Variation in GABAergic Interneuron Morphology in Relation to Anxiety

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Abstract: The amygdala, particularly the basolateral amygdala, has been implicated in the manifestation of several anxiety-related disorders, such as generalized anxiety disorder and PTSD. The amygdala itself is necessary to form associations with frightful stimuli and emotional responses to them. Decreased GABA receptor activity in BLA has been associated with increased severity of fear responses and vice versa. With the recent discovery that distinct GABA interneuron morphologies exist, questions have arisen concerning their effect on BLA activity and subsequent anxiety-like behavior. If a relationship does exist, GABA interneuron morphology could become a marker by which at-risk individuals could be identified and provided preventative treatment. This study hypothesized that Wistar rats with more spiny compared to aspiny GABAergic interneurons would freeze more when administered a fear conditioning task. Our data supported our hypothesis as well as its inverse. This can be explained by the large spine density of spiny interneurons allowing for more feedback inhibition to BLA. However, the data provided by this study is only correlational, and it is not possible to tell which factor is impacting the other. A third variable may also be implicated in affecting both. Future research should also explore potential sex differences.

Introduction

Disorders attached to apparent hyperactive amygdala activity are abundant and manifest in a variety of permutations. There is plenty of evidence that the amygdala is implicated in the formation of disorders like PTSD and generalized anxiety disorder; however, the mechanisms by which these disorders arise are not well known. Continuing research into the functions of the amygdala, specifically the basolateral amygdala (BLA), can provide information that can facilitate the development of more effective treatments as well as technology or methods to identify at-risk individuals.

While both the hippocampus and the amygdala have roles in general emotional acquisition and expression, there is evidence that the amygdala is more strongly associated with responses to stressful or frightful stimuli and the formation of fear associations. Lesions to the amygdala disrupted freezing responses to both explicit and contextual cues; lesions to hippocampus interfered only with contextual fear responses and cued CS responses were comparable to non-lesioned subjects (Phillips and LeDoux, 1992). This implies that the amygdala is necessary for the creation of fear associations to specific stimuli.

Decreased GABA receptor activity, which mediates inhibition within BLA, has been linked to increased severity of fear responses. A study done in 2005 explored the effects of previous stressors on fear responses and basolateral amygdala in Wistar rats. In their first experiment, they found that previously restrained rats experienced comparatively lower activity in the GABA receptors of BLA during a fear conditioning task (Manzanares et al., 2005). There is also evidence that high anxiety may indicate a deficiency in the systems that mediate activity of GABA in BLA. Lehner and her colleagues found that rats treated with midazolam (MDZ) experienced decreased severity in fear responses, expressed in this experiment as fewer and shorter freezing during a contextual fear test (2010). Dopamine D3-like receptors in BLA have

been implicated in decreased activity in GABAergic interneurons; conversely administering dopamine D3 antagonists to BLA lead to increased GABAergic transmission and less severe fear responses (Diaz et al., 2011). On the other hand, a related study found that noradrenergic facilitation of GABA transmission was specifically attenuated by fear conditioning (Skelly et al., 2016). It seems that outside stimuli can affect activity in GABAergic interneurons, but the opposite is also true, with circulating neurotransmitters affecting responses to frightful stimuli. It is more than likely the case that a feedback loop makes this possible.

A vast majority of the literature concerning the functions of the BLA has concerned activation. While there has been coverage of the broad inhibitory mechanisms within it, mechanisms on the neuronal level are not well understood. However, a recent study concerning the basolateral amygdala has found two distinct morphological categories of GABAergic interneuron morphology: spiny and aspiny (Klenowski et al., 2015). Prior to this experiment, it was believed that interneurons had far fewer, if any, spines in comparison to principal neurons in BLA. However, this study found that some interneurons, dubbed "spiny," had spine densities comparable to nearby principal neurons (Klenowski et al., 2015). Further *in vivo* studies indicated that different interneuron types have distinct spiking behaviors in response to different brain states and stimuli (Vereczki et al., 2016). In conjunction, these findings imply that GABA interneurons may have more impact on BLA than previously expected, and their morphology may have implications concerning variation in BLA inhibition across individuals. If so, exploring the possible effects of GABAergic interneuron morphology could offer another route to prescreen individuals and enact preventative measures.

This study seeks to explore the relationship between anxiety and inhibitory synaptic connectivity using animal models. In particular, we aim to find associations between GABAergic

interneuron morphology in the basolateral amygdala and recorded individual anxiety level. We hypothesize that individuals with more spiny compared to aspiny interneurons in BLA, they will express less severe fear responses. Likewise, if an individual has an aspiny-biased interneuron composition, they will exhibit more severe fear responses.

Methods

Subjects

The subject pool used in this experiment will be as similar to the sample used in the original Klenowski et al. study from 2015 to ensure that if the morphological differences are not found, it is not related to the species or previous treatment of the sample.

The sample consisted of forty 8-week-old Wistar rats. The sex ratio of the sample was not calculated. The rats were paired in cages with one other same-sex member in a 12 hour light/dark cycle. Food and water were provided *ad libitum*.

Materials

The fear conditioning portion of this experiment required a rodent conditioning chamber enclosed by a sound-attenuating cubicle. The experimenters provided no discerning markings or textures inside the conditioning chamber outside of those innate in the chamber itself. The sound-attenuating cubicle is necessary for providing the conditioned stimulus.

A metal bath filled with high-Mg2+ Ringer solution kept at an ice-cold temperature was used to preserve the brains once they were removed and the coronal sections once they were cut. The sections themselves were taken using a vibratome while the sections were still submerged.

Neurobiotin (NB) was used to clear away cell membrane and fill the interneurons for further study.

Procedure

Fear Conditioning Task

We began by categorizing the subjects as either high anxiety (HA) or low anxiety (LA). Fear responses were measured specifically using an aversive classical conditioning experiment and recording the freezing response across trials. High anxiety subjects experience the least amount of change in freezing response across trials while low anxiety subjects experience a significant amount of change across trials.

The subjects are placed inside the conditioning chamber alone across a total of six days and one day to preceding the trials to get them used to the chamber without the stimulus; this pre-trial only lasts about 20 minutes. On the first and second day of trials, the subjects are placed inside the chamber for about 45 seconds, the last 500 milliseconds of which a tone is played. Two trials happen each day with a delay of about 90 seconds between each trial. The third through sixth days of trials are extinction trials which take a total of 45 seconds. There is only one trial on each day. Those with comparatively long periods of freezing across all trials are labelled high anxiety, and those with comparatively short periods of freezing are labelled low anxiety.

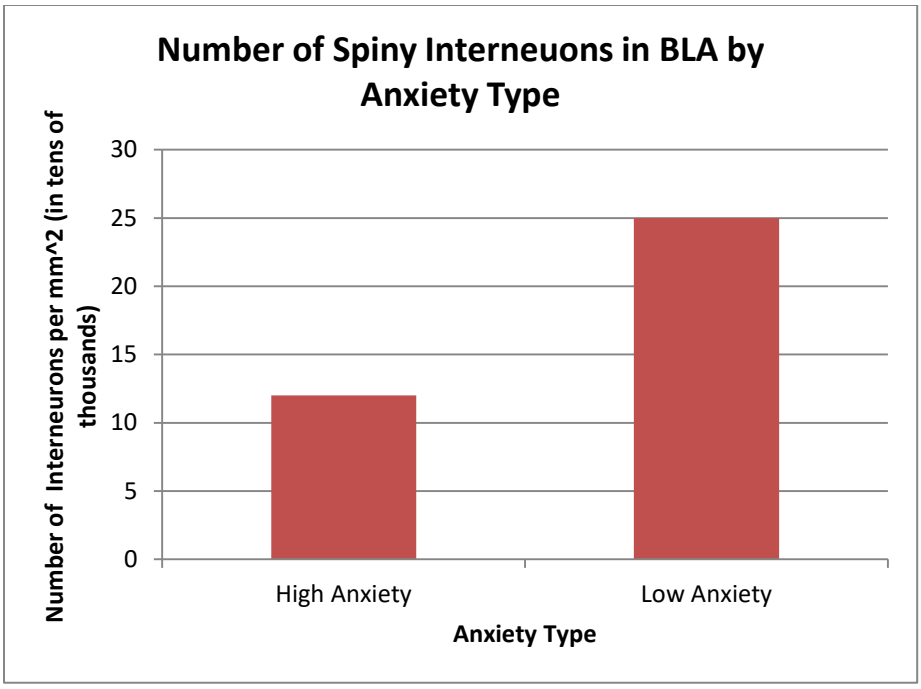
NB Electroporation and Imaging

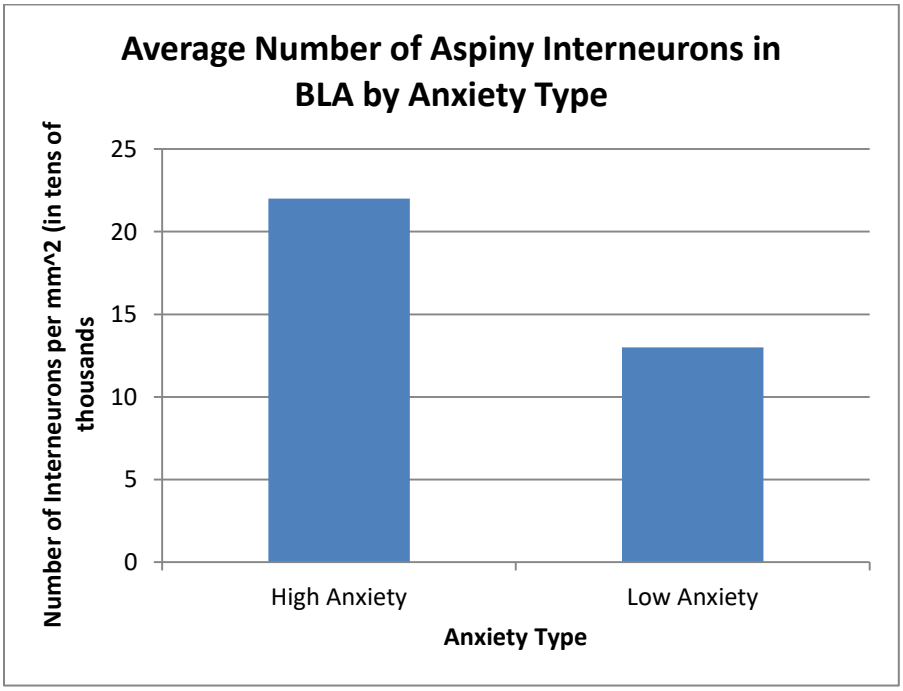
One day after the last extinction trial, the subjects were anesthetized then sacrificed via decapitation. The brain was then removed and cut sagittally down the midline of the forebrain. It is then submerged in the Mg2+ Ringer solution for dissection. Coronal sections approximately 300 μm thick were then taken using a vibratome. One of the sections (the same cut from each brain) underwent an NB electroporation procedure and then was analyzed using Neurolucida software to get the aspiny-spiny interneuron ratio of each subject. We then calculated correlations between anxiety score and aspiny-spiny ratio across the sample. Interneurons were measured by number of cells per mm^2.

Hypothesized Outcome and Discussion

We predict that our hypothesis will be proven correct with this experiment; we expect to find that high anxiety rats will have far fewer spiny GABAergic interneurons compared to their low anxiety counterparts. Conversely, high anxiety rats will also have far more aspiny neurons compared to those with low anxiety scores. We anticipate that high anxiety rats will have an aspiny-spiny ratio of 1:0.55, and low anxiety rats will have an aspiny-spiny ratio of 1:1.92 approximately.

If we assume a direct relationship in the direction we predicted, this phenomena could be explained by interneurons being the mechanism by which BLA inhibits itself; because spiny interneurons being larger and capable of releasing more inhibitory transmissions, they more efficient in inhibiting BLA in response to frightful stimuli. Conversely, aspiny interneurons have a much smaller dendritic spine density on average, and as a result they are not able to produce as much inhibition to BLA as spiny interneurons.





We believe these findings are possible because this would not be the first instance that morphological changes to memory-critical brain areas would be connected with an increased vulnerability to anxiety-related disorders. Research done by Gilbertson and his colleagues found

a relationship between severe stress and decreased hippocampal volume (2002). Using a monozygotic twin study, they determined that comparatively low hippocampal can serve as a predictor for developing an anxiety disorder, particularly PTSD, in response to a traumatic experience (Gilbertson et al., 2002). It is possible that this can be true of other morphological changes in that they may be able to predict anxiety disorder vulnerability.

However, this study has several potential confounds. Firstly, these findings are purely correlational; the relationship between these two variables could be the inverse of what was described previously, and exposure to frightful or traumatic stimuli could change GABA interneuron morphology. We were not able to count the aspiny-spiny ratio prior to the fear conditioning task so there is no way to know if the morphology could have changed in response to the conditioning. Future researchers should consider a separate sample reared in the same conditions, but sacrificed without exposure to a fear conditioning task to serve as a control. While this still harbors its own confounds, it may be able to offer some insight into the possibility of interneuron morphology changing in response to trauma. It could also be possible that a third underlying variable or process affects both of these variables that was not measured in this study. Researchers should consider studies concerning the difference in activity between spiny and aspiny interneurons in response to both neutral and frightful stimuli.

This study and the original Klenowski et al. study also ignored the possible effects of sex on our results. It is well documented in the literature that women are more likely to develop general anxiety disorder and PTSD compared to men. If GABA interneuron morphology does indicate risk to develop an anxiety-related disorder, it would be worth exploring potential sex differences in terms of interneuron morphology. It may serve as a good measure to retest the

hypothesis of this study if we can find that women have more aspiny GABA interneurons in BLA compared to men.

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