THE NR2B SUBUNIT AND DIFFERENTIAL REARING: THE ROLE OF THE AMYGDALA AND HIPPOCAMPUS IN THE ACQUISITION OF PAVLOVIAN CONDITIONED FEAR

by

EMILY K. REINHARDT

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Approved by:

Major Professor
Dr. Mary Cain

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Abstract

Research has demonstrated that an enriched rearing environment improves learning in many tasks. However, growing evidence suggests that an enriched environment may not provide the same benefits during a fear conditioning paradigm. In fact, it appears that an isolated rearing environment may facilitate acquisition of fear to an aversive stimulus. The neural mechanisms responsible for this disparity in fear learning among differentially reared animals are currently unknown. The NR2B subunit of the NMDA receptor has been shown to be involved in the acquisition of fear and influenced by differential rearing, making it a prime candidate to begin investigating these underlying neural mechanisms. Therefore, this study assessed the expression of the NR2B subunit in brain regions important for the acquisition of fear (amygdala and hippocampus) among differentially reared rats. Rats were reared in an enriched, an isolated, or a standard condition for 30 days. They received four tone-footshock pairings, after which their brains were removed and expression of the NR2B subunit was quantified in the basolateral amygdala (BLA), central nucleus of the amygdala (ACe), and the CA3 region of the hippocampus. Analyses found that the isolated rats began to acquire fear to the aversive stimulus faster than the enriched and standard housed rats. However, the isolated rats showed the least amount of NR2B expression in the BLA while there were no rearing differences in expression within the ACe or the CA3. The results from this study provide further insight to the importance of the rearing environment in learning and memory, especially the learning of fear, and its central neural basis.

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Chapter 1 - Introduction

Anxiety is a growing concern in our society today. Millions of people living in the United States are affected by anxiety disorders while billions of dollars are spent each year to cover their costs (Greenberg et al., 1999). Due to its severe impact on lives, a vast amount of research has been devoted to understanding anxiety (and fear) in an attempt to find better ways to help individuals combat and cope with anxiety (Legerstee, Garnefski, Jellesma, Verhulst, & Utens, 2010; Mällo et al., 2007; Ströhle, 2009). However, an interesting aspect of fear and anxiety is that people appear to learn about and cope with fear and anxiety in different ways. These differences may begin to explain why some individuals struggle with anxiety, depression, phobias, post-traumatic stress disorder, etc., and why others do not (Meaney, 2001; Weinberger, Schwartz, & Davidson, 1979). As such, research has been done to explicate the factors that contribute to these differences. One of these factors appears to be the rearing environment. Specifically, the environment is a strong influence that moderates neurological and behavioral changes in individuals. Studying these changes in humans introduces procedural issues, potential confounding variables, and ethical restrictions; therefore an animal model is frequently used to study the relationship between the environment and the differences associated with learning about and coping with fear and anxiety.

Differential Rearing

One way to study the effect of the environment on neurological and behavioral changes in animals, such as rats, is to raise them in different environments. A frequently used differential rearing paradigm is based on early work conducted by Renner and Rosenzweig (1987), which includes the use of an enriched condition (EC) and an isolated condition (IC). Rats raised in the

enriched environment are housed with other rats in a large cage that includes novel objects that are changed daily and the rats receive daily handling during the rearing period. Rats raised in the isolated environment are singly housed in hanging wire cages where they do not have access to novel stimuli or to other rats and they are not handled during the rearing period. In addition to these two rearing environments, a standard rearing environment is often used for comparison. This standard condition (SC) typically includes the housing of two rats together in plastic shoe-box style cages where they have no access to novel stimuli and are only handled during weekly bedding changes. This standard rearing environment is often included in the rearing paradigm to act not as a control for the enriched and isolated conditions but rather as a laboratory standard, as this environment is the suggested housing condition as defined by the National Institute of Health (National Research Council, 2011). This differential rearing paradigm, including the use of the standard environment, was employed in the current study to better understand the relationship between the rearing environment and rats' abilities to learn about aversive stimuli.

Learning and Memory

There has been extensive research investigating the role of the rearing environment in learning and memory (Fone & Porkess, 2008; Renner & Rosenzweig, 1987; Simpson & Kelly, 2011). Wood and Rebec (2009) found that rats reared in enriched conditions learned a discriminative learning task faster than rats reared in an isolated condition. During operant tasks, Gill and Cain (2011) showed that enriched rats learned faster than isolated or socially housed rats when a light cue was present. Additionally, it appears that an enriched environment facilitates performance in spatial memory tasks (Frick & Fernandez, 2003; Harati et al., 2012; Van Waas & Soffié, 1996). Woodcock and Richardson (2000) found that enriched rats spent less time freezing in a context that had not previously been associated with a fearful stimulus compared to

standard housed rats, while Duffy, Craddock, Abel, and Nguyen (2001) observed that enriched mice froze more when returned to a fearful context compared to control mice. Similarly, Barbelivien et al. (2006) showed that enriched rats discriminated between a fearful conditioning context and a neutral context while standard housed rats did not. However, standard housed rats appeared to freeze more to a tone that had previously been paired with a footshock, compared to enriched rats (Barbelivien et al., 2006). These findings suggest that an enriched rearing environment improves learning and memory compared to other rearing conditions. They also indicate that contextual cues mediate the improvements associated with enrichment whereas tone cues do not.

Interestingly, there is additional evidence suggesting that the enhancements associated with enrichment may be task-dependent. Specifically, while some research indicates that enrichment improves learning about and remembering appetitive tasks (Wood & Rebec, 2009; Wood, Siegel, & Rebec, 2006), these same enrichment benefits may not be seen when learning about an aversive task, especially when compared to an isolated rearing environment. Walasek, Wesierska, and Werka (2002) examined the conditioned suppression of bar pressing during a fear conditioning paradigm. They showed that individually housed rats suppressed their bar pressing during the presentation of a conditioned auditory stimulus (CS) paired with a mild footshock. Enriched rats, however, demonstrated impairments in their ability to discriminate their fear; they showed suppression before, during, and after the presentation of the CS. Freeman and Ray (1972) observed that rats raised in an isolated environment learned a passive avoidance response to an aversive stimulus faster than rats in an enriched environment. Similarly, Reinhardt and Cain (2015b) found that isolated rats acquired fear to an aversive stimulus at a faster rate than enriched rats. It is worth noting, however, that the isolated environment used by

Freeman and Ray (1972) resembles the standard condition that is proposed for the current study. In fact, much of the learning and memory research that studies the effects of the rearing environment on an aversive task fails to use a true isolated environment, as discussed by Renner and Rosenzweig (1987). Therefore the use of a true isolated environment is an important and necessary contribution to the aversive learning and memory research. Overall, these findings provide evidence that the rearing environment has an important impact on learning and memory. More specifically, it appears that while environmental enrichment enhances appetitive learning, an isolated environment may enhance aversive learning.

The Role of the NMDA Receptor in Learning and Memory

A major contributor to the neurological mechanisms of learning and memory is the excitatory neurotransmitter glutamate (Maren, 2005; Walker & Davis, 2002). There are several glutamatergic receptor types (e.g., mGluR, AMPA) that have been implicated in learning and memory (Riedel, 1996; Walker & Davis, 2002), as well as in long-term potentiation (Levenson et al., 2002; Malinow & Malenka, 2001; Nicoll & Roche, 2013; Shepherd & Huganir, 2007). The N-methyl-D-aspartate (NMDA) receptor has been shown to be important for learning and memory (Cain, 1997; Collingridge et al., 2013), particularly for the acquisition of fear memory. Goosens and Maren (2004) determined that administration of the competitive NMDA antagonist ±-3-(2-carboxypiperazin-4-yl) propyl-1-phosphonic acid (CPP) impaired the acquisition of freezing during an auditory fear conditioning stimulus while Fanselow and Kim (1994) determined that the administration of the NMDA receptor antagonist D,L-2-amino-5-phosphonovaleric acid (APV) attenuated the acquisition of Pavlovian conditioned fear to contextual cues. Campeau, Miserendino, and Davis (1992) also found that the use of the competitive NMDA antagonist DL-2-amino-5-phosphonopentanoic acid (AP5) blocked the

acquisition of fear-potentiated startle. Similarly, antagonism of the NMDA receptor with the non-competitive antagonist MK-801 prevented the suppression of rats' lever pressing for food pellets when presented with a tone paired with a mild footshock (Hoehn-Saric, McLeod, & Glowa, 1991). In general, these results suggest that the inhibition of the NMDA receptor leads to impairments in the acquisition of various forms of fear.

While antagonism of the NMDA receptor seems to impair learning, enhancement of the receptor appears to facilitate learning and memory. The NMDA receptor partial agonist GLYX-13 enhanced learning in rats during trace eye blink conditioning, the Morris water maze, and the T-maze (Burgdorf et al., 2011). Ren et al. (2013) found that administration of the partial NMDA agonist d-cycloserine (DCS) directly into the hippocampus facilitated extinction learning of a tone that had previously been paired with a footshock, while Walker, Ressler, Lu, and Davis (2002) demonstrated that the infusion of DCS into the BLA enhanced the extinction of fear-potentiated startle in rats. This research indicates that while inhibition of the NMDA receptor impedes the acquisition of fear learning, the use of an NMDA agonist to enhance the functioning of the receptor improves learning, including the acquisition and extinction of fear.

NMDA and Differential Rearing

It is clear that both the NMDA receptor and the rearing environment are influential in learning and memory. It should then be no surprise that both enriched and isolated rearing environments have different effects on the NMDA receptor, especially in learning and memory tasks. Duffy et al. (2001) demonstrated more robust LTP in hippocampal tissue from environmentally enriched mice compared to control mice. They also showed significant deficits in LTP when APV (an NMDA receptor antagonist) was introduced into the tissue, suggesting that the hippocampal LTP was dependent upon NMDA receptors. However, Whitaker,

Degoulet, and Morikawa (2013) found that synaptic plasticity of NMDA receptors in the ventral tegmental area (VTA) was improved in socially isolated rats, compared to group housed rats. The researchers hypothesized that due to their reduced activation in a socially isolated environment and the involvement of NMDA receptors in dopamine activity (Overton & Clark, 1997; L. P. Wang et al., 2011), dopaminergic neurons compensated by increasing their ability to undergo synaptic strengthening and plasticity via the enhancement of NMDA receptors. These studies demonstrate that the rearing environment, both enriched and isolated, has an impact on the NMDA receptor and its plasticity in the brain.

Additionally, some of the differential rearing effects are specific to individual subunits of the NMDA receptor. The NMDA receptor is composed of many subunits which are thought to belong to three families: the NR1, NR2, and NR3. Interestingly, it appears while NR1 subunits are generally expressed throughout the adult brain, the NR2 subunits (NR2A-D) show regional specificity in their distribution patterns (Monyer, Burnashev, Laurie, Sakmann, & Seeburg, 1994; Wenzel, Fritschy, Mohler, & Benke, 1997). In the adult rat brain, the NR2A and NR2B subunits show their greatest expression in the cerebral cortex, the hippocampus, and the olfactory bulb, the NR2C subunit predominates in the cerebellum, and the NR2D subunit, while present during post-natal development, is virtually non-existent in adulthood (Monyer et al., 1994; Wenzel et al., 1997). This regional specificity may explain why the four subunits in the NR2 family play a major role in determining the functionality of the NMDA receptor (Gonda, 2012). As a result of this subunit specificity, the NR2 subunit has become a prime candidate for investigating its relationship with the rearing environment, especially among the NR2A and NR2B subunits. It appears that environmental enrichment upregulates the NR2A subunit mRNA in the CA2, CA3, CA1, and the dentate gyrus (DG) regions of the hippocampus (Andin, Hallbeck, Mohammed, &

Marcusson, 2007). Similarly, Bredy, Zhang, Grant, Diorio, and Meaney (2004) found that an enriched rearing environment could reverse the damaging effects of reduced maternal care in the hippocampus, as rats raised in an enriched environment had increased mRNA expression of the NR2A and NR2B subunits of the NMDA receptor when compared to rats raised in a standard environment. However, Turnock-Jones et al. (2009) found enhanced expression of NR2A subunit mRNA in the mPFC of rats reared in an isolated environment compared to rats reared in a social condition. These findings suggest that the effects of the rearing environment on the NMDA receptor, especially the NR2 subunits, may be unique to individual brain regions.

Differentially reared animals also show behavioral changes associated with the NMDA receptor. Rampon et al. (2000) selectively deleted the NR1 subunit of the NMDA receptor in the CA1 region of the hippocampus of mice (CA1-KO mice). They found that by knocking out this subunit, the mice showed impaired freezing in a context in which they had previously received footshock presentations, compared to control mice. These hippocampal-dependent impairments were reversed in CA1-KO mice that had been exposed daily to an enriched environment. Similarly, Y.-P. Tang, Wang, Feng, Kyin, and Tsien (2001) demonstrated that transgenic (Tg) mice that overexpressed the NR2B subunit of the NMDA receptor showed not only improved hippocampal LTP compared to wild-type mice, but also enhanced learning and memory in a fear conditioning paradigm. More specifically, wild type mice that received exposure to an enriched environment and non-enriched Tg mice showed similar improvements in their contextual fear conditioning, cued fear conditioning, and their extinction of fear, compared to non-enriched wild type mice. The researchers suggest that these findings provide evidence that environmental enrichment and the genetic modification of the NMDA receptor have overlapping mechanism(s), such that exposure to environmental enrichment is just as beneficial as genetically enhancing the

NR2B subunit (Y.-P. Tang et al., 2001). In summary, it appears that both enriched and isolated environments influence the NMDA receptor and, in turn, the learning and memory of a fearful stimulus.

Fear Learning and the NR2B Subunit

Much of the early work investigating NMDA receptors did not attempt to disentangle functional differences in the acquisition of fear from the expression of fear. However, more recent research has been attempting to localize these differences and it appears that the NR2B subunit of the NMDA receptor is particularly involved in fear learning (Bermudo-Soriano, Perez-Rodriguez, Vaquero-Lorenzo, & Baca-Garcia, 2012; Parkes & Westbrook, 2011). As previously discussed, Ya-Ping Tang et al. (1999) demonstrated that the overexpression of the NR2B subunit facilitated the enhanced retrieval of contextual and cued fear conditioning. To further disentangle differences in acquisition and expression of fear, Rodrigues, Schafe, and LeDoux (2001) gave systemic injections, as well as infusions into the lateral nucleus of the amygdala (LA), of the selective NR2B antagonist ifenprodil. They found that, in both forms of administration, ifenprodil dose-dependently impaired the acquisition of conditioned fear while preserving the expression of previously learned fear. Additionally, Walker and Davis (2008) showed that the infusion of the NR2B selective antagonist CP101,606 into the ACe and the BLA disrupted the acquisition of fear-potentiated startle but did not impair the expression of fear. These results suggest that the NR2B subunit is necessary for the acquisition of fear.

Though NR2B appears to be important for fear learning, less is known about the role of NR2B among differentially reared animals. E. H. Y. Lee, Hsu, Ma, Lee, and Chao (2003) found that the infusion of ifenprodil (an NR2B antagonist) into the CA1 region of the hippocampus prevented the improvements in spatial learning via the Morris water maze among rats exposed to

an enriched environment. To explore further the role of the NR2B subunit in differentially reared animals and their involvement in fear learning, A pilot study was conducted and showed that peripheral administration of 1mg/kg ifenprodil facilitated the acquisition of fear to a tone paired with a footshock in enriched and isolated rats but not in standard housed rats while 3mg/kg ifenprodil had no effect on fear acquisition, regardless of rearing environment (Reinhardt & Cain, 2015a). Isolated rats suppressed their lever pressing behavior faster than enriched rats, indicating that isolated rats learned about the aversive stimulus faster than enriched rats. Interestingly, standard housed rats acquired fear to the stimulus faster than both the enriched and isolated rats which may explain why ifenprodil had no effect on the standard rats' behavior (Reinhardt & Cain, 2015a). Additionally, Zhao et al. (2009) demonstrated that rats raised in social isolation showed significantly more upregulation of NR2B mRNA in the hippocampus than rats raised in social groups. While these socially housed rats did not have access to novel stimuli, they were raised in larger groups with four rats per group. Given the implications of the NR2B subunit in fear learning, this finding provides additional evidence that rats individually housed are better equipped to learn about a fearful stimulus than rats housed in larger social groups. However, it is evident that more research is still needed to understand better the effect of the rearing environment on the NR2B subunit, especially in the learning of fear.

The Role of the Amygdala in Fear Learning

It is clear that animals exposed to different environments display behavioral differences in learning and memory, especially fear learning, as well as neurological differences associated with the NMDA receptors. Specifically, there is preliminary evidence to suggest that the NR2B subunit may be associated with the environmental differences associated with fear learning (Reinhardt & Cain, 2015b). Therefore, it is likely that these NR2B differences are found in

regions of the brain that are involved in the learning and memory of fear, such as the amygdala and the hippocampus (J. J. Kim & M. W. Jung, 2006; Maren, 2001).

The amygdala is a nucleus found in the limbic system that has been extensively studied for its role in emotional states, including fear (Paré, Quirk, & LeDoux, 2004; Walker & Davis, 2002). In an early study, Blanchard and Blanchard (1972) demonstrated that lesioning of the amygdala induced impairments in the freezing to and avoidance of fearful stimuli while Kim, Rison, and Fanselow (1993) found that amygdaloid lesions disrupted the acquisition of freezing to a footshock. Similarly, Vazdarjanova and McGaugh (1999) inactivated the BLA in rats with a reversible sodium channel blocker (lidocaine) following the presentation of unsignaled footshocks. Twenty-four hours later, the rats were returned to the context they originally received the footshocks but no shocks were presented. They found that the rats displayed significantly lower levels of freezing compared to control rats that did not receive lidocaine infusions. Interestingly, they also activated the BLA with the infusion of oxotremorine, a cholinergic muscarinic agonist, following the presentation of unsignaled footshocks (Vazdarjanova & McGaugh, 1999). When rats were returned to a context where they had originally received unpaired presentations of a footshock, they spent more time freezing than control-infusion rats. Together, these findings demonstrate that damage to or inactivation of the amygdala attenuates fear responses toward aversive stimuli while activation facilitates these responses.

The Basolateral Amygdala (BLA)

It has been well established that the amygdala receives sensory inputs from many regions in the brain (e.g., thalamus, hippocampus, olfactory cortex). These regions project sensory information to both the lateral and basal nuclei of the amygdala, which together make up the

BLA (Jeansok J. Kim & Min Whan Jung, 2006; LeDoux & Schiller, 2009; Maren, 2001). The association between a conditioned stimulus and an unconditioned stimulus in a fear conditioning paradigm appears to be established and stored within the BLA (LeDoux & Schiller, 2009; Maren, 2001). As such, the BLA is crucial for the acquisition of fear. BLA lesions prior to fear-potentiated startle training decreased the startle response to both auditory and visual conditioned stimuli (Campeau & Davis, 1995), indicating that the learning of the fearful stimulus was attenuated. Meanwhile, Muller, Corodimas, Fridel, and LeDoux (1997) showed that temporary inactivation of the lateral and basal nuclei of the amygdala with muscimol (a GABAa agonist) impaired the acquisition of freezing to a tone that was paired with a footshock.

Another important aspect of the BLA is the large number of glutamatergic inputs into the BLA (LeDoux & Schiller, 2009). This provides further evidence for the importance of the amygdala in fear learning, as the relationship between fear learning and the glutamatergic NMDA receptor has been extensively discussed above. In particular, the NR2B subunit has been investigated in the BLA for its role in the acquisition of fear. Rodrigues et al. (2001) showed that the infusion of ifenprodil (an NR2B antagonist) into the LA in rats prior to the presentation of tone-footshock pairings decreased the expression of fear (compared to control rats) in response to the tone as well as the conditioning context. However, when ifenprodil infusions occurred after the conditioning sessions but prior to the tone-only and context-only tests, freezing was not impaired. These findings suggest that LA infusions of ifenprodil impair the acquisition of conditioned fear but do not affect the expression of a previously learned fear. More recently, Walker and Davis (2008) found similar results with a fear-potentiated startle paradigm; infusion of CP101,606 (an NR2B antagonist) into the BLA prior to the pairing of a light with a footshock impaired the fear-potentiated startle in rats. However, when the infusions occurred following the

initial light-shock training, the expression of fear-potentiated startle was not attenuated. While there is evidence that NR2B subunits in the BLA are important for the acquisition of fear, less is known about this relationship among differentially reared animals.

The Central Nucleus of the Amygdala (ACe)

While the BLA has been largely implicated in the acquisition of fear, the ACe has been identified as an output region of the amygdala (LeDoux & Schiller, 2009; Maren, 2001; Paré et al., 2004). Specifically, it is the output region for the expression of the conditioned fear response, such as freezing, startle, increased heart rate, blood pressure, respiration, and glucocorticoid release. The ACe projects to various brain areas that are necessary for these responses to occur (e.g., hypothalamus, periaqueductal gray, medulla, bed nucleus of the stria terminalis). Historically, the BLA has been considered the site for synaptic plasticity in fear conditioning due to its role in CS-US inputs and associations (Paré et al., 2004) while the ACe has been viewed simply as a passive relay of fear response outputs and not as vital in the acquisition of fear (LeDoux, 2000). Fanselow and Kim (1994) demonstrated that the infusion of APV (an NMDA receptor antagonist) into the ACe of rats prior to the presentation of footshocks did not impair their levels of freezing when returned to that same context for a shock-free contextual test. This finding suggests that the ACe is more involved in the expression of the fear response than the acquisition of fear.

However, there is emerging evidence that suggests the ACe does indeed play a more predominant role in the acquisition of fear than had previously been thought (Paré et al., 2004). Goosens and Maren (2003) showed that the infusion of APV, an NMDA antagonist, into the ACe prior to auditory fear conditioning significantly impaired freezing during the acquisition session compared to control rats that did not receive APV. Similarly, Wilensky, Schafe,

Kristensen, and LeDoux (2006) found that the inactivation of the ACe with infusions of muscimol prior to presentations of a tone paired with a footshock significantly attenuated their levels of freezing during the acquisition phase. Ciocchi et al. (2010) also demonstrated that the ACe is involved in the acquisition of fear by inactivating the ACe with infusions of the GABAA receptor agonist, muscimol-bodipy (BPY), prior to the presentation of an auditory stimulus paired with a mild footshock. These findings suggest that the inactivation of the ACe attenuated the acquisition of fear to the aversive stimulus. While it appears that the ACe is also involved in the acquisition of fear, less is known about the role of the ACe in the fear learning differences among differentially reared animals. Additionally, there is a lack of research investigating the NR2B subunit in the ACe and its involvement in the acquisition differences among animals raised in different environments.

The Role of the Hippocampus in Fear Learning

Similar to the amygdala, the hippocampus is a structure in the limbic system that has been largely implicated in fear conditioning (Maren, 2001; Sanders, Wiltgen, & Fanselow, 2003). The hippocampus, however, has been heavily studied for its importance in memory formation and storage, including contextual and spatial fear memories (Tsien, Huerta, & Tonegawa, 1996; Zhang et al., 2008). Kim and Fanselow (1992) demonstrated that lesions to the hippocampus following the learning of a tone-footshock association significantly impaired freezing levels in rats that were returned to the conditioning context for a context-only retention test. Interestingly, they also found that the more time that occurred from the conclusion of the conditioning sessions and the lesioning of the hippocampus, the higher the levels of freezing which suggests that more of the contextual memory was retained. The hippocampus has also been shown to be involved in the acquisition of fear. Phillips and LeDoux (1992) found that

lesions to the hippocampus prior to the presentation of tone-footshock pairings impeded the acquisition of freezing to the context (prior to the onset of the CS) but did not affect the acquisition of freezing to the cue (during the CS), compared to non-lesion control rats. This finding suggests that lesions to the hippocampus impaired the acquisition of contextual fear.

The CA3 Region of the Hippocampus

One specific region in the hippocampus that has been identified as being involved in the acquisition of fear is the CA3 region. The CA3 region is proposed to have the capacity to receive hippocampal inputs, make rapid associations among the inputs, and then store the associations for a short period of time (McClelland & Goddard, 1996; Treves & Rolls, 1994; Wiebe, Stäubli, & Ambros-Ingerson, 1997). Therefore, the CA3 is an ideal location for the acquisition of conditioned fear to be studied. I. Lee and Kesner (2004) demonstrated that neurotoxic lesions to the CA3 region impaired the acquisition of freezing when presented with tone-footshock pairings; specifically, freezing differences were found early in the acquisition phase. Hunsaker and Kesner (2008) obtained similar results when they lesioned the dorsal portion of the CA3. Rats with dorsal CA3 lesions showed initial impairments in fear acquisition to tone-footshock pairings while rats with ventral CA3 lesions did not show acquisition impairments. Additionally, McHugh and Tonegawa (2009) found that CA3-NR1 KO mice (genetically lacking the NR1 subunit of the NMDA receptor in the CA3 region of the hippocampus) showed deficits in their acquisition of conditioned fear to a tone that co-terminated with a mild footshock. These findings all illustrate the role that the CA3 region of the hippocampus has in the acquisition of conditioned fear.

There is research that suggests the rearing environment has differential effects on the CA3 region of the hippocampus. As previously discussed, Andin et al. (2007) found that

environmentally enriched rats displayed increased levels of NR2A mRNA in the CA3 region compared to standard housed rats while Segovia, Yagüe, García-Verdugo, and Mora (2006) demonstrated that enriched, aged rats had increased basal levels of glutamate in the CA3 compared to isolated, aged rats as well as both enriched and isolated young rats. Interestingly, Leger et al. (2012) demonstrated that environmental enrichment enhanced recent aversive memories in a passive avoidance task compared to a standard environment. Additionally, while they found greater brain metabolic changes in the frontal, motor, and prelimbic cortices in enriched mice compared to standard mice, standard mice showed greater metabolic activity in the basolateral amygdala and hippocampus, including the CA3 region. These findings illustrate that the rearing environment does have an effect on the CA3 region yet more work needs to be done to disentangle what these effects are for each of the rearing environments. Additionally, less is known about the relationship between differential rearing and the NR2B subunit in the CA3. In fact, to our knowledge, there has been no prior research that has examined the role of the rearing environment on the NR2B subunit in the CA3, especially using a fear conditioning paradigm.

Hypotheses for the Current Study

The current study investigated the relationship between the rearing environment and the NR2B subunit in an attempt to understand the fear learning differences among differentially reared animals. Therefore, differentially reared rats underwent Pavlovian fear conditioning to examine their fear learning of an aversive stimulus. Their expression of the NR2B subunit was then quantified in the BLA, the ACe, and the CA3 regions. Based on the literature and previous work in our lab, it was hypothesized that rats reared in isolated and standard environments would acquire fear to the aversive stimulus faster than rats reared in an enriched environment.

Additionally, isolated and standard housed rats were predicted to show more NR2B subunit expression compared to enriched rats in the BLA, given its importance in the acquisition of fear. Similarly, if the ACe is a site of plasticity in fear conditioning and plays a role in the acquisition of fear, then isolated and standard housed rats would express more NR2B compared to enriched rats.

Finally, it was hypothesized that there would be greater expression of NR2B in the CA3 region of the hippocampus in isolated and standard housed rats compared to enriched rats, as the CA3 has been shown to be involved in the acquisition of conditioned fear. While Zhao et al. (2009) showed greater hippocampal NR2B mRNA upregulation in isolated rats compared to socially housed rats, it is important to recognize that their socially housed rats were housed in larger groups (four rats per group) than the proposed standard condition (two rats per group). Therefore, I expected to find similar results such that isolated (and standard housed rats) would show greater expression of the NR2B subunit than the enriched rats, which were reared in larger groups. An alternative hypothesis for the proposed study was that enriched rats would show greater NR2B expression in the CA3 compared to isolated or standard housed rats. Andin et al. (2007) found greater levels of NR2A mRNA in the CA3 of environmentally enriched rats compared to standard rats. While there is some evidence to suggest that the NR2A and NR2B subunits may have unique roles in postnatal development due to differences in expression (Laurie, Bartke, Schoepfer, Naujoks, & Seeburg, 1997; Monyer et al., 1994; Wenzel et al., 1997), there is conflicting evidence that indicates that this uniqueness is maintained into adulthood (Bannerman et al., 2008; Li et al., 2009; von Engelhardt et al., 2008). For example, there is some evidence to suggest that the NR2A subunit is necessary for LTP (Dalton, Wu, Wang, Floresco, & Phillips, 2012) and the NR2B subunit is vital for long-term depression (LTD;

Duffy, Labrie, & Roder, 2008), while other research indicates that such distinction and selectivity does not exist (Berberich et al., 2005). If the NR2A and NR2B subunits do in fact share similar roles and levels of expression in adulthood, then it was predicted that we would see the upregulation of the NR2B subunit in the CA3 of environmentally enriched rats compared to standard housed rats, similar to the upregulation of NR2A mRNA found by Andin et al. (2007).

Chapter 2 - Methods

Animals

Male Sprague Dawley rats (Charles River, Portage, MI, USA) arrived in the laboratory at 21 days of age and were randomly assigned to one of three environmental rearing conditions (EC, IC, or SC). Rats had ad libitium access to food and water. The colony room was maintained at 22° C, humidity ranged from 30-70%, and the light cycle was kept on a 12-hr light/dark cycle (lights on from 0700 to 1900 hours). All procedures were approved by the Institutional Animal Care and Use Committee at Kansas State University and were in compliance with National Research Council guidelines (National Research Council, 2011).

Environmental Conditions

Twenty-one day old rats arrived in the lab and were randomly housed in EC, IC, or SC groups. They reared in these conditions for 30 days prior to testing. EC rats (n=18) were housed with other rats in a large metal cage (60 cm × 120 cm × 45 cm) lined with sandy chip bedding. Fourteen novel stimuli (children's toys and PVC pipe) were placed in the cage; seven of these objects were replaced and rearranged daily. All fourteen objects were replaced twice weekly with new stimuli. EC rats also received daily handling during the rearing period. IC (n=18) rats were housed individually in hanging metal cages (17 cm × 24 cm × 20 cm) with solid sides, backs, and tops and wire mesh floors and front panels. IC rats had no access to novel stimuli and were not handled during the rearing period. SC rats (n=18) were housed in pairs in standard plastic shoebox cages (20 × 43 × 20 cm) lined with sandy chip bedding. SC rats were handled during weekly cage changes.

Apparatus

Standard two-lever operant conditioning chambers (30.5 cm × 24.1 cm × 21 cm; Model ENV-007; Med Associates Inc., St. Albans, VT, USA) enclosed in sound attenuating compartments were used. The front panel of each chamber included two levers (12.5 cm from floor) and a magazine (3 cm × 4 cm × 4 cm; 2.5 cm from floor) located between the levers. Sucrose (20% concentration) was delivered to the magazine via a dipper cup (0.1 mL). Each magazine was equipped with a dipper cue light (1 watt; 4.1 mm in diameter). Rats' lever press responding was recorded using MED-PC IV (Med Associates). Each chamber also contained a Quick Disconnect shock grid floor, a Standalone Aversive stimulator/scrambler (Med Associates), and a Cage Tweeter tone generator (Med Associates). Compressed pellet bedding (Shepherd Specialty Papers) lined the waste pans in each chamber.

Procedure

Baseline Training

The procedures for baseline training and acquisition phase were based on previous studies conducted in our laboratory (Reinhardt & Cain, 2015b). Following the 30 day rearing period, rats were gradually food deprived to 85% of their free-feed body weight, between 51-57 days of age. EC rats were separated into pairs for a 1 hr feeding each day. At 58 days of age, all rats began magazine training and shaping in the operant chambers. During this time, the rats learned to associate pressing the active lever with a four second sucrose presentation (20% sucrose, dipper cue light illuminated). All rats were hand shaped to press the active lever until they receive approximately 100 sucrose deliveries. An inactive lever was present and did not result in a consequence if pressed; the active and inactive levers were counterbalanced across the rearing conditions and across each operant chamber. All rats then received five sessions of baseline training. On day 1 of baseline training, rats began lever pressing on a one response

fixed-ratio schedule of reinforcement (FR1) that lasted for 30 min. Training on day 2 was a 15 s variable interval (VI-15) schedule, followed by a VI-45 schedule on day 3, and concluded with a VI-90 schedule on days 4 and 5. Training sessions on days 2-5 each lasted 60 min.

Acquisition

Baseline training was followed by one session of Pavlovian fear conditioning (acquisition). Rats only received one session of acquisition as Reinhardt and Cain (2015a) found that ifenprodil produced the most robust effects during the first session of acquisition. During the acquisition session, rats lever pressed for sucrose on a VI-90 schedule and received four presentations of a tone CS (60-s tone, 3000-Hz, 80 dB) and a scrambled footshock US (0.5-s, 0.6-mA). Half of the rats from each rearing condition were placed in the "paired group", meaning they experienced the termination of the CS immediately followed by the onset of the US. The other half of the rats was placed in the "unpaired group"; they received random presentations of the CS and the US such that the CS was not predictive of the US. All rats received a total of four conditioning trials, with each conditioning session lasting 90-min. The first trial always occurred approximately 16 min after the start of the session. The intertrial interval (ITI) was variable with a mean of 14 min. Due to an equipment malfunction during the acquisition phase, the affected animals were removed from all further experimentation and analyses (EC, n = 3; IC, n = 3; SC, n = 4).

Immediately following the end of the acquisition session, rats were deeply anesthetized with a sodium pentobarbital injection (390-780 mg/kg sodium pent; 0.5-1.0 ml i.p.). Transcardial perfusions with a saline rinse followed by a 4% paraformaldehyde fix were conducted to fix the brains, which were then removed and stored in a 20% sucrose cryoprotectant at room temperature.

Immunohistochemistry

As previously demonstrated in our lab (Mersmann, Gregg, & Cain, 2013), a general avidin-biotin-peroxidase complex (ABC) procedure was used to process the tissue samples. The frozen brains of all rats (EC = 15, IC = 15, SC = 14) were sliced into serial transverse sections (40μm) using a Thermo Scientific Microm HM550 cryostat. Tissue sections containing the BLA (anterior-posterior, -2.8 mm; medial-lateral, ±5.0 mm; dorsal-ventral, -8.7 mm), the ACe (anterior-posterior, -1.88 mm; medial-lateral, ±3.8 mm; dorsal-ventral, -8.2 mm), and the CA3 region (anterior-posterior, -3.3 mm; medial-lateral, ±2.6 mm; dorsal-ventral, -3.8 mm) were collected (Paxinos & Watson, 1998). The free-floating tissue sections were incubated in a blocking solution of 5% normal goat serum diluted in 0.2% Triton X-100 in phosphate buffered saline (PBS-TX; 50 mM mixed phosphate buffer, 150 mM NaCl, pH 7.4) for 30 min. The tissue was then rinsed with PBS-TX and then reacted with rabbit anti-NMDAR2B antibody overnight at 4^{o}C (1:500, Thermo Scientific – Pierce Antibodies). The tissue was again washed with PBS-TX, placed in the biotinylated secondary antibody (3 hr, room temperature), washed again with PBS-TX, placed in the ABC (3 hr, room temperature), and washed with PBS-TX. Finally, the NR2B subunit was stained with 0.01% H_2O_2 and 0.05% diaminobenzide (DAB). The tissue sections were then mounted onto gelatinized slides and cover slipped with VectaMount (Vector Labs). Based on the large quantity of tissue, the tissue was divided and processed in two different batches.

A light microscope (Olympus BX41) using bright field illumination was used to examine the cells visualized with DAB. Images of the tissue were captured using a SPOT Idea^{TM} CMOS camera and SPOT Advanced imaging software. The tissue was then quantified and scored by automatic counting of the cells stained for the NR2B subunit, using ImageJ software. Cells

stained for NR2B were collected and counted from a minimum of two different sections (anterior, medial, posterior plates) within the entire structure of each brain region to capture a representation of NR2B from the whole structure. For all regions, the sample area quantified from each plate was 0.38mm × 0.51mm (Figure 1). The sections used for each area were as follows (Paxinos & Watson, 1998): BLA plates 29, 32, and 34; ACe plates 26, 27, 29; CA3 plates 27, 33, 36, 37, 38, and 39. Given the large size of the hippocampus, more plates were included in the CA3 region. However, some of the tissue within the hippocampus was damaged during the plating process and therefore, some of the plates could not be scored for each rat. To address this loss in tissue, a minimum of three different plates were included in the counting of NR2B in the CA3. The average count of the plates within each region was used for data analysis. To prevent bias in the data collection, the tissue was coded and experimenters were blind to the experimental conditions of the tissue until all of the tissue was processed and quantified.

Statistical Analyses

The behavioral data was assessed with the calculation of a suppression ratio. The suppression ratio was the dependent measure for the behavioral data, as it was used to measure the level of fear in the rats. It was calculated by dividing the total number of active lever bar presses during the 60-sec CS period (C) by the total number of active lever bar presses during the 60-sec pre-CS period (P) and the number of active lever bar presses during the CS period (C).

$$Suppression\ Ratio\ = \frac{C}{P + C}$$

A suppression ratio of 0 indicates complete suppression of all responding during the presentation of the CS, suggesting that the rat experienced a maximum amount of fear.

However, a suppression ratio of 0.5 indicates that there was no suppression of responding during the presentation of the CS, suggesting that the rat did not experience any fear.

To determine the effect of the rearing environment on the acquisition of fear, suppression ratios for the acquisition phase were analyzed with a 2 × 3 × 4 mixed factorial analysis of variance (ANOVA). The learning group (paired or unpaired) and the rearing environment (EC, IC, or SC) were the between-subjects factors while the acquisition trials (4 trials) was the within-subjects factor. Additionally, slope analyses were conducted on suppression ratio scores as a function of trial for each learning group (paired or unpaired) within each rearing environment (EC, IC, or SC). Slope estimates were then analyzed with one-sample t-tests, such that slope estimates significantly different from zero reflect significant increases (or decreases) in suppression ratios across trials.

Immunohistochemistry data was analyzed with separate 2 × 3 between subjects ANOVAs for each of the three specified brain regions. The learning group (paired or unpaired) and the rearing environment (EC, IC, or SC) were the between-subjects factors while the mean cell counts (for each brain region) were the dependent measure. Simple effects analyses were used to probe interactions.

Finally, correlational analyses, using Pearson's r, were conducted between the suppression ratios of the last acquisition trial and NR2B expression in each of the three brain regions to examine the relationship between the levels of fear at the end of the acquisition session and the expression of the NR2B subunit in the BLA, ACe, and CA3, respectively. For all analyses, the alpha level was set to .05.

Chapter 3 - Results

Behavioral Results

Lever-Press Training

The number of active lever presses during lever press training was analyzed to ensure that there were no significant differences in lever press behavior between rearing conditions. While there were differences in active lever presses among the rearing conditions and the two learning groups early in the VI training (during VI-15 training, VI-45 training, and the first day of VI-90 training, Fs(1, 43) = 3.26 – 4.89, ps < .05, η_p^2s = .11-.16), there were no significant differences between rearing conditions in active lever presses by the last day of VI-90 training. It was expected that there would be differences early in VI training and that once the rats' active lever-pressing stabilized there would be no differences, as demonstrated on the last day of VI-90 training.

Fear Acquisition

A 2 × 3 × 4 repeated measures ANOVA was conducted to determine the effects of the learning group and rearing environment on the acquisition of fear at each trial. A significant main effect of learning group was found, $F(1, 38) = 5.71$, $p < .05$, $\eta_p^2 = .13$, with the paired rats having significantly lower suppression ratio scores than the unpaired rats.

Slope analyses were conducted on suppression ratio scores as a function of trial for each learning group within each rearing environment in order to evaluate each group's speed of learning. The slope estimates for each group of rats were then analyzed using one-sample t-tests, such that the slope estimates were compared to zero. A slope of zero indicates that there was no change in suppression ratios across the acquisition trials. Therefore, a slope estimate that is

significantly different from zero indicates that there was a significant change in suppression ratios across trials; a negative difference indicates a decrease in suppression ratio scores and more fear learning. The slope estimate for paired, isolated rats was significantly less than zero, $t(6) = -3.52$, $p < .05$, such that their suppression ratio scores significantly decreased across trials (see Figure 2A). The slope estimates for all other groups of rats did not significantly differ from zero, indicating that their suppression ratio scores did not change across trials (see Figures 2B and 2C).

Based on these fear acquisition findings, the pre-CS active lever pressing data was analyzed to ensure that the effects were not due to baseline differences in active lever pressing. Repeated measures ANOVAs were conducted for each of the learning groups. Significant main effects of trial were found for the paired and unpaired rats, Fs(3, 57) = 3.69-9.84, ps < .05, η_p^2 = .16-.34 (see Figure 3). For the paired and unpaired rats, the pre-CS active lever pressing significantly decreased as the acquisition trials progressed. However, no other effects were found. These findings indicate that there are no baseline differences in pre-CS active lever pressing among the different rearing groups, suggesting that the significant findings in the fear acquisition data are not confounded by baseline group differences in lever press behavior.

NR2B Results

BLA Expression

A 2 × 3 between-subjects ANOVA was conducted to determine the effects of the rearing environment and the learning group on the expression of NR2B in the BLA. A significant main effect of rearing condition was found, $F(2, 43) = 3.79$, $p < .05$, $\eta_p^2 = .17$. This indicates that the

expression of NR2B in the BLA was dependent upon the rearing environment the rats were raised. Simple effects analyses revealed that IC rats had significantly lower levels of NR2B expression than EC rats, $F(1, 41) = 4.08$, $p < .05$, $\eta_p^2 = .16$. (see Figure 4). There was no significant difference in NR2B expression between the paired and unpaired rats; therefore, the differences seen among the rearing environments may demonstrate their baseline levels of NR2B.

Correlational analyses were conducted, using Pearson's *r*, to examine the relationship between fear acquisition and mean NR2B expression in the BLA. Suppression ratio scores on the last acquisition trial were correlated with the mean level of NR2B expression within the BLA, for each rearing condition. No significant correlations were found, suggesting that there was no significant relationship between the rats' level of fear at the end of the acquisition session and their levels of NR2B expression within the BLA.

ACe Expression

A 2 × 3 between-subjects ANOVA was conducted to determine the effects of the rearing environment and the learning group on the expression of NR2B in the ACe. No significant effects were found (see Figure 5), indicating that the rearing condition of the rats and the learning group to which they were assigned did not have a significant impact on their levels of NR2B in the ACe.

Correlational analyses were conducted, using Pearson's r, to examine the relationship between fear acquisition and mean NR2B expression in the ACe. Suppression ratio scores on the last acquisition trial were correlated with the mean level of NR2B expression within the ACe, for each rearing condition. No significant correlations were found, indicating that there was no

significant relationship between the rats' level of fear at the end of the acquisition session and their levels of NR2B expression within the ACe.

CA3 Expression

A 2 × 3 between-subjects ANOVA was conducted to determine the effects of the rearing environment and the learning group on the expression of NR2B in the CA3. No significant effects were found (see Figure 6), indicating that the rearing condition of the rats and the learning group to which they were assigned did not have a significant impact on their levels of NR2B in the CA3.

Correlational analyses were conducted, using Pearson's r, to examine the relationship between fear acquisition and mean NR2B expression in the CA3. Suppression ratio scores on the last acquisition trial were correlated with the mean level of NR2B expression within the CA3, for each rearing condition. A significant positive correlation was found among the SC rats, $r = .63$, $p < .05$ (see Figure 7), indicating that lower suppression ratio scores on the last acquisition trial were associated with lower levels of NR2B expression in the CA3. No significant correlations were found among EC or IC rats, suggesting there was not a significant relationship between the EC or IC rats' level of fear at the end of the acquisition session and their respective levels of NR2B expression within the ACe.

Chapter 4 - Discussion

This study examined the effect of the rearing environment on the acquisition of fear, as well as the expression of the NR2B subunit in the basolateral amygdala (BLA), the central nucleus of the amygdala (ACe) and the CA3 region of the hippocampus. Isolated condition rats (IC) showed faster fear acquisition than enriched condition (EC) and social condition (SC) rats. Given the implicated role of NR2B in fear acquisition, it was expected that IC rats would show the greatest levels of NR2B expression compared the EC or SC rats. However, IC rats showed the least amount of NR2B expression in the BLA while there were no significant effects of the rearing environment on NR2B expression in the ACe or the CA3. Overall, this indicates that the rearing environment does affect fear learning while also altering NR2B expression within the BLA.

An Isolated Rearing Environment Facilitates the Acquisition of Fear

The current experiment was designed to further understand the role of the NR2B subunit in differentially reared animals and its implications in the acquisition of conditioned fear. It was expected that rats reared in the isolated and standard environments would show a significant decrease in suppression ratio scores across the acquisition trials compared to enriched rats. As predicted, this experiment found that rats reared in an isolated rearing environment learned about an aversive stimulus faster than rats raised in enriched and standard environments. These findings are opposite of previous work that has demonstrated enhanced appetitive learning among animals raised in enriched environments compared to isolated and standard housed animals (Frick & Fernandez, 2003; Gill & Cain, 2011; Wood & Rebec, 2009). However, this supports previous fear conditioning experiments conducted in our laboratory that found that

isolated rats acquired fear faster than enriched rats (Reinhardt & Cain, 2015a, 2015b). Similarly, the fact that no learning differences were seen among the enriched and standard housed rats supports the findings of Barbelivien et al. (2006), in which they demonstrated no differences among enriched and standard housed rats in their levels of freezing during the initial acquisition phase.

Rats that received predictive pairings of the CS and the US began to acquire fear, while rats that received unpaired presentations of the CS and US did not acquire fear. This finding was expected as this indicates that the paired rats learned the CS-US association and began to fear the presentation of the CS while that unpaired rats did not learn to fear the presentation of the CS. This is a commonly used learning paradigm that is used to ensure that changes in behavior are due to learning the CS-US association given a perfect positive contingency and not just the mere presence of the stimuli (Rescorla, 1967). More specifically, this experiment found that during just one fear acquisition session, only the paired rats reared in the isolated environment began to acquire fear to the CS. While prior research has found that enriched and standard housed animals can acquire fear if given enough training (Reinhardt & Cain, 2015a), this experiment highlights the impact the rearing environment has on initial fear learning.

In terms of learning and memory, the rearing environment has also been suggested to affect conditioning to a discrete cue versus an experimental context. There are discrepant findings regarding the influence of the rearing environment on cued fear conditioning. Barbelivien et al. (2006) demonstrated that standard housed rats appear to freeze more to a tone that had previously been paired with a footshock, compared to enriched rats while Tang et al. (2001) found that enrichment facilitated freezing to an auditory stimulus that was associated with a footshock. While this would have predicted that enriched or standard conditioned rats would

show greater amounts of fear, the current study found that only the isolated rats acquired fear to the aversive cue, in accordance with previous research (Reinhardt and Cain, 2015b). Similarly, Walasek et al. (2002) found that individually housed rats acquired fear to an auditory cue that was paired with a footshock while enriched rats didn't discriminate their fear specifically to the cue. In contrast to cued conditioning, enriched animals show better memory for fearful conditioning contexts (Duffy et al., 2001; Y.-P. Tang et al., 2001) as well as better discrimination between aversive and non-aversive contexts compared to standard housed animals (Barbelivien et al., 2006; Woodcock & Richardson, 2000). Accordingly, the differences in cued versus contextual fear conditioning as a function of rearing environment warrants further investigation.

NR2B Expression in the BLA is Lowest in Isolated Rats

Based on the expectation that isolated and standard housed rats would display faster acquisition of conditioned fear, it was predicted that isolated and standard housed rats would show greater expression of the NR2B subunit in the basolateral amygdala (BLA) than enriched rats. Interestingly, the results from the current study demonstrated the opposite of this prediction. Isolated rats showed the lowest level of NR2B expression while enriched rats showed the greatest level of expression. This finding was unexpected given prior research demonstrating NR2B is vital for fear acquisition (Rodrigues et al., 2001; Ya-Ping Tang et al., 1999; Walker & Davis, 2008). Specifically, isolated rats began to fear the CS but they showed the least amount of NR2B expression. Given the BLA's role in storing CS-US associations in fear learning paradigms (LeDoux & Schiller, 2009; Maren, 2001) and NR2B's role in the acquisition of fear (Rodrigues et al., 2001; Walker & Davis, 2008), it is puzzling that NR2B expression was the greatest in the group of rats that did not acquire fear (enriched) and the lowest in the group of rats that actually began to acquire fear (isolated). This finding supports research

that demonstrated a significant down regulation of NR2B (and NR2A) protein levels within the amygdala following conditioned fear-potentiated startle (Zinebi et al., 2003). It is important to highlight that there were multiple fear conditioning sessions and NR2B protein analysis occurred 24 hours after the final day of fear learning. So, it is possible that Zinebi et al. (2003) witnessed a down regulation in NR2B due to the consolidation or even reactivation of these fear memories rather than due to initial acquisition of fear. However, this study clearly illustrates that NR2B is down regulated as a result of fear conditioning, which supports the current findings.

Another explanation for this finding is that the previous studies that have examined the role of NR2B in the initial stages of fear learning have not directly examined the expression of NR2B but rather have manipulated NR2B activation with the use of antagonists (Campeau et al., 1992; Day, Cooper, Markham, & Huhman, 2011; Fanselow & Kim, 1994; Rodrigues et al., 2001). Given that this is one of the only studies to examine NR2B expression early in fear acquisition, there may be a function of NR2B expression early in aversive learning that research has not yet been able to identify. Alternatively, another possible explanation for this contradictory finding may be due to the methods employed in this experiment. Following the fear conditioning session, the rats were immediately perfused and their brains collected. While this timeline supports prior research (Burghardt, Sigurdsson, Gorman, McEwen, & LeDoux, 2013), there is other literature that has indicated a delay in brain collection following the behavioral paradigm; Zhao et al. (2009) waited 2 hours before removing brains, Zinebi et al. (2003) waited 24 hours, while Gourley, Kedves, Olausson, and Taylor (2009) and S. H. Wang, de Oliveira Alvares, and Nader (2009) waited 48 hours. However, it is important to note that while all of these studies were quantifying NR2B expression, none of these studies were attempting to isolate a change in NR2B expression to fear acquisition. So, it is possible that not

enough time was given following the fear conditioning session to allow for the related changes in NR2B expression within the BLA to occur. The differences we are currently observing may just be baseline expression and not representative of the expression following the fear conditioning experience. This explanation is further supported by the fact that there was no difference in NR2B expression between the paired and unpaired rats. If more time had been given between the end of the fear conditioning session and the collection of brains, it is possible that there would have been a difference in NR2B expression among the paired and unpaired rats as well as differences in expression among the different rearing environments that better aligns with their behavioral data.

NR2B Expression in the ACe is Not Impacted by the Rearing Environment

There is evidence that indicates that the central nucleus of the amygdala (ACe) is important for the acquisition of fear (Paré et al., 2004). Therefore, it was hypothesized that if the ACe is important for the acquisition of fear, then the isolated rats would show more NR2B expression in the ACe compared to the enriched and standard housed rats. This hypothesis was not supported by the current set of data, as there was a difference in rates of fear acquisition among the rearing environments while there was no difference in NR2B expression in the ACe. The isolated rats began to acquire fear to the aversive stimulus while the enriched and standard rats did not acquire fear. While NR2B is present in the ACe (Sah, Faber, Lopez de Armentia, & Power, 2003), the ACe is a highly GABAergic region (Ehrlich et al., 2009; Sah et al., 2003). It appears that GABA-facilitated inhibition within the ACe contributes to the acquisition of fear (Ciocchi et al., 2010; Rodriguez Manzanares, Isoardi, Carrer, & Molina, 2005; Wilensky et al., 2006). GABA functioning has also been shown to be moderated by the environment (Caldji, Francis, Sharma, & Plotsky, 2000; Harte, Powell, Swerdlow, Geyer, & Reynolds, 2007). It is

possible that the acquisition differences seen among the differentially reared rats may be due in part to differences in GABA neurotransmission within the ACe, rather than NR2B expression. Therefore, understanding better the relationship between GABA and glutamate (specifically the NR2B subunits) in the ACe should provide a clearer understanding of the ACe's role in the acquisition of conditioned fear (Mahan & Ressler, 2012).

While there is evidence that the ACe is important for fear acquisition (Paré et al., 2004), there is research that indicates that the ACe is simply a passive output station for the expression of emotional responses (LeDoux & Schiller, 2009; Maren, 2001; Paré et al., 2004). Therefore, the rearing induced differences in fear learning coupled with the absence of rearing induced differences in NR2B expression in the ACe may suggest that this expression was simply a result of the exposure to the aversive stimulus (i.e., the footshock). All of the rats experienced the aversive stimulus (i.e., the footshock) regardless of whether they were in the paired or unpaired group. As the ACe is activated a in response to aversive events (Merali, McIntosh, Kent, Michaud, & Anisman, 1998), just being exposed to the footshock may facilitate NR2B expression within the ACe. This could explain why there were no differences in NR2B expression among the different groups of rats. However, the current experiment did not contain the necessary control groups to fully evaluate this hypothesis. Therefore, the absence of NR2B differences in the ACe among the rearing environments may indicate that NR2B within the ACe is not involved in the acquisition of fear but rather the passive relay of fear response outputs, but additional experiments are necessary to test this possibility.

Differential Rearing Does Not Alter NR2B Expression in the CA3

The CA3 region receives inputs into the hippocampus, makes associations among these inputs, and then temporarily stores these associations (McClelland & Goddard, 1996; Treves &

Rolls, 1994; Wiebe et al., 1997). Therefore, the CA3 has been identified as a region involved in the acquisition of fear (I. Lee & Kesner, 2004). It was predicted that isolated and standard housed rats would acquire fear faster than enriched rats; therefore, the isolated and standard rats would show greater levels of NR2B expression compared to enriched rats. Even though isolated rats did begin to acquire fear while standard and enriched rats did not, there was no significant difference in NR2B expression in the CA3 among the three rearing environments. While this finding in the CA3 was surprising given the differences in fear acquisition among the rearing environments, there are contradictory findings in the literature regarding the kind of impact the environment has on NR2B in the hippocampus. Environmental enrichment appears to upregulate the NR2B and NR2A subunits within the hippocampus (Andin et al., 2007; Bredy et al., 2004) while Zhao et al. (2009) found that an isolated environment upregulated NR2B gene expression within the hippocampus compared to group housed rats. Therefore, it is possible that there are baseline differences in NR2B expression among differentially reared rats and these differences were washed-out following fear conditioning, resulting in no differences in NR2B expression seen among the rearing environments.

There was a significant positive correlation for standard condition rats between their suppression ratios on the last acquisition trial and their level of NR2B expression within the CA3. This indicates that lower suppression ratio scores (more fear) on the last acquisition trial were associated with lower levels of NR2B expression. However, this correlation was driven by two outlier rats so future research is needed to verify the robustness of the relationship between fear conditioning suppression ratio scores and NR2B expression within the CA3.

Additionally, while research investigating the CA3 has found that NMDA is important for the acquisition of contextual fear (Cravens, Vargas-Pinto, Christian, & Nakazawa, 2006; I.

Lee & Kesner, 2004), evidence suggesting that NMDA in the CA3 is involved the acquisition of cued fear is lacking. However, what is currently known about the role of the CA3 in cued fear acquisition has been determined through the use of lesion studies (Hunsaker & Kesner, 2008; I. Lee & Kesner, 2004), reversible inactivations (Daumas, Halley, Francés, & Lassalle, 2005), and the development of genetically modified mice (McHugh & Tonegawa, 2009). These studies did not target the specific effects of NR2B on cued fear acquisition in the CA3. Therefore, the role of NR2B within the CA3 in fear acquisition still needs to be determined. All together, the findings in the CA3 from the current study illustrates the need for future research to understand better the role the rearing environment has on fear acquisition and NR2B expression within the CA3 region of the hippocampus.

Future Directions and Implications

The current study provides additional information about the role of NR2B expression on the acquisition of fear. While there was only one significant difference in expression within the BLA of enriched rats and one significant relationship between expression and suppression ratio within the CA3 of standard rats, the overall lack of differences offers us some insight into the function of NR2B and raises even more questions about its role in fear learning and relationship with the rearing environment. As previously discussed, there was no difference in NR2B expression among the paired and unpaired rats. Therefore, it is possible that changes in NR2B expression within the amygdala and hippocampus are not present immediately following a fear conditioning paradigm but may show changes several hours after. Changing the length of time that is allotted from the end of the fear conditioning session to the removal of brains may result in differences in NR2B expression among the rearing environments as well as among the paired and unpaired rats. So, it would be important for future studies to determine the timeline of these

changes so as to better understand the role of NR2B in the acquisition of fear to an aversive stimulus.

The fact that isolated rats were the only rats to acquire fear coupled with the fact that there was no difference in NR2B expression within the ACe suggests that NR2B within the ACe may be influenced by the mere presence of the footshock and is therefore involved in the passive relay of fear response outputs rather than the acquisition of fear. While the design of the current experiment controlled for the presence of the CS and the US in the fear acquisition session (i.e., paired and unpaired groups), all rats in the experiment were exposed to the footshock (US). Therefore, in order to tease apart the relationship between NR2B expression and the presence of the footshock, it would be beneficial for future studies to examine and compare the levels of NR2B expression within the ACe among rats reared in the three rearing environments that were not exposed to the footshock. Given the lack of research surrounding NR2B expression within the ACe, such research would provide additional insight into the influence differential rearing has on fear learning and processing. This would offer a baseline level of NR2B expression that would control for the presence of aversive stimuli and would offer additional insight into why there were no rearing differences in NR2B expression within the ACe.

Research has demonstrated that the rearing environment has an impact on the regulation and expression of the other NMDA receptor subunits, including the NR2A subunit (Andin et al., 2007; Bredy et al., 2004; Turnock-Jones et al., 2009). There is also evidence to suggest that these other NMDA subunits play important roles in the acquisition of fear (Cui et al., 2013; Walker & Davis, 2008). Many studies examine the relationship between NR2A and NR2B through the use of the NR2A:NR2B ratio (Cui et al., 2013; Law et al., 2003; Xu et al., 2009). While there is plenty of evidence that suggests that a genetic overexpression of the NR2B

subunit results in enhanced learning and memory (Cao et al., 2007; Jacobs & Tsien, 2012), genetic overexpression of the NR2A subunit appears to have the opposite effect. Overexpression of the NR2A subunit leads to long-term memory constraints and impairments in synaptic plasticity (Cui et al., 2013). Given these functional differences between the NR2A and NR2B subunits, knowing the relationship between NR2A and NR2B and its effect on fear acquisition among differentially reared rats may provide a clearer understanding for the results of the current study.

Overall, the results from the current study suggest that an isolated rearing environment facilitates learning about an aversive stimulus. More specifically, it indicates that being raised in an aversive and anxiety-inducing environment (isolation; Weiss, Pryce, Jongen-Rêlo, Nanz-Bahr, & Feldon, 2004) enables the learning of other aversive and anxiety-inducing stimuli. The NR2B findings are less clear with the only significant differences occurring within the BLA; the enriched rats show more NR2B expression than the isolated or standard housed rats. However, as this is one of the first studies to begin to compare the neurobiological mechanisms associated with fear learning among isolated and enriched rearing environments, it is not surprising that more questions are raised than are addressed. It is clear that more work needs to be done to unravel this tangled web, especially since the ultimate goal is to understand the relationship between the rearing environment and fear/anxiety disorders. Prior research has suggested that the NMDA receptor and its varied subunits be investigated for the development of therapies for a number of neuropsychiatric disorders (Cui et al., 2013; Gonda, 2012). So, this study adds to the current body of research that will aid the development of pharmaceutical compounds and therapies used to treat anxiety disorders. In total, these current findings help to further our

understanding of the rearing environment and its effect on fear and anxiety, especially the neural underpinnings of the acquisition of fear.

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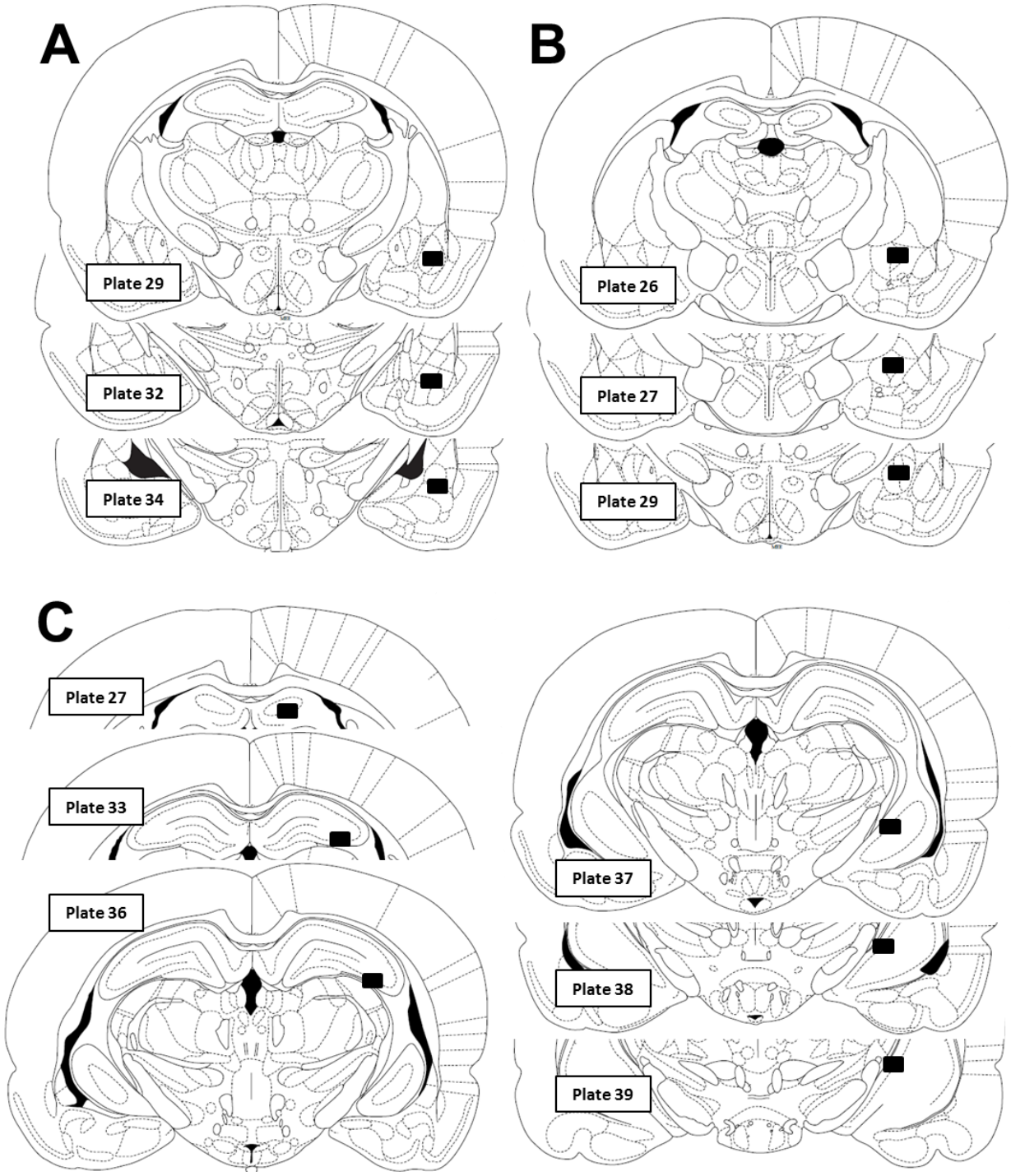
Figure 1 Graphic representation of areas analyzed within the BLA, ACe, and CA3.



Note: Graphic representations within (A) the BLA, (B) the ACe, and (C) the CA3 (Paxinos & Watson, 1998)

Figure 2 Mean ± SEM suppression ratio scores for trials 1-4 of acquisition.

Note: Mean suppression ratio scores for (A) IC, (B) SC, and (C) EC rats. An asterisk (*) indicates that the slope estimate for paired, isolated rats was significantly less than zero.

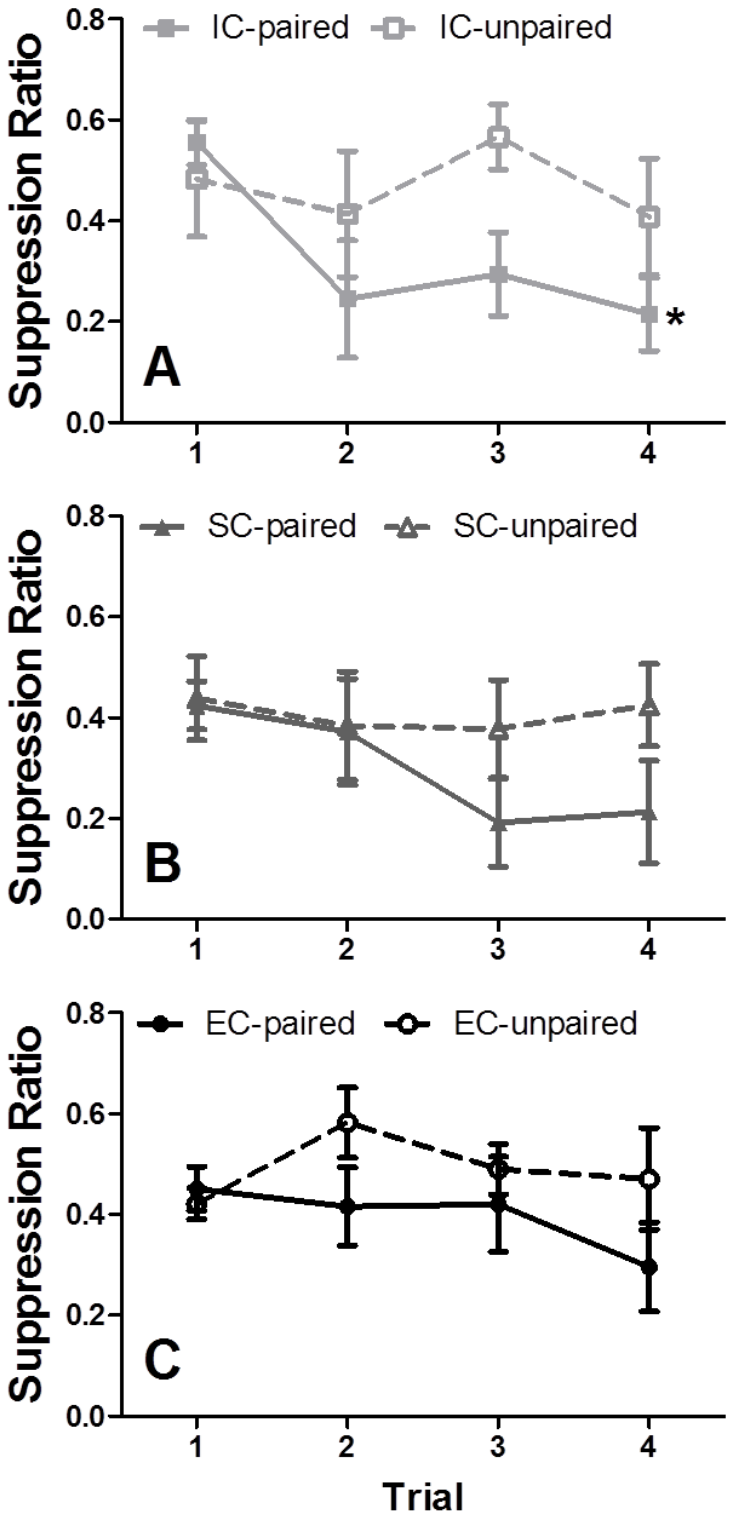
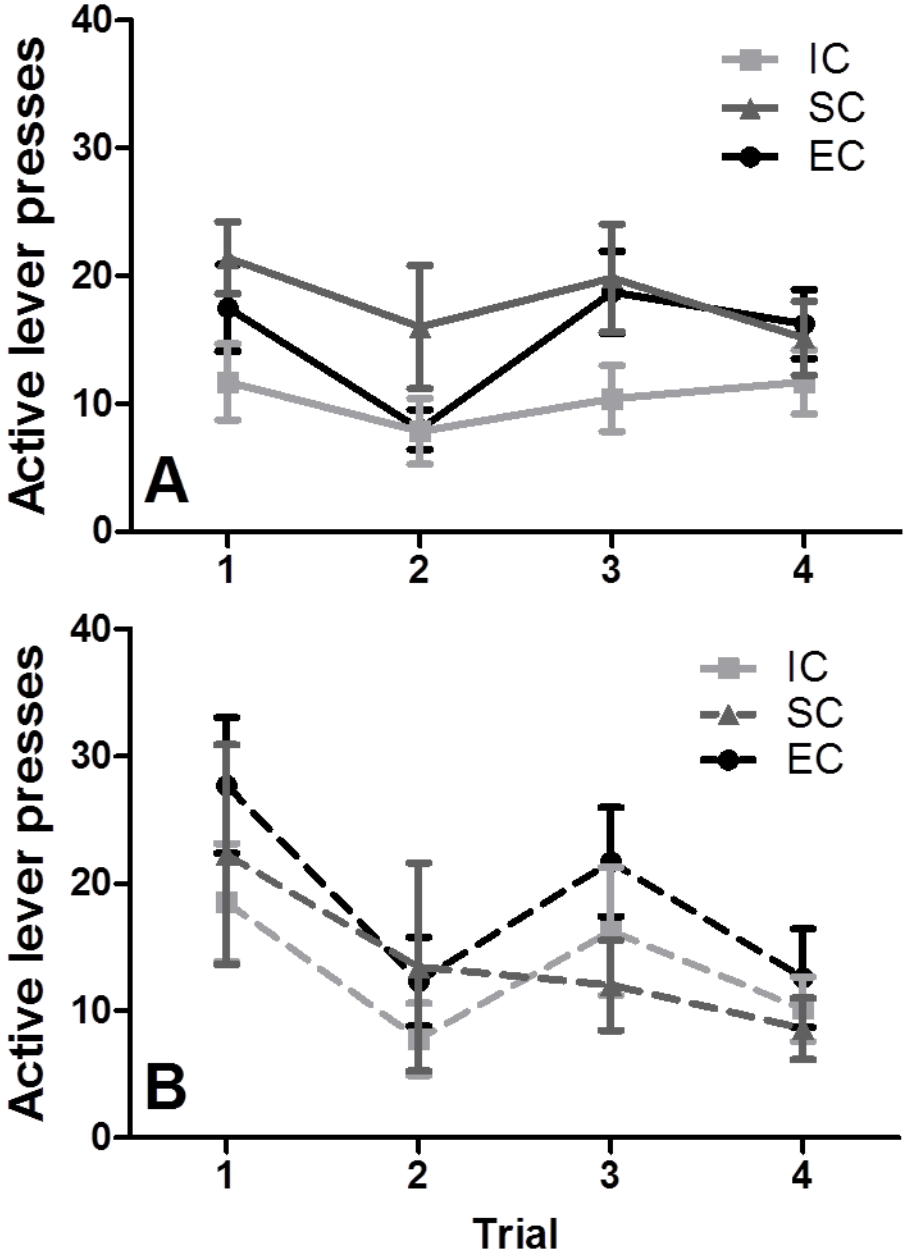
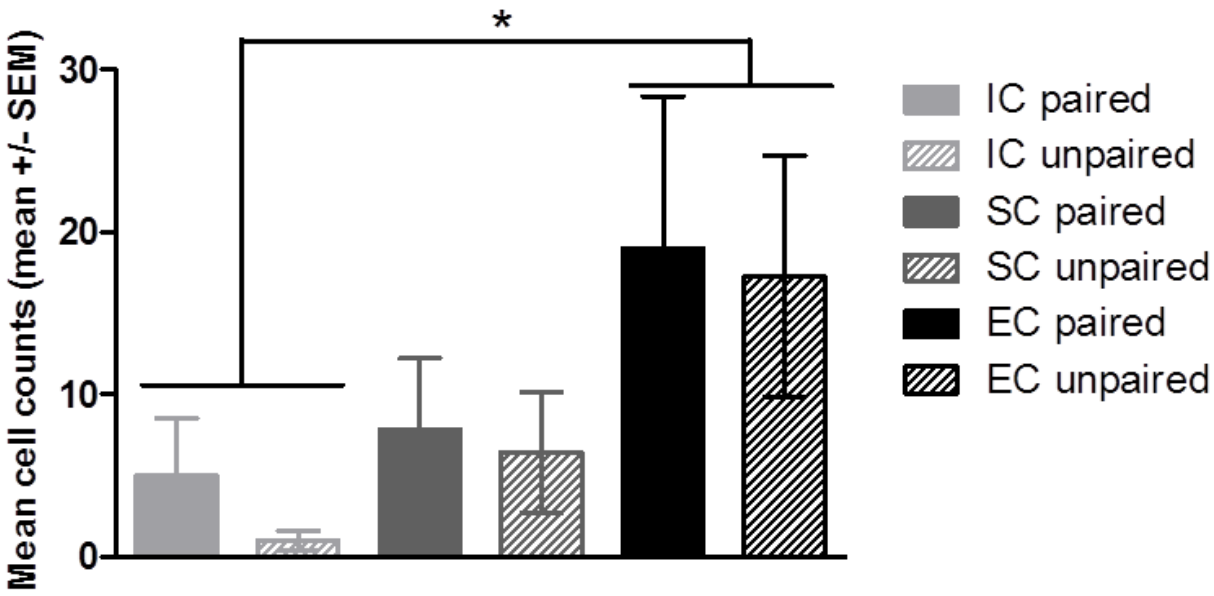


Figure 3 Mean ± SEM active lever pressing during the pre-CS period for trials 1-4 of fear acquisition.



Note: Mean active lever pressing during the pre-CS period for (A) paired and (B) unpaired rats.

Figure 4 Mean ± SEM number of cells labeled for NR2B in the BLA.



Note: An asterisk (*) indicates that IC rats had significantly fewer cells labeled for NR2B than EC rats, $p < .05$.

Figure 5 Mean ± SEM number of cells labeled for NR2B in the ACe.

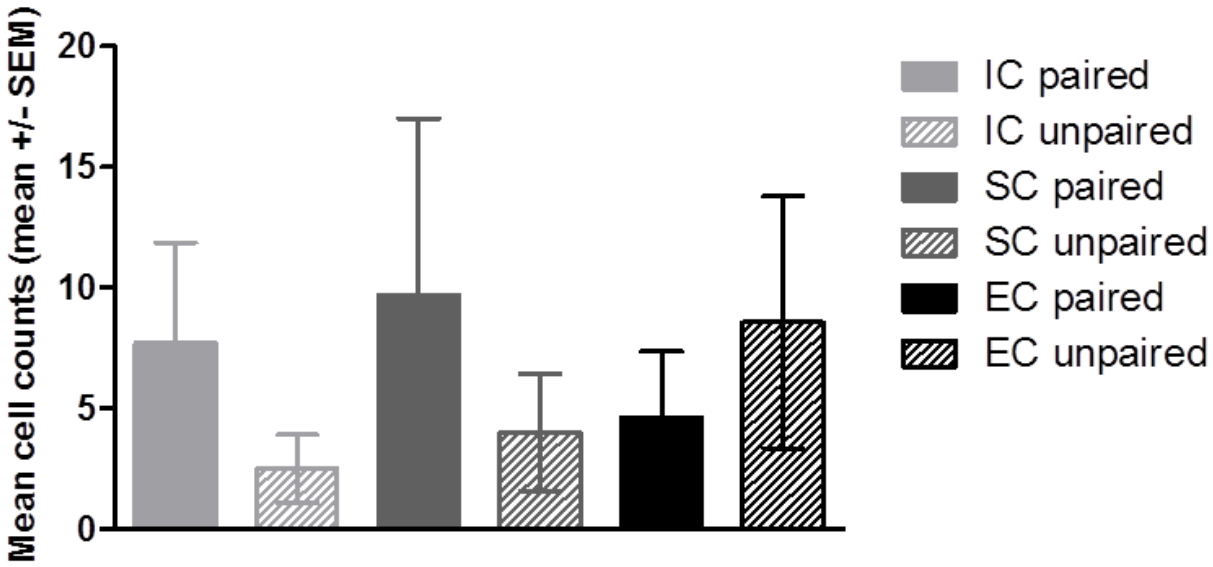


Figure 6 Mean ± SEM number of cells labeled for NR2B in the CA3.

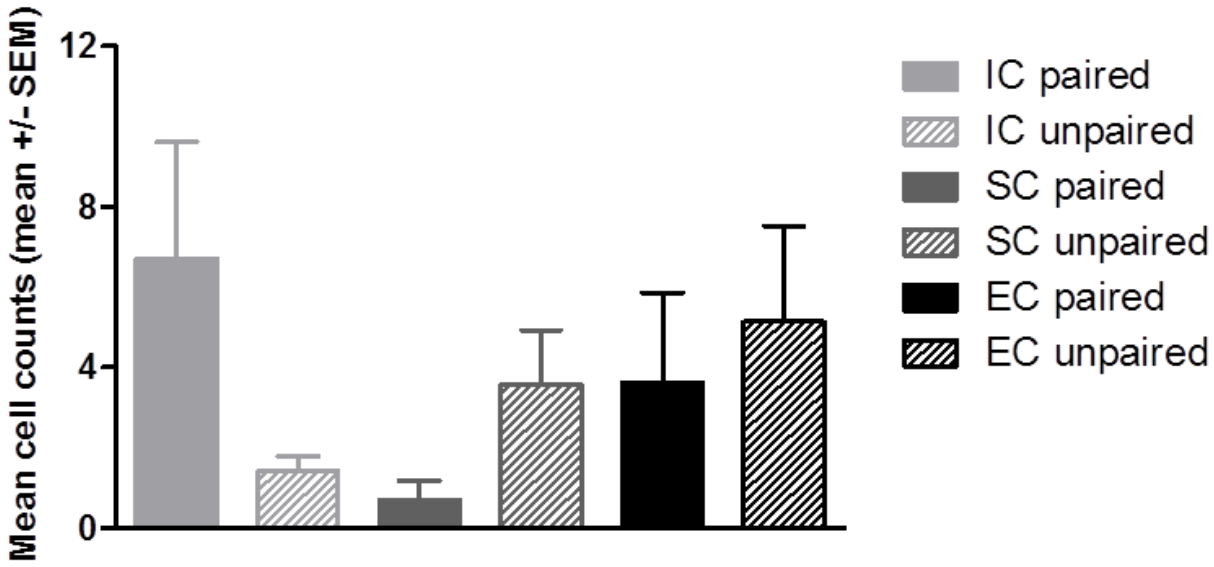


Figure 7 Relationship between suppression ratios on the last fear acquisition trial and the mean level of NR2B expression in the CA3 among SC rats.

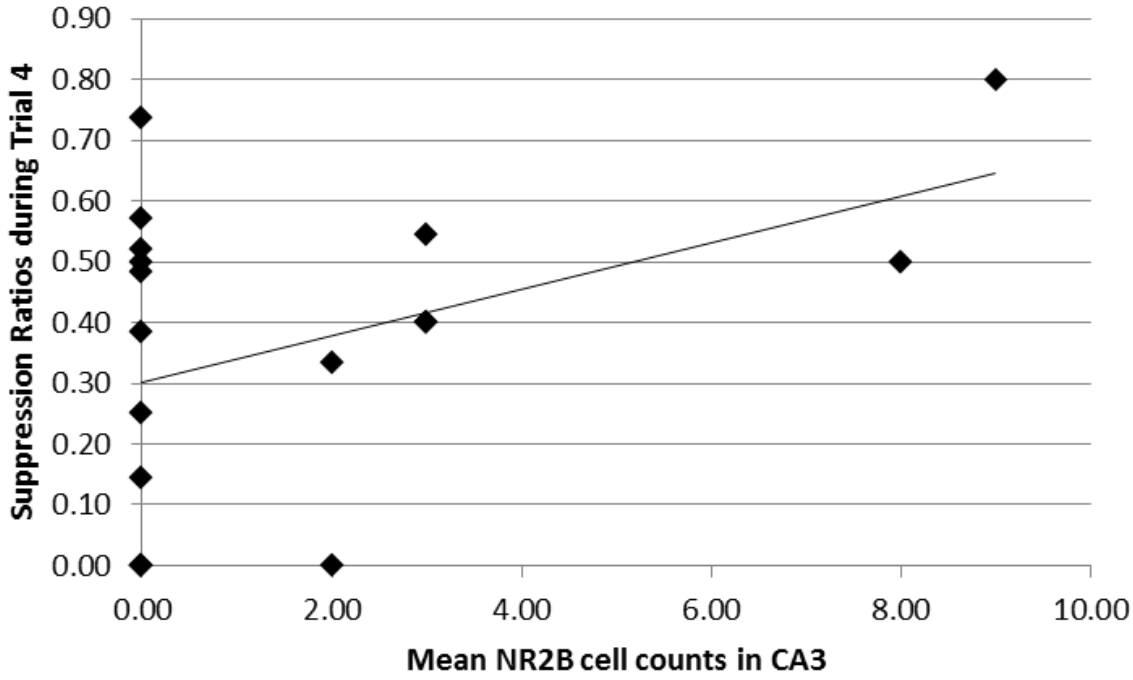
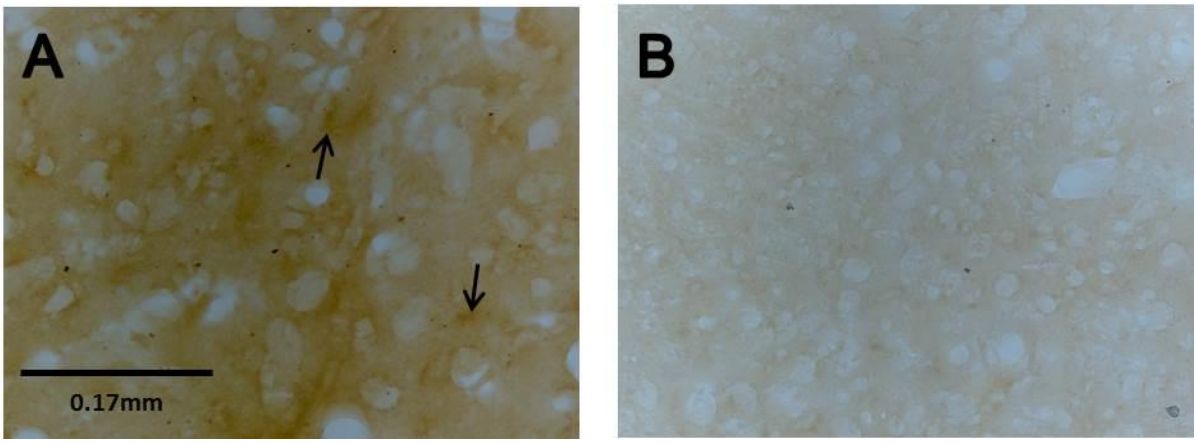


Figure 8 Representative images of tissue processed for NR2B expression.



Note: (A) BLA image with NR2B expression; examples of labeled cells marked by arrows. (B) BLA image with no NR2B expression.