt 1995; Bussey et al. 1996; Allman et al. 2001; Jones, Groenewegen, and Witter 2005). In studies in rodents that have targeted the ACC during fear conditioning, lesions have targeted a range of locations. For example, Farr and colleagues targeted the coordinates AP: .5 mm, ML: .5 mm, DV: -1.8 mm while Tang and colleagues targeted the coordinates AP: .62 mm, ML: .5 mm, DV: -.9 mm (Farr et al. 2000; Tang et al. 2005). In our optogenetic experiments we saw expression in both dorsal and ventral components and there was certainly a range of anterior-posterior expression. However, we target the coordinates AP: 1 mm, ML: .25 mm, DV:-2.1 - -2.3 mm. This is anterior to Farr and Tang's coordinates and covers much more of the ventral portion of ACC. In addition, our coordinates are similar to those used by Jeon et al, who also showed that their ACC lesions, while impairing contextual observational fear did not affect classical fear conditioning (Jeon et al. 2010). Resolving whether these differences in results can be explained by the differences in target region of the ACC calls for a systematic investigation of observational and classical fear conditioning along the AP and DV axis of the mouse ACC.

A second potential reason for these results is that we utilize optogenetics in order to inhibit a specific projection from the ACC only during the CS. Previous studies have not utilized this tool to target specific downstream targets of the ACC and instead have utilized more traditional approaches to inhibit cell bodies and axons of passage in the ACC in a manner that was not temporally defined to a particularly discrete period. It is

possible that inhibition of specific ACC projections may lead to different behavioral outcomes than non-specific inhibition of cell bodies in the ACC with varying downstream targets. This idea has been experimentally tested in other brain regions such as the amygdala and VTA (Tye et al. 2011; Lammel et al. 2012). For example, Tye and colleagues showed that optogenetic inhibition of BLA input to CeA lead to anxiogenesis, while non-specific inhibition of BLA cell bodies did not have an anxiogenic effect (Tye el al. 2011).

On a more theoretical level, while it is clear that although there are likely overlapping brain regions, such as the amygdala, involved in both observational and classical fear conditioning, there must also be regions that are not involved in both. Although observational learning likely utilizes similar mechanisms of association and plasticity necessary for learning, there are certainly different routes of processing involved, some of which are specific to social information. This becomes clear when considering the nature of the US in both paradigms. In classical fear conditioning the US is usually a footshock. This is a discrete unimodal sensory stimulus that is consistent across trials and processed through thalamic structures such as the ventral posterolateral (VPL) and ventral posteromedial nuclei (VPM) as well as the PAG (Romanski et al. 1993; Shi and Davis 1999; Lanuza, Moncho-Bogani, and LeDoux 2008; Jeon et al. 2010; Johansen et al. 2010). In contrast, in observational learning the US is the behavior of another animal. This is a multimodal stimulus that is variable across trials and is likely processed through a combination of auditory, visual, and olfactory cues. It is currently still a matter of debate what the key features of the social

US are and studies have shown results suggesting visual, auditory, and olfactory cues can be utilized to drive fear behavior in another animal (Kavaliers, Choleris, and Colwell 2001; Bredy and Barad 2009; Kim et al. 2010). There has not been a thorough characterization of the neural basis underlying how social USs are detected and processed. It seems likely that although certain elements of a social US may independently and innately drive defensive behaviors, cortical regions such as the ACC may be necessary to bind all of the various inputs that comprise the social US in order to create a complete representation of the social US that is then used to drive learning. Despite the clearly divergent manner in which the USs are processed in the brain, there is likely convergence at some critical node in the processing pathway as the resulting behavioral output of both types of learning is the same. The results from this study suggest that the amygdala may be the first site of this convergence. Future studies may tease apart whether individual neurons in the amygdala respond to both the social US as well as a direct US such as shock experience. These neurons would have to have input from regions involved in observational conditioning such as the ACC as well as regions involved in classical fear conditioning, but not observational conditioning, such as the VPL and VPM (Jeon et al. 2010).

One caveat of our interpretation of this data is that the ACC might not be specifically involved in observational conditioning because of its social component, but rather might be involved because it is a weaker form of conditioning. It is true that animals in classical fear conditioning demonstrate higher overall levels of freezing than animals in observational conditioning. This suggests that it is indeed a weaker form of

conditioning. We do not directly test whether the ACC is also necessary for weaker forms of classical fear conditioning, so this is a possibility. One study demonstrated that lesions in the rostral ACC of the rat leads to deficits in classical fear conditioning, however if animals are exposed to 12 trials as opposed to 6 they are still able to learn (Bissiere et al. 2008). This suggests that although the ACC may be involved in weaker forms of classical fear conditioning, it is not necessary for classical fear conditioning. In our paradigm mice received 30 trials and were still unable to learn without ACC input to the amygdala. This suggests that in contrast to classical fear conditioning, observational conditioning cannot occur without the ACC.

The question as to where neural circuits involved in social cognition diverge from neural circuits involved in other non-social forms of cognition is ongoing. The answer to this question is critical for defining the "social brain". Here we provide some evidence that ACC input to the BLA may be specific for utilizing social information to drive behaviors important for learning and social interaction. However it is clear the ACC is also involved in non-social behaviors. Defining which inputs and outputs from the ACC are specific for social information will allow us to further define the social brain and give us clues as to which circuits may be important for correcting aberrant social behaviors.

Understanding deficits in social behavior in autistic mouse models

In Chapter 1 I outlined the translational motivation for this project. Here, I want to suggest one way in which the work accomplished for this dissertation can be utilized to begin understanding social deficits seen in psychiatric disease. It is clear that there are

genetic strains of mice that have deficits in social interaction and are unable to learn through observation, while other strains can undergo observational learning while still having profound deficits in social interaction (Keum et al. 2016). This suggests that different genetic manipulations can alter social interaction and observational learning in a dissociable way. We provided evidence that ACC input to the BLA is critical for observational fear learning as well as social interaction in wildtype C57/Bl6 mice However, it is unknown how this circuit functions in animals with social interaction deficits, such as those seen in mouse models of autism (Crawley 2007; Silverman et al. 2010; Peça et al. 2011). In addition, it continues to be a point of contention whether or not autistic patients have the ability to learn through observation[4] with some studies showing deficits, while others show a conserved ability (Varni et al. 1979; Egel, Richman, and Koegel 1981; Ledford et al. 2008; Leaf et al. 2012; Nadel et al. 2011; Taylor and DeQuinzio 2012).

Whether or not autistic patients have deficits in observational learning has implications for where we believe the deficit in social cognition lies. An inability to learn through observation implies that deficits are at the most fundamental level of social cognition whereas a conserved ability for observational learning implies that basic forms of social cognition are intact and that the deficit arises due to higher order social cognition. This would suggest that basic mechanisms of social processing could potentially be harnessed for therapeutic benefit. If this is true, then targeted

[4] As autism is currently defined as a spectrum of disorders, it is possible that along the spectrum there is variation in observational learning ability. Thus higher functioning patients with autism may learn through observation, while patients with more severe cases may not be able to. Genetic analyses of these different populations may reveal key genetic determinants underlying variation in social cognition.

pharmacological intervention or stimulation of the regions involved in social processing could potentially rescue some of the social deficits seen in autism and other psychiatric disorders. In light of the results shown here, this is an intriguing hypothesis, given the aberrant ACC activity that has been found in autistic patients (Thakkar et al. 2008; Dichter, Felder, and Bodfish 2009).

Our characterization of the ACC-BLA circuit provides a basis that we can use to test whether or not this circuit functions aberrantly in mouse models of autism that are unable to learn through observation while being conserved in mouse models of autism that can learn through observation. This provides unique opportunities to ask about how various genetic manipulations known to be involved in autism in humans affects circuit function in this critical node for both observational learning as well as social interaction. If it is true that mouse models that are unable to learn through observation have deficits in this circuit, we can use the information we have provided in this dissertation to directly test whether enhancing or restoring proper function using optogenetic or pharmacogenetic tools can rescue observational learning and social interaction deficits. These experimental lines of questioning have the potential to provide unique and powerful insights about how genetic mutations may alter circuit function and where those deficits in circuit function arise.

Back to the clinic: optogenetics as a tool for discovery and healing

I have always considered how the tools that I use to understand the neural basis of social behavior in rodent models might be directly used to improve the lives of

patients with psychiatric disorders. It has become increasingly clear, that the current approach to treating psychiatric disease is one that must move towards a more biologically based methodology (Hyman 2007; Craddock and Owen 2010; Kupfer et al. 2011). Diseases are currently diagnosed and treated based on constellations of symptoms that often overlap diagnostic boundaries. This system is a remnant from a time when there was not a sufficient biological understanding of the brain that could inform a mechanistic basis for diagnosis and treatment (Zigler and Phillips 1961). As research efforts have improved, the neuroscience community has made strides towards the goal of being able to understand the pathogenesis of mental illness. What we have learned increasingly suggests that mental illnesses are not as segregated as might be suggested by current standards (Dichter, Damiano, and Allen 2012; Cross-Disorder Group of the Psychiatric Genomics Consortium 2013). Various animal studies have demonstrated how clinical and human data can be combined with the power of optogenetic approaches to elucidate the circuitry that govern the behaviors seen in psychiatric diseases such as depression (Tye et al. 2012; Ramirez et al. 2015), anxiety (Tye et al. 2011; Ada C. Felix-Ortiz et al. 2013), and ADHD (Wells et al. 2016) and how this information can then be used to provide further direction for clinical and human research.

Indeed, using optogenetics in rodents has provided marvelous insights into neural circuitry that has been useful from both a basic as well as translational perspective. However, this is likely only the beginning of a path towards an eventual more direct use of optogenetics in humans. Researchers have already had success

demonstrating both the safety and ability of optogenetics to perturb neuronal activity in rhesus and macaque monkeys (Han et al. 2009; Han 2012). The behavioral effect of stimulation in monkeys is currently much more limited than that seen in rodents (Diester et al. 2011). However, groups of researchers have been able to create a deficit in saccadic eye movements through optogenetic inhibition of superior colliculus cells (Cavanaugh et al. 2012), induce changes in saccade latencies through optogenetic stimulation of the arcuate sulcus (Gerits et al. 2012), and induce saccades themselves through activation of visual cortex (Jazayeri, Lindbloom–Brown, and Horwitz 2012). These studies are a clear demonstration of the potential to affect non-human primate behavior through optogenetics. This combined with emerging technologies for creating transgenic non-human primates provides attractive possibilities for future modeling and optogenetic study of psychiatric disease in non-human primates (Yang et al. 2008; Sasaki et al. 2009).

Lastly, there is the potential for optogenetics to someday play a directly therapeutic role in humans. It has already been shown that the adeno-associated virus vector that is commonly used to deliver opsin genes for optogenetics is safe and tolerable for use in humans (Kaplitt et al. 2007; LeWitt et al. 2011). It has also been shown ex vivo that human retinas with light insensitive photoreceptors can be re-activated through the use of halorhodopsin and that in mouse models of retinitis pigmentosa this same strategy can be used to restore phototransduction and resulting retinal circuit function (Busskamp et al. 2010). This has produced optimism about the ability of optogenetics to be used as a therapeutic tool in retinitits pigmentosa

(Busskamp et al. 2012). If optogenetics can find its way into human studies as a therapeutic medium, the framework for its application would already exist. The concept of using stimulation to affect changes in the behavior of patients has been practiced through the use of Deep Brain Stimulation (DBS). Various studies have shown the ability of DBS to treat Parkinsons, Depression, OCD, Tourette's, and Anxiety (Mayberg et al. 2005; Hamani and Temel 2012). Optogenetics could replace DBS as a more sophisticated approach to using stimulation of brain regions to affect behavioral and affective change. Whether this ultimate realization of the capability of optogenetics occurs or not, it is clear that it represents a great technological advancement for neuroscientists. It has lead to a more thorough and precise understanding of how basic neural mechanisms underlie complex behavior. Continued emphasis on translational and reverse translational approaches will continue to leverage this powerful tool to provide the kind of insights that will lead to meaningful changes in the way we approach, diagnose, and treat psychiatric disease.

As an MD/PhD candidate, my career goal is to use the tools of basic neuroscience to explore mechanisms of psychiatric disease in a manner that will lead to better therapeutic interventions for patients. As a future physician scientist, I plan to run a research program that answers cutting-edge questions about neuropsychiatric disorders informed by the clinical care of psychiatric patients, in order to facilitate the delivery of progressive care to psychiatric patients. I also hope to take the lessons we learn from the lab and clinic and find ways to apply them on a policy level to improve the overall mental health of disadvantaged communities. Social justice and service to the

community are also priorities. As an undergraduate, medical, and graduate student, I actively planned and participated in events aimed at education, diversity in science, and health disparities. As a scientist, I plan to continue engaging in these important issues as well. This dissertation is a first step towards achieving these goals. Here, we utilize cutting edge basic science tools in order to understand a neural circuit that is necessary for a fundamental social behavior. This will hopefully inspire future work aimed at delineating how circuits underlie social behaviors, how those circuits malfunction in psychiatric disease, and how we can utilize that information to provide better and more effective treatments for patients.

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