Levels of Perineuronal Nets in the Basolateral Amygdala Are Correlated with Sex Differences in Fear Learning

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ABSTRACT

Although the correlation between traumatic events and the development of posttraumatic stress disorder (PTSD) is by no means one to one, trauma and exposure to extreme stressors greatly increases a person's vulnerability to developing mental illness. The symptoms of PTSD, though incredibly diverse, are generally characterized by prolonged and heightened reaction to traumatic events. Despite the fact that women are more than twice as likely to develop PTSD, much of the research on this disorder has largely relied on the use of male subjects. While one could argue that this gender discrepancy can be attributable to various social factors, investigation of the biological basis of such sex differences may prove to be crucial component in the development of novel and more targeted treatment options. This paper will review putative roles of steroid hormones, alterations in the neural circuits involving fear, and changes in neuroplasticity in the sex differences seen in PTSD and fear-related learning. Using a standard fear conditioning paradigm, our group has found that while female rats show similar levels of conditioned inhibition compared with that of their male counterparts, females also exhibited superior fear discrimination in both conditioning and recall tests. Analysis of specialized extracellular matrix structures called perineuronal nets (PNNs) revealed that females displayed a much higher density of PNNs in the basolateral amygdala (BLA) than males, but not in the prefrontal cortex.

INTRODUCTION

Traumatic events or other significant uncontrollable stressors increase a person's vulnerability to developing a mental illness, particularly anxiety and stress-related disorders. Posttraumatic stress disorder (PTSD) is typified by a constellation of symptoms (e.g. flashbacks, nightmares, dissociation, insomnia, etc.) that cause significant distress and interference with daily activities for those living with the disorder. According to the U.S. Department of Veteran Affairs, PTSD has a lifetime prevalence of around seven percent and affects over five million people in the United States alone. However, not everyone who undergoes a traumatic experience will develop PTSD. Risk factors that increase the chance of developing PTSD include prior history of mental illness, history of traumatic brain injury, nature of the trauma experienced, and gender. Women have nearly double the risk of developing PTSD compared to males, and while this may be partially explained by a higher rate of assaultive trauma towards women, social factors alone cannot explain this discrepancy (Breslau et al., 1999b; Fennema-Notestine et al., 2002; Kessler et al., 1995). For such a large variation, there must be a host of biological processes at the origin.

Indeed, the difference in prevalence of PTSD between males and females is mediated by a number of factors including differing gonadal hormone levels, dysregulated neural circuits, and altered neurophysiology. Though discussion of the complete neural networks underlying fear-related learning is beyond the scope of this paper, it will nonetheless examine recent literature on fear circuitry, neurobiology, neuroplasticity, and sex differences found in these domains. Additionally, this paper will review our group's recent experimental findings regarding sex differences in fear discrimination in male and female rats.

Fear Conditioning and Discrimination

Much of fear-related learning relies on classical conditioning and Hebbian learning mechanisms. In fear conditioning models, a neutral stimulus (CS+) is paired with an aversive stimulus (US) such that the CS+ eventually elicits a conditioned response even when presented without the US. For both humans and animals, a complex network of interconnected brain areas helps to mediate fear conditioning, as presentation of the CS+/US activates a number of structures crucial to the acquisition and expression of learned fear (Greco & Liberzon, 2015; Kim & Jung, 2006).

As a structure crucial to the formation of emotional memories and a driver of physiological responses to stressful situations, the amygdala contains numerous afferent and efferent connections to various brain areas. The different processes performed by the amygdala are mediated by different regions, with the central amygdala (CeA) largely mediating behavioral output and the basolateral amygdala (BLA) mediating much of the sensory information processing (Orsini and Maren, 2012). Presentation of dangerous or aversive cues not only activates the amygdala as a whole, but induces neurophysiological changes that help drive fear learning. For example, Ostroff et al. (2010) demonstrated that fear conditioning causes an increase in synapse size, spine size, and post-synaptic density size in a subset of dendrites within the lateral amygdala (LA). These impressive morphological alterations are also accompanied by alterations in resultant neural activity and synaptic strength (Christianson et al., 2012). Both synapse and dendritic spine size influence neuroplasticity, with larger spines associated with enhanced long term potentiation (LTP) and smaller spines associated with enhanced long term depression (LTD) (Matasuzaki et al, 2004; Zhou et al., 2004). Fear conditioning also increases polyribosome numbers and dendritic spine apparatuses, a type of smooth endoplasmic reticulum

structure. These neuroplastic changes suggest that traumatic experiences may influence neurobiology by altering cellular capacity for protein synthesis, as well as dendritic and synaptic morphology.

PTSD patients often show considerable difficulty differentiating between safe and unsafe conditions, leading to exacerbation of hyperarousal symptoms (Jovanovic et al., 2012). Impaired fear discrimination capabilities can result from a number of different factors such as altered activity levels in various brain areas and aberrant neurotransmitter signaling. The orbitofrontal cortex (OFC) is critical to the processing of emotional events, and its inactivation has been correlated with impaired recall of safety signals (Sarlitto, Foilb, and Christianson *unpublished observations*). Surprisingly, the ventral hippocampus, which is involved in the acquisition and modulation of learned fear, is not crucial for fear discrimination nor recall of fearful stimuli (Bast et al., 2001; Chen et al., 2016). Within the amygdala, separate populations of neurons have been found to preferentially respond either safety cues or danger cues (Sangha, 2015). Although extinction training reversed this bias in some neurons, these results provide a direct cellular link to the excitatory-inhibitory balance needed for appropriate discriminative behaviors.

With regards to sex differences, numerous studies have found functional and morphological differences in various brain areas (Labrenze et al., 2015; Lebron-Milad et al., 2012). Within the infralimbic-basolateral amygdala (BLA), males with high levels of freezing following fear conditioning exhibit increased dendritic spine density and shorter spines, whereas females do not show these changes regardless of freezing level (Gruene et al., 2015b). Additionally, males show increased contextual conditioning and higher levels of perforant path LTP within the hippocampus than females. (Maren et al., 1994). Several studies have shown that males show superior context discrimination, whereas females tend to generalize their fear

responses to neutral contexts (Day et al., 2016; Lynch et al., 2013). However, when exposed to chronic stress, females show significantly less extinction impairment than chronically stressed males, and females not exposed to chronic stress (Baran et al., 2009; Ribeiro et al., 2010, Yuen et al., 2016). Serotonin (5-HT)-depleting agents such as para-chlorophenlalanine (PCPA) have been shown to abolish sex differences in contextual fear conditioning and exert anxiolytic effects in males but not females (Naslund et al., 2013; Pettersson et al., 2016). Other data from our group has found that 5-HT_{2C} receptor antagonism enhances fear discrimination and recall of safety signals (Foilb et al., 2016b). Taken all together, this provides a basis for sexually divergent neurotransmitter modulation of neural activity. Furthermore, these data support the idea of developing more targeted therapies that account for sex differences such as these.

Safety Learning

Another hallmark of PTSD is reduced safety learning (Jovanovic et al., 2012). Safety learning, or conditioned inhibition, is the ability to attenuate a fear response when a danger and a safety cue are simultaneously presented (Christianson et al., 2012). Because safety cues (CS-) are not paired with aversive stimuli, they come to predict the absence of danger and thus inhibit fearful behavior.

The various regions of the amygdala show divergent responses to safety signals. The BLA undergoes increased expression of immediate early genes after presentation of safety cues, and the LA has been shown to be both necessary and sufficient for conditioned inhibition (Kong et al., 2014). The CeA, however, is not required for conditioned inhibition (Falls et al., 1995; Kong et al., 2014). This aligns well with the fact that the BLA mediates information processing of emotional environmental stimuli and that the CeA mediates behavioral output of fearful responses. Additionally, conditioned inhibition reduces dendritic spine size and synaptic density

in the LA, thereby decreasing synaptic strength (Ostroff et al., 2010). Electrophysiological recordings obtained from LA neurons show decreased activity when fear-trained mice were simultaneously presented with danger and safety cues (Rogan et al., 2005). These results indicate that conditioned inhibition is at least partially mediated by reduced amygdala excitation and output in the presence of safety signals. Thus, as hyperactivity of the amygdala results in enhanced fearful behavior, a reduction of such activity may underlie and help combat the impaired conditioned inhibition seen in PTSD patients (Jovanovic et al., 2010, 2012).

Although the amygdala is heavily involved in both the consolidation and acquisition of safety signals, it does not act in isolation (Christianson et al., 2012; Kong et al., 2014). Several neural circuits contribute to safety learning, and brain regions involved in conditioned inhibition include the extended amygdala complex, insular cortex (IC), ventromedial prefrontal cortex (vmPFC), basal ganglia, and hippocampus (Christianson et al., 2012). Furthermore, the IC has been highly implicated in the pathology of anxiety disorders and PTSD (Hughes and Shin, 2011; Lin et al., 2013). This finding comes as no surprise considering that the IC receives a wealth of somatosensory information and has wide-reaching afferent and efferent connections with other cortical, thalamic, and amygdala regions (Nieuwenhuys, 2012; Rodgers et al., 2008). The posterior insular cortex, or sensory insula (Si), is especially necessary for safety learning to take place (Foilb et al., 2016a). Normally, the presence of safety signals increases levels of social exploration and mitigates the deleterious consequences of uncontrollable stressors (Christianson et al., 2008, 2011). However, pharmacological lesioning of the Si reduces the beneficial behavioral and physiological effects of safety signals after stressor exposure. Interestingly, the IC does not seem to be critical for the acquisition of fear learning, which suggests that, though similar, both safety and fear learning operate via independent processes (Shi and Davis, 1999).

Sex Hormones and Fear

Female gonadal hormones are known to greatly influence behavior as a whole; however, there is little agreement on their exact effects on fear learning and stress behavior. On one hand, estrogens have been linked to lower levels of freezing (i.e. reduced fear expression), enhanced hippocampal neurogenesis, increased dendritic spine density, and reduced contextual conditioning (Barha et al., 2010; Gupta et al., 2001; Woolley et al., 1993). Intact females have been shown to have nearly double the hippocampal dendritic spine density of males and intact females, with spine density peaking in the proestrus phase (Farrell et al., 2015; Woolley et al., 1993). Additionally, ovariectomized (OVX) female rats treated with estrogen implants often perform comparably to intact females, while OVX females not supplemented with estrogen tend to resemble males in terms of increased freezing behavior (Gupta et al., 2001). Similar to the results reported by Baran et al. (2009), elevated estrogen in the prefrontal cortex (PFC) seems to protect against cognitive impairment during exposure to chronic stress (Yuen et al., 2016). On the other hand, these findings do not help to explain the increased incidence of PTSD and anxiety disorders among females. Several other groups have demonstrated that estrogens impair inhibition of fear, leading to increases in overall freezing levels (Lynch et al., 2013; Toufexis et al., 2007). OVX females not given estrogen replacement exhibit significantly less freezing to neutral contexts, while OVX + estrogen and intact females show substantial context generalization (Lynch et al., 2013). Intact males and OVX females without estrogen replacement have greater hippocampal LTP than OVX females supplemented with estrogen (Gupta et al., 2001). Moreover, estrogens have been correlated with increased capacity for norepinephrine (NE) synthesis and decreased NE degradation (Bangasser et al., 2016). Female rats have not only been shown to have more NE neurons in the locus coeruleus (LC), but also more complex LC

dendritic morphology. The resulting distortion in overall NE tone could lead to heightened autonomic activation and at least partly mediate the morphological and behavioral changes that follow exposure to stressful stimuli. While these findings substantiate claims that sex hormones exert discrete changes within the brain, it may be possible that the role of estrogen in fear behaviors cannot be described in a general sense due to the widespread distribution of estrogen receptors throughout the body. Therefore, a clear link between estrogen and the neural mechanisms that mediate fear and stress-related disorders cannot be drawn at the present time.

In addition to the estrogen controversy, several other counterarguments can be made regarding the validity of studies citing sex differences as a potential factor in the development of PTSD. For one, the accuracy of current animal models to study sex differences in fear expression has been called into question as male and female rats sometimes show very different responses to fearful stimuli (Gruene et al., 2015b). Researchers have recently found that while many females show the same type of freezing behavior that males do, some females are also prone to have more physically active responses (i.e. darting behavior). This observation may explain some of the discrepancies between studies reporting correlations between estrogen and fear, yet this cannot be definitively concluded as none of the studies previous to Gruene et al. (2015a) analyzed darting behavior.

Perineuronal Nets

While not technically part of the neuronal machinery, specialized extracellular matrix (ECM) structures have emerged as important players in the regulation of neural activity. One such type of structure, termed perineuronal nets (PNNs), serves a number of functions within the central nervous system, but is especially associated with the restriction of synaptic plasticity and stabilization of existing synaptic connections (Härtig et al., 1992; Miao et al., 2014; Sorg et al.,

2016). PNNs are characterized as a dense mesh surrounding the soma and proximal dendrites, and preferentially localize to fast-spiking parvalbumin (PV)-containing GABAergic interneurons. However, they have been known to form around glutamatergic pyramidal neurons (Alpár et al., 2006; Carstens et al., 2016; Wegner et al., 2003). PNN levels have been positively correlated with levels of both PV and Kv3.1b subunit-containing neurons, suggesting that PNN clustering around fast-spiking elements has functional significance (Härtig et al., 1999, 2001). Increased PNN expression around GABAergic neurons helps to regulate regional excitatory-inhibitory tone, and prevent activity-induced toxicity via mechanisms described below.

The general structure of PNNs is relatively consistent, with hyaluronan (HA), tenascins (Tn-R), chondroitin sulfate proteoglycans (CSPGs), and linker proteins being the main constituents (Miao et al, 2014). Membrane-bound CSPG receptors help anchor CSPGs to the cell, while Tn-R and linker proteins facilitate and stabilize CSPG-HA cross-linkages. Although, it is worth noting that despite the regular overall structure of PNNs, their *specific* molecular makeup is subject to wide-variation (Sorg et al., 2016). The majority of PNN-associated CSPGs are made up of aggrecan protein cores connected to polysaccharide glycosaminoglycan (GAG) chains, however some can contain a combination of aggrecan, brevican, versican, and phosphocan (Kitagawa & Miyata, 2016; Sorg et al., 2016). Additionally, the number and length of the GAG chains is also highly variable, though the exact reason behind this variation has yet to be elucidated.

The dense mesh formed by these protein aggregates typically emerges 2-4 weeks postnatal, and fully stabilize 6-7 weeks postnatal. During PNN formation, CSPGs undergo changes in sulfation patterns that increase the degree of inhibition on the neuron (Sorg et al., 2016). The 6-O-sulfation typically seen in juvenile brains allows for higher levels of plasticity

compared to the 4-O-sulfation seen in adults, creating more permissive conditions for axonal regeneration after spinal cord lesioning (Lin et al., 2011). The shift towards increased 4-O-sulfation products and resultant decrease in plasticity suggests that sulfation patterns at least partially mediate the closure of critical periods.

Clearly, this modulatory relationship between neurons and PNNs is not purely unidirectional. Sensory deprivation has been shown to extend critical period plasticity and postpone activity-dependent formation of PNNs (Dityatev et al., 2007; Pizzorusso et al., 2002). Dark-rearing of juvenile rats, for example, leads to increased ocular dominance plasticity and inhibits PNN maturation in primary visual cortex. Additionally, inhibition of neurotransmitter release and blockade of Ca^{2+}-permeable AMPA receptors have been shown to decrease the amount of pericellular CSPG aggregation, further supporting the notion of activity dependent formation (Dityatev et al., 2007). Furthermore, implantation of embryonic interneurons into the BLA has been shown to interfere with PNN formation and eliminate already existing PNNs (Yang et al., 2016). This reduced expression of PNNs results in enhanced plasticity of amygdalar circuitry and recovery of fear erasure capabilities commonly seen in juveniles. Unique behavioral phenotypes resulting from juvenile-like plasticity can also be brought about via chemical digestion of PNNs. The enzyme chondroitinase ABC (ChABC) abolishes PNNs in the area of administration, and the PNNs do not fully reform until 60 days after ChABC administration (Lensjø et al., 2016). Gogolla et al. (2010), demonstrated that PNN digestion within the BLA impaired consolidation of fear memories and increased fear memory erasure. Furthermore, PNN destruction in the prefrontal cortex and hippocampus has been shown to cause impaired consolidation of fear memories and context specific memory, respectively (Hylin et al., 2013). One possible explanation is that the altered intrinsic excitability levels resulting from

ChABC administration promote decoupling of the synaptic connections established during fear conditioning, thus facilitating full memory erasure (as opposed to extinction) and reducing memory consolidation.

PNNs are by no means static structures, and despite the stabilization that takes place after critical period closure, they are still subject to alteration. Chronic fluoxetine treatment, for example, has been shown to reduce both PV and PNN levels in the frontal cortex of adult mice (Ohira et al., 2015). Similarly, recurrent binge drinking in young adult mice leads to increased PNN expression in the insular cortex, but not primary motor cortex or anterior cingulate cortex (Chen et al., 2015). Studies on the role of PNNs in critical period plasticity provide a basis for modification of mature PNNs after the synaptic connections and the ECM have been established. Moreover, these results support the notion that neural activity continues to influence PNN expression even after maturation is complete. Although kainite-induced seizures have been found to increase levels of endogenous enzymes known to degrade PNNs, few studies have examined the effect of normal neural activity on mature PNN morphology or expression (Wang & Fawcett, 2012). One group has found increased levels of cells double-labeled for c-Fos and PNNs after exposing rats to a drug-associated context (Slaker et al., 2013). They posit that the increase in c-Fos in these PNN-associated neurons may be a compensatory response to the heightened pyramidal cell firing brought on by the context, however the long-term effects of increased c-Fos on PNNs were not examined. Perhaps long term changes in neural activity lead to the production of molecules that promote PNN remodeling, however, because the exact mechanisms mediating neuronal modulation of PNN formation are not well understood, more research is needed to clarify this relationship.

PNNs exert their effects on synaptic plasticity and neural activity in a multitude of ways. In addition to serving as a physical barrier to the formation for new synaptic contacts, PNNs have been found to impede neurite outgrowth and axonal sprouting (Wang & Fawcett, 2012; Wang et al., 2011). ChABC treatment in animals with spinal cord lesions increased generation of new axonal branches and enhanced functional recovery. The restriction of axon collateral generation is, in part, due to the fact that CSPGs impair axonal mitochondria function, a key part of the regulation of actin dynamics and filopodia formation (Sainath et al., 2016). CSPGs cause slight depolarizations in the membrane of axonal mitochondria, which leads to downstream inhibition of actin polymerization and transport of contactin. Acetyl-L-carnitine administration has been shown to counteract the deficits induced by CSPGs, presumably by upregulating mitochondrial respiration. PNNs also influence neural activity by acting as an ionic buffer around fast-spiking neurons (Härtig et al., 2001, 1999). CSPGs are highly negatively charged and inhibit free diffusion of cations. Creation of a buffering environment in the immediate vicinity of areas with high concentrations of ion channels (e.g. initial axon segment) allows CSPGs to act as cation exchangers and circumvents the need for other compensatory mechanisms against free ion diffusion. In addition to serving as a repository of sorts for Na^+ and K^+ ions, PNNs help to sequester proteins known to affect plasticity. PNNs facilitate the transport of neuronal activity-regulated pentraxin (Narp) across the synaptic cleft and PNN digestion has been shown to interfere with Narp-PNN colocalization (Kitagawa & Miyata, 2016). Narp binds to GluR4 receptors post-synaptic to PV-positive cells, which results in subsequent upregulation of GluR4 expression and increased availability of sites for excitatory synaptic inputs. The chemorepulsive axon guidance molecule semaphoring 3A (Sema3A) is another plasticity-modulating molecule and binds to CSPGs (Winter et al., 2016). High levels of pericellular

Sema3A are thought to impair the formation of new synaptic contacts and thus restrict plasticity. Finally, PNNs have been shown to inhibit lateral diffusion of AMPA receptors (Frischknecht et al., 2009; Sorg et al., 2016). Restriction of AMPA receptor mobility may contribute to stabilization of already existing synapses, as well as reduce short-term plasticity and the ability to consolidate new synaptic contacts.

A growing body of evidence has implicated PNN malformation and dysfunction in a number of disease pathologies such as schizophrenia, Alzheimer's disease, bipolar disorder, autism spectrum disorder, and epilepsy (Kitagawa & Miyata, 2016). Many of the aforementioned mechanisms of restricted synaptic plasticity also serve neuroprotective or important regulatory functions crucial to the formation and maintenance of healthy neural connections. With respect to Alzheimer's disease, neurons surrounded by PNNs are less likely to be damaged by β-amyloid or neurofibrillary tangle accumulation, and areas expressing high levels of PNNs tend to show less degeneration than areas with lower expression levels (Kitagawa & Miyata, 2016). It is thought that the structural barrier formed by PNNs helps to counteract the oxidative properties of the β-amyloid protein accumulations and that the antioxidant properties of aggrecan-containing CSPG chains help to reduce levels of reactive oxygen species. Moreover, limiting the intrinsic excitability of GABAergic cells helps prevent activity-induced toxicity and oxidative stress. Yet it should be noted that PNNs do not provide absolute protection from the neurodegenerative effects of Alzheimer's disease. Findings by Li et al. (2017) have demonstrated that tau pathology enhances disruption and reorganization of the ECM. Not only does this counteract PNN's neuroprotective properties, but also leads to subsequent inhibition of PNN formation and neural plasticity.

Despite the literature supporting the role of PNNs in fear-learning, little research has been conducted on their role in mental illnesses such as PTSD or anxiety. Though it's unlikely that widespread PNN digestion will be used as a therapeutic intervention given their varying roles in neural function and structural support, treatments promoting graded reductions in PNN expression could one day have clinical relevance. The resultant increase in synaptic plasticity may allow patients to overcome anxiety-inducing associations and bolster a rewiring of the aberrant neural circuitry underlying their symptoms. Thus, continued study of the relationship between extracellular matrix structures and neuronal activity, as well as the effects exogenous drug administration on such structures will provide invaluable insight into modulation of neural circuitry and creation of effective therapies.

Research on ECM modulation of neural activity has expanded substantially within the past decade, although few studies have examined sex differences in PNN levels. One study by Cornez et al. (2015) examined PNNs in zebra finches and found that males expressed higher levels of PNNs in certain nuclei mediating song-production. However, no other animal studies have analyzed similar sex differences. In our experiments, we sought to relate known sex differences in fear learning to PNN expression in areas that have been highly implicated in PTSD pathology. Based on pilot study data and literature suggesting that males exhibit greater amounts of conditioned fear than females, we hypothesized that males would show higher levels of PNN expression in the amygdala, but lower levels in the PFC compared to females (Barker et al., 2010; Gruene et al., 2015b). To do this, intact male and normally cycling female rats were given fear discrimination training, followed by summation testing 24 hours later. At the conclusion of our behavioral experiments, animals were sacrificed and PNN expression was analyzed in the BLA, PL, and IL.

MATERIALS AND METHODS

Animals

Male and female Sprague-Dawley rats (M = 34, F = 34) were housed in plastic tub cages (2 animals per cage) under a 12:12 light/dark cycle. Males and females were kept in the same vivarium and were allowed 7-10 days to acclimate to new environment. Food and water were given *ad libitum*. All procedures were approved by the Boston College Institutional Animal Care and Use Committee and complied with National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Apparatus

All behavioral conditioning and testing was conducted in black plastic chambers with wire-mesh lids (10 x 11 x 6-in; L x W x H) and stainless steel shock grids (Model H10-11R-TC-SF, Coulbourn Instruments, Whitehall, PA). A 15 x 12 x 27-in (L x W x H) enclosure surrounded each chamber, with ventilation and masking noise (~55dB) provided by a small fan. Each enclosure was equipped with two infrared LED lights (CMVision Model IR30) and one overhead camera (Model VX-5000, Microsoft, Redmond, VA). Cameras were modified with infrared passing filters to allow for the detection of infrared light, and freezing level analysis using ANY-Maze software (version 4.98, Stoelting, Wood Dale, IL). A flashing white LED light array (Model LPL620WTHD) provided the visual cues and speakers affixed to the top of the enclosure provided the auditory cues.

CS+/CS- Conditioning

Fear conditioning consisted of 15 presentations of both the CS+ (paired with footshock) and CS- (unpaired) cues in a quasi-random order so that no cue was presented more than 2 times in a row. A 5s 1kHz tone (75dB) was played before each CS presentation, after which a 15s presentation of the CS took place. 15s auditory (white noise pips, duration = 10 ms, rate 3 Hz, 75 dB) or visual (flashing Led light, 264.0 Lux, 20 ms on/off) cues served as the CS. Footshock (500ms 1.2 mA) coterminated with each presentation of the CS+. An inter-trial interval of 70s separated each CS-trial, such that the conditioning sessions lasted 45 minutes. All conditioning sessions occurred in the morning.

Summation Testing

A summation test was used to assess both fear discrimination and conditioned inhibition. After 2 minutes of baseline context exposure, CS+, CS-, and CS+/- (simultaneous presentation of the CS+ and CS-) cues were presented for 30s in quasi-random order. Experiments 1-4 consisted of 15 presentations of each cue. Experiments 5-6 consisted of 3 presentations of each cue. All summation tests were given during the afternoon, approximately 24 hours after training.

Perineuronal Net Analysis

Animals were anesthetized with tribromoethanol and transcardially perfused with 4% formaldehyde solution. Brains were removed and stored in 30% sucrose solution. Brains were flash-frozen in 2-methylbutane in dry ice and 40 μm sections (B+5.26mm—B-6.12mm) were obtained with a cryostat (-20ºC). 1 out of 10 slices was placed on slides and coverslipped. Immunohistochemistry was performed at room temperature on BLA (B-1.56mm—B-3.36mm) and PFC (B+3.72mm—B+2.7mm) slices. Sections were washed in PBST (10% stock) and blocked in 5% normal goat serum. Sections were then incubated in WFA (1:400) overnight at 4ºC. Sections were mounted onto unsubbed slides with Vectashield Hardset with DAPI. Tiled

20x (BLA) or 10x (PFC) images were acquired with a Zeiss AxioImager Z2 microscope, and PNN numbers were obtained with ImageJ software (National Institutes of Health). To measure a PNN density, a PNN : area ratio was calculated by dividing the number of PNNs by the area (pixels) of the region in question. In a select number of sections, stain intensity was analyzed using the ROI method detailed in Slaker et al. (2016). Due to the small subset of sections that were eligible for ROI method implementation, these data were not used in our interpretation.

Statistics

Two-way analysis of variance (ANOVA) was used to analyze by-trial behavioral data, as well as freezing scores averaged across entire testing and conditioning sessions, with sex as a between-subjects variable and cue as a within-subjects variable. For by-trial data, freezing levels to the CS+, CS-, and CS+/- were averaged in trial blocks from conditioning and testing sessions. Data was aggregated and sorted by cue presentation and each block contained data from 45s of cue presentation. During summation testing, cues were presented for 30s and data was analyzed in a similar fashion as above. For analysis of PNN density and discrimination index data, a student's t-test was used. Statistical analyses were performed using GraphPad Prism 7.

RESULTS

Fear Discrimination

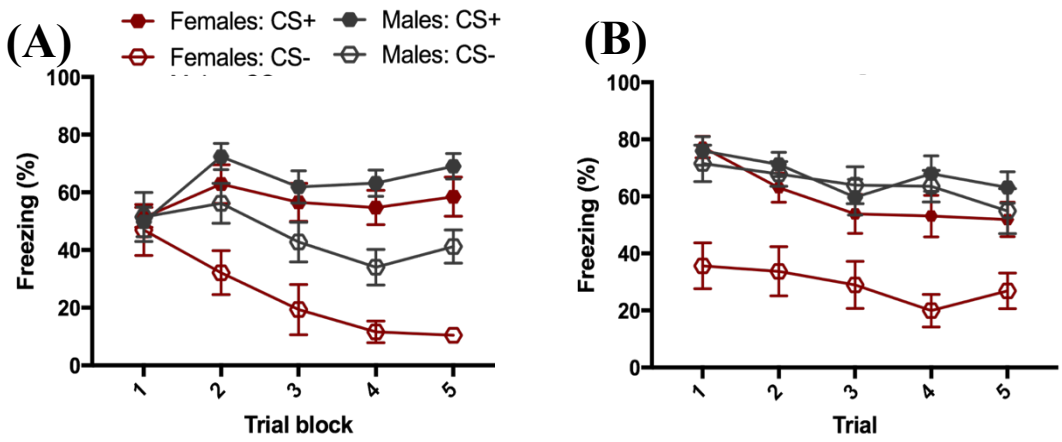

Figure 1. ***(A)*** *Conditioning Session 1: Freezing averages (+SEM) to the CS+ and CS- averaged across trial blocks (3 cue presentations). Females rapidly reduce freezing to the CS- compared to males.* ***(B)*** *Conditioning Session 2: Freezing averages (+SEM) to the CS+ and CS- averaged across trial blocks (3 cue presentations). Females show consistently low freezing to the CS-, and males do not inhibit fear to the CS+.*

Time spent freezing was analyzed in trial blocks for each 15s CS presentation, with each session containing 5 blocks. Each trial block consisted of 3 cue presentations, and freezing was determined by comparing freezing levels to the CS+ compared to the CS-; a significant difference in freezing between the two indicates the presence of fear discrimination. On Day 1 of conditioning (Figure 1A), discrimination was significant in females in trials 2-5 (T2 $p = 0.0032$; T3 $p = 0.0002$; T4, T5 $p < 0.0001$). Males showed discrimination in trials 4-5 (T4 $p = 0.0059$; T5 $= 0.0094$). On Day 2 of conditioning (Figure 1B), females discriminated throughout the training session (T1 $p < 0.0001$; T2 $p = 0.0079$; T3 $p = 0.0336$; T4 $p = 0.0019$; T5 0.0326). Males, however, did not show discrimination in any trial.

The data from Day 1 of conditioning across all experiments were aggregated (Figure 2) and analyzed in trial blocks in the same manner as the data in Figure 1. Females showed discrimination in all trials (T1 $p = 0.0419$; T2-T5 $p < 0.0001$), and reduced freezing to the CS- compared to males (T1 $p = 0.0233$; T2-5 $p < 0.0001$). Males discriminated in trials 2-5 (T2 $p = 0.0127$, T3-5 $p < 0.0001$). Females also showed reduced freezing to the CS+ in trial 5 ($p = 0.0136$).

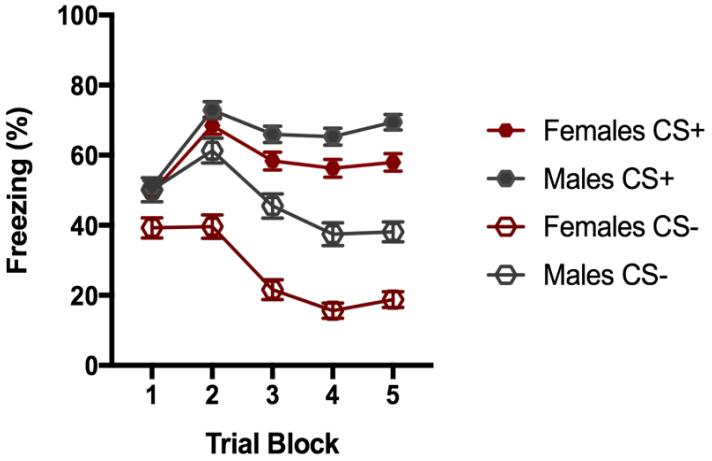

Figure 2. Aggregated freezing averages (+SEM) for Day 1 of conditioning to the CS+ and CS- (across all experiments).

On Day 1 of recall testing, freezing scores averaged across the testing session (Figure 3A) show that females show reduced fear to the baseline context compared to males ($p < 0.0001$) and the CS- ($p = 0.0003$). Freezing scores to the CS+ and CS- were compiled from all experiments and averaged across trial blocks (Figure 3C). Females exhibited robust discrimination across all trials (T1-T10 $p < 0.0001$), and males showed discrimination in trials 2-10 (T2 $p = 0.0047$; T3 $p = 0.0031$; T4 $p = 0.0004$; T5 $p = 0.0021$; T6-T10 $p < 0.0001$). Females also showed reduced freezing to the CS- compared to males on trials 1-6 (T1-T2 $p < 0.0001$; T3 $p = 0.0049$; T4 $p = 0.0011$; T5 $p = 0.0001$; T6 $p = 0.0105$) and on trial 8 ($p = 0.021$). A discrimination index was computed by dividing the freezing scores (averaged across test

sessions) to the CS- by the CS+ (Figure 3B). Higher discrimination indices indicate impaired ability to differentiate between danger and safety cues, while lower discrimination indices indicate greater fear discrimination, and safety signal recall. The data from our female animals falls into the latter category, and further supports the data in Figures 3A and 3C.

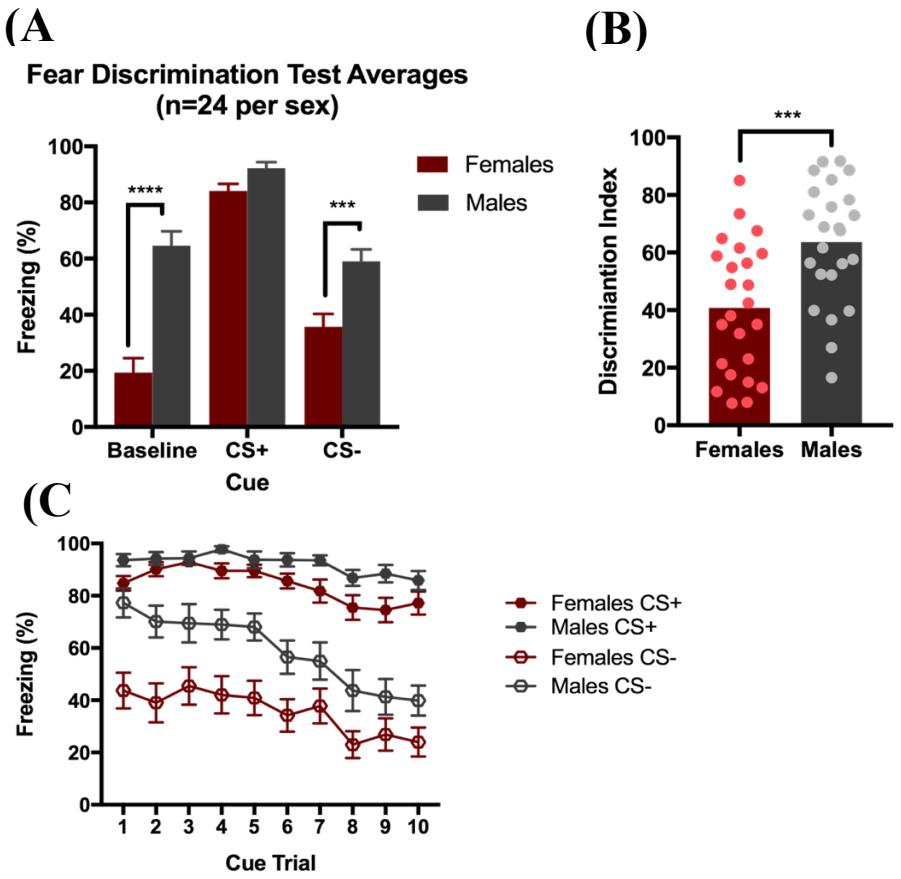

Figure 3. ***(A)****Aggregated freezing averages (+SEM) for day 1 of recall testing to the baseline context, CS+ and CS- (across all experiments). Females show less freezing to baseline context and the CS-.* ***(B)*** *Discrimination index across day 1 of all recall tests.* ***(C)*** *Aggregated freezing averages (+SEM) to the CS+ and CS- of day 1 of recall testing averaged across trial blocks.*

Summation Testing (ST)

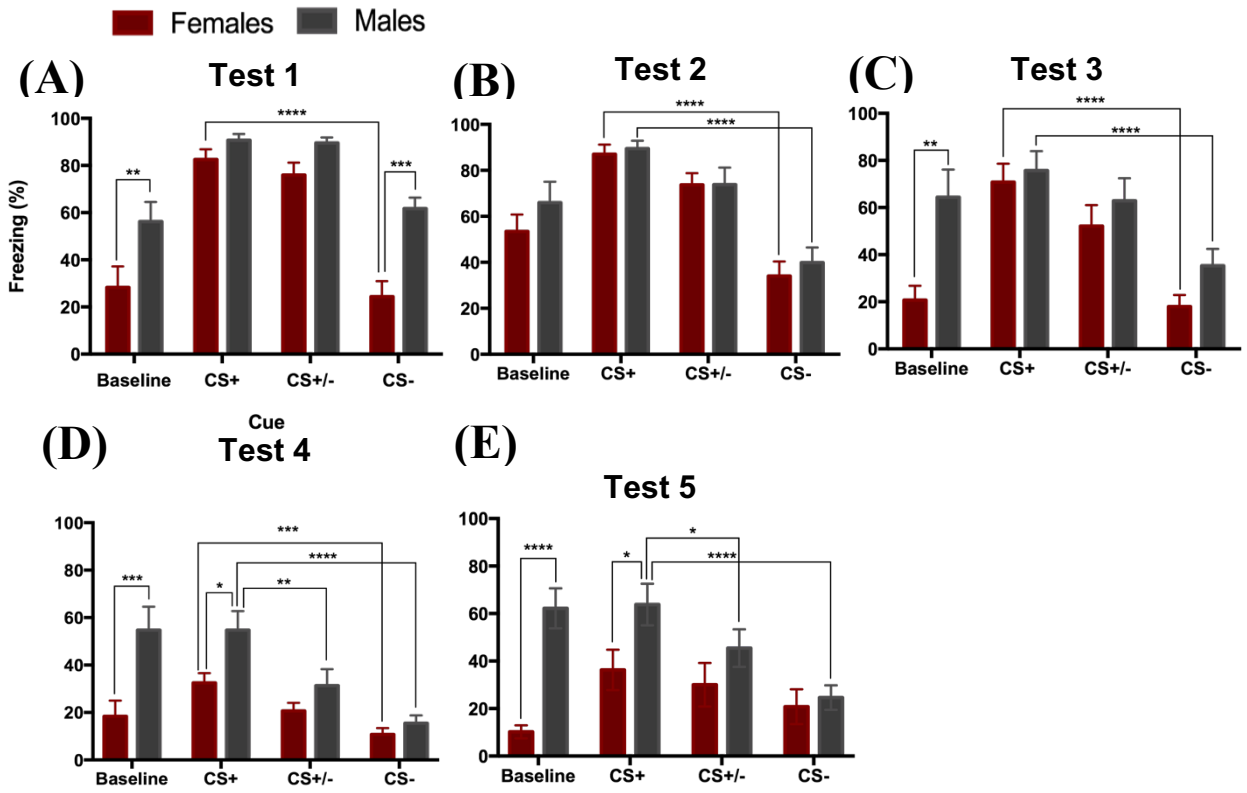

Figure 4. ***(A-E)*** *Freezing averages (+SEM) to the baseline context, CS+, CS-, and CS+/- cues for each summation test.*

In summation tests (Figure 4A-4E), females show superior recall of safety signals and reduced contextual fear conditioning, mainly during the initial tests. In test 1 and tests 3-5, females froze significantly less to the baseline context (ST1 $p = 0.0045$; ST3 $p = 0.0015$; ST4 $p < 0.0001$; ST5 $p = 0.0003$) males. Females froze much less to the CS-, but only during test 1 ($p < 0.0001$). Females exhibited discrimination in all tests, except test 4 (ST1-ST3 $p < 0.0001$; ST5 $p = 0.009$), while males showed discrimination in all tests except test 1 (ST2-5 $p < 0.0001$). In later sessions (tests 4 and 5) females show reduced freezing to the CS+ compared to males (ST4 $p = 0.047$; ST5 $p = 0.048$). Males showed conditioned inhibition, but only during tests 4 and 5 (ST4

p = 0.0224; ST5 p = 0.0043). Females, however, did not show conditioned inhibition in any testing session.

Perineuronal Net Density

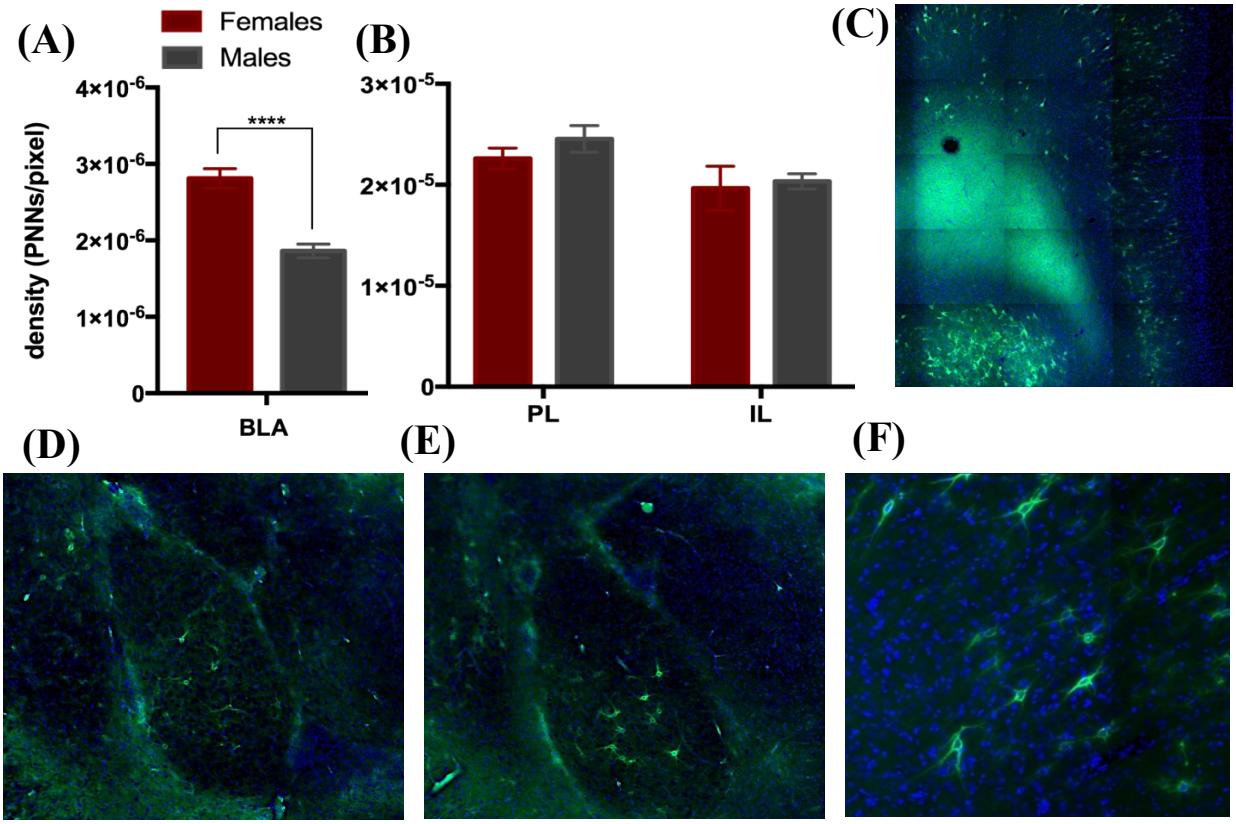

Figure 5. ***(A)*** *PNN density in the BLA calculated as PNNs per square pixel.* ***(B)*** *PNN density in the prelimbic (PL) and infralimbic (IL) cortices.* ***(C)*** *10x Tiled images of sample PFC section including PL/Il. Nuclei stained with DAPI (blue). PNNs and nonspecific white matter stained with WFA (green).* ***(D-E)*** *20x tiled images of male (D. left) BLA and female BLA (E. right) showing sex difference in PNN (green) density.* ***(F)*** *PNNs (green) surrounding cell bodies, proximal dendrites, and initial axon segments in PL.*

Immunohistochemisty in PFC and BLA revealed successful labeling of PNNs and nuclei (Figures 5C, 5F). Student t-tests revealed that females had a higher PNN density in the BLA compared to males ($p < 0.0001$), but no sex difference was observed in the PL or IL (Figure 5A-B). This sex difference is also evident in the sample BLA sections shown in Figure 5D and 5E.

DISCUSSION

After only a few cue presentations, our data show that females are able to distinguish between danger and safety cues with more accuracy than males while still demonstrating comparable levels of fear to the CS+ (Figures 1A & 2). This reduced freezing persists into following conditioning sessions (Figure 1B) and recall tests (Figures 3 & 4). Day et al. (2016) similarly reported that females acquire safety signals more rapidly than males did, but also found that females later generalized their fear whereas males did not. One could interpret the females' rapid decrease in freezing to the CS- during the first conditioning session as evidence of differential learning between males and females. However, one significant caveat is that these results could also reflect a difference in the ability to express learned safety rather than a difference in acquisition and/or recall capabilities.

Previous work by our group has found that conditioned inhibition does not take place until later testing sessions (Foilb et al., 2016a). In the results of this study, males showed conditioned inhibition during tests 4 and 5, but this effect was not seen in females during any testing session (Figure 4). This absence of conditioned inhibition in females may be partly due to a floor effect and the generally low levels of freezing in later testing sessions. During summation testing, all animals decreased their freezing to each cue with each progressive testing session, which may be due to extinction (Figure 4). It is possible that the animals learned that the absence of foot shock at the beginning of the session indicated that no shocks would be administered at all, almost as its own safety cue. The fact that females showed significant fear discrimination but not conditioned inhibition supports the notion that these processes occur via different neural mechanisms and that these mechanisms operate differently in males and females.

While a significant body of evidence has implicated PNNs in the neurobiology of fear, and while numerous studies have established sex differences in fear learning, few groups have proposed a possible link between the two. Our results show that while no sex difference was observed in the either of the PFC subdivisions, males showed a significantly lower density of PNNs within the BLA compared to that of females. These results ran contrary to our original hypothesis that the impaired acquisition and recall of safety cues seen in males would be correlated with higher PNN expression. In this line of thinking, it was supposed that higher PNN levels (which were also assumed to be mostly co-localized to GABAergic interneurons) would result in increased inhibition of these inhibitory cells, thus releasing other excitatory elements from inhibitory control. The resultant increase in excitation would have provided a reasonable explanation for the increased freezing levels seen in males. In light of our actual data, this reasoning obviously no longer stands.

Given the wide-reaching behavioral and cellular effects of PNNs, the sex differences in PNN density seen in our results may be due to a multitude of factors. One potential explanation for the sex differences in PNN density and fear learning is that increased PNN expression in females causes a reduction in neuronal intrinsic excitability and subsequent alteration in excitatory-inhibitory tone. Upregulation of inhibitory elements may help to reduce “background noise” and facilitate superior fear discrimination. Additionally, it is possible that due to the reduced excitability of PNN-associated cells, safety cues are less likely to cause sufficient excitation of these GABAergic interneurons. The resulting decrease in inhibition would then release post-synaptic glutamatergic neurons from inhibitory control. As mentioned before, the BLA is important for the processing of danger and safety cues rather than just the output of fear responses (which is mostly mediated by the CeA and brainstem structures). Increases in

glutamatergic transmission within the BLA could facilitate superior Hebbian learning processes and LTP in non-PNN associated neurons. Additionally, it may be possible that danger cues, which are much more salient than safety cues, override PNN-mediated inhibition in the BLA, and interfere associative learning processes about safety cues. In short, PNNs may act as a dimmer on a light rather than a simple on-off switch. By inducing graded inhibition, PNNs may create favorable conditions for better processing of environmental stimuli and discrimination between danger and safety.

Other putative cellular mechanisms may also help to explain our behavioral results, but likely do not act in isolation. As previously mentioned, estrogen has been shown to augment dendritic spine density, which is correlated with improved learning capabilities (Farrell et al., 2015; Woolley et al., 1993). Furthermore, PNN formation is thought to be highly activity dependent (Dityatev et al., 2007). Therefore, higher levels of neural activity due to increased spine density may exert positive effects on PNN number, density, and thickness. Again it should be noted that the effects of estrogen are highly disputed, and no studies to date have been conducted on gonadal hormone regulation of ECM structures. Even if estrogens were shown to affect PNN numbers and morphology, PNN modification past critical period closure is still a poorly understood topic, so it is difficult to make conjectures on their precise effects. Another putative cellular mechanism involves PNN sequestration of Narp to its associated neuron. Since Narp is eventually transported across the synaptic cleft and upregulates GluR4 expression on the postsynaptic neuron (which is mostly likely a glutamatergic pyramidal neuron), increased PNN expression could cause increased glutamatergic transmission via increased GluR4 expression on excitatory neurons (Kitagawa & Miyata, 2016). Although, as previously mentioned, Narp is not the only plasticity-modulating molecule sequestered by PNNs. Other molecules such as Sem3A

would likely attenuate the effects of Narp, so it is unlikely that increased Narp localization to PNN-associated neurons would produce the sex differences seen in our behavioral data.

Though numerous studies have tied PNNs to reduced neural plasticity, several groups have demonstrated that PNNs are actually necessary for adequate consolidation and acquisition of certain types of memories. For example, both Hylin et al. (2013) and Gogolla et al. (2009) showed that PNNs support the consolidation of fear memories. Furthermore, destruction of PNNs in the medial prefrontal cortex (mPFC) has been shown to impair the acquisition and maintenance of cocaine-induced conditioned place preference, as well as decrease inhibition of pyramidal neurons (Slaker et al., 2016). Moreover, the pattern of holes in PNNs are thought to contribute to the stabilization of memories, although this effect was only analyzed in the context of very-long term memories (Tsien et al., 2013). These findings refute the general notion that the altered plasticity caused by PNNs leads to reduced learning and provides a potential explanation for the rapid acquisition of safety signals seen in our females.

It should be emphasized that PNNs are modulators of synaptic plasticity, not absolute controllers. Likewise, the findings regarding PNNs' effects on neural activity are not in total agreement. PNN removal has been correlated with increases in fast-rhythmic activity in inhibitory interneurons in the anterior cingulate cortex (ACC) (Steullet et al., 2014). On the contrary, PNN removal in the primary visual cortex (V1b) has also been linked to reduced activity of inhibitory neurons and increase spiking variability (Lensjø et al., 2016). This suggests not only that the consequences of increased PNN expression are region specific, but that there may be other factors contributing to the overall effect of PNNs on neurons and neural circuitry.

Nonetheless, mammalian fear circuitry is not limited to the BLA, PL, or IL. Follow-up studies on PNN expression in other areas like the IC, periaqueductal gray (PAG) and

hypothalamus would expand our current understanding of the role of PNNs in fear and anxiety behaviors (Canteras, 2002; Watson et al., 2016). Additionally, a major assumption of most experiments (including our own) is that only a negligible amount of PNNs will co-localize with non-inhibitory, PV-negative neurons. In order to totally rule out the contribution of glutamatergic-PNN associations, immuno-assays for GABA, PV, and/or glutamate would need to be conducted. Although the majority of PNNs will localize to GABAergic cells, co-localization with glutamatergic cells occurs throughout the cortex and subcortical regions (Alpár et al., 2006). PNNs surrounding excitatory pyramidal neurons also tend to be thinner and more diffuse than PNNs surrounding inhibitory interneurons (Wegner et al., 2003). These morphological differences undoubtedly produce differential effects on cellular activity and the attenuated thickness of the pyramidal PNNs likely exerts less inhibitory control over their associated cells. The specific morphology of the PNNs in our experiment was not examined, yet in conjunction with data from immuno-assays, the results of such analyses would allow for the formulation of a more concrete mechanism of PNN modulation of fear expression. Moreover, additional follow-up experiments on the effects of PNNs on LTP and action potential kinetics would provide a more complete understanding the overall contribution of PNNs to learning processes.

While it is tempting to reduce PTSD to the product of a few aberrant neurobiological processes, the etiology of this disorder is enormously complex, drawing from genetic mechanisms, trauma severity, environment, and several dysregulated neural circuits. Our knowledge of the biology of PTSD as a whole is still incomplete; however, several studies suggest that altered activity levels of various cortical and subcortical areas may play a large role. Still, this does not explain the greater prevalence of PTSD among women compared to men.

In addition to the obvious differences in gonadal hormone levels between males and females, numerous studies, including our own, have provided neurobiological evidence of sex differences in behavior and brain activity tied to mental illnesses. The altered serotonergic and noradrenergic mediated activity can exacerbate anxiety-like behaviors and negative behavioral responses to stressful stimuli, which may explain the hyperarousal symptoms more frequently reported by women with PTSD. Aside from the fact that estrogen does have an effect on neurobiology, there is little agreement within the scientific community regarding the link between estrogen and fear-related behaviors. Female sex hormones do play a part in neuroplasticity, and the reduced hippocampal neuroplasticity due to gonadal hormone levels may contribute to the impaired fear extinction and inhibition present in individuals with PTSD. Finally, specialized extracellular matrix structures have shown to be powerful regulators of synaptic plasticity and activity. The fact that some findings regarding their mechanism of action seem to be at odds reflects a lack of complete understanding of their effects on neurobiological systems. Our findings regarding sex differences in PNN density in the BLA are among the first to demonstrate sex-dependent expression of these structures. In light of these results and well-established behavioral data showing differential fear learning between males and females, subsequent studies on the effects of PNN digestion are needed to fully elucidate their role in fearful behaviors. In conjunction with continued research on the etiology of PTSD and fear-learning in females, such studies may pave the way for future pharmacological therapies.

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