Dendritic morphology of amygdala and hippocampal neurons in more and less predator stress responsive rats and more and less spontaneously anxious handled controls

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Abstract

We investigated the neurobiological bases of variation in response to predator stress (PS). Sixteen days after treatment (PS or handling), rats were grouped according to anxiety in the elevated plus maze (EPM). Acoustic startle was also measured. We examined the structure of dendritic trees of basolateral amygdala (BLA) output neurons (stellate and pyramidal cells) and of dorsal hippocampal (DHC) dentate granule cells of less anxious (LA) and more (extremely) anxious (MA) stressed animals (PSLA and PSMA). Handled controls (HC) which were less anxious (HCLA) and spontaneously more anxious (HCMA) equivalently to predator stressed subgroups were also studied. Golgi analysis revealed BLA output neurons of HCMA rats exhibited longer, more branched dendrites with higher spine density than the other groups of rats, which did not differ. Finally, spine density of DHC granule cells was equally depressed in HCMA and PSMA rats relative to HCLA and PSLA rats. Total dendritic length of BLA pyramidal and stellate cells (positive predictor) and DHC spine density (negative predictor) together accounted for 96% of the variance of anxiety of handled rats. DHC spine density was a negative predictor of PSMA and PSLA anxiety, accounting for 70% of the variance. Data are discussed in the context of morphological differences as phenotypic markers of a genetic predisposition to anxiety in handled controls, and a possible genetic vulnerability to predator stress expressed as reduced spine density in the DHC. Significance of findings for animal models of anxiety and hyperarousal comorbidities of PTSD are discussed.

Keywords

Stress; Amygdala; Hippocampus; Morphology; Resilience; Vulnerability

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1. Introduction

Individuals respond to stress and trauma differently. In some, traumatic experience leads to post traumatic stress disorder (PTSD) while others are less affected [1–3]. Relatively little is known about the molecular and neural substrates of individual differences in response to trauma [1]. However, correlational behavioral research implicates a variety of factors, including personality traits [4,5] as well as interaction of genetic factors and experiential factors, such as reduced functioning polymorphisms in the serotonin transporter (5-HTTLPR), and life stress or social support at the time of stress [6–8]. Moreover, the 5-HTTLPR exerts a potent modulatory effect on amygdala reactivity to environmental threat [6]. Since this genetically driven effect exists in healthy subjects, Hariri and colleagues suggest that the 5-HTTLPR may represent a susceptibility factor for affective disorders by biasing the functional reactivity of the human amygdala in the context of stressful life experiences and/or deficient cortical regulatory input [6]. Support for this idea comes from studies associating PTSD with 5-HTTLPR [8–13]. So, factors affecting functional amygdala reactivity may be important contributors to vulnerability to stress. In this context, it is important to note that right amygdala reactivity to both trauma reminders and general negative stimuli is enhanced in humans diagnosed with PTSD [14,15].

One way to identify putative causal substrates is to study the effects of stress on brain and behavior of more and less stress vulnerable animals. A useful paradigm in this regard is exposure of rodents to brief predator stress, a putative model of hyperarousal and generalized anxiety characteristics of PTSD [16–18]. Domesticated strains of laboratory rats retain the fear of predators like a cat, even if they have never been exposed to predators [19,20]. On exposure to a cat (predator stress – PS) or cat stimuli (predator scent stress – PSS), laboratory rats and mice develop long-lasting (3 weeks or longer) hyperarousal (enhanced acoustic startle response) and anxiety [18,21–25]. Like humans following a traumatic event, not all stressed animals respond similarly. Some remain unaffected, showing little fear sensitization [18,26,27].

Consistent with human data, serotonin transporter gene knockout mice are more vulnerable to predator stress [7,28]. This finding provides an interesting parallel to the human clinical literature involving the 5-HTTLPR, and in that context, implicates modulation of amygdala function in vulnerability to predator stress. It is of interest in this regard that predator stress induces a lasting enhancement of excitability of right rodent amygdala, detected as a potentiation of afferent and efferent transmission in basolateral (BLA) and central amygdala [29,30]. Furthermore, degree of anxiogenic effect of predator stress is tightly predicted by degree of potentiation in amygdala circuitry [31]. Moreover, it has been inferred from electrophysiological evidence that one mechanism mediating predator stress potentiation of amygdala circuitry could be changes in dendritic morphology [29]. In these studies, potentiation of afferent input to BLA from the ventral hippocampus was accompanied by an increase in electrophysiological estimates of maximal receptor binding (B_{max}). Among the possible explanations of this finding discussed was an alteration in dendritic morphology resulting in increased receptor binding due to synaptic restructuring or synaptic proliferation. Structural variation which alters neural transmission in BLA could then alter fearful response which highly correlates with BLA transmission.

Indeed, variation in dendritic arbors of BLA neurons are related to the ability of restraint stress to generate anxiety [32,33]. Anxiety generated by stress and stress hormone is accompanied by BLA dendritic hypertrophy [32–34], and experimental reduction of dendritic length results in reduction of anxiety [35]. Moreover, once generated, BLA dendritic hypertrophy is as long lasting as stress induced anxiety [33]. Finally recent findings suggest that dendritic hypotrophy in principle output neurons (stellate and pyramidal cells) of the right BLA may be a resilience marker against lasting anxiogenic effects of predator stress [27].

Studies in the dorsal hippocampus (DHC) add to our understanding of individual differences in response to severe stress. In DHC (area CA1), up regulation of ARC gene expression (mRNA) was found in rats unaffected by PSS [36], whereas down regulation of BDNF and up regulation of TrkB receptors was observed in rats made extremely fearful by PSS [37]. These data implicate the dorsal hippocampus in vulnerability to predator stress. Moreover, reduced hippocampal volumes in humans have been implicated as a predisposing factor to PTSD in twin studies [38]. In animal models of stress effects on hippocampal morphology, the loss of hippocampal volume arises from losses in the neuropil involving losses of dendrites and synapses [39] suggesting a substrate for loss of hippocampal volume in humans. In addition, psychosocial and restraint stress induce dendritic atrophy in DHC CA3 neurons of animals [40]. Finally, chronic immobilization stress in rats induces dendritic atrophy and debranching in dorsal hippocampal area CA3 neurons, while inducing hypertrophy of dendrites of BLA stellate and pyramidal neurons. Both effects are associated with increased anxiety in the elevated plus maze. In contrast chronic unpredictable stress had no effects on plus maze anxiety and no effects on hippocampal cells, while producing dendritic atrophy in amygdala bipolar cells [32]. These results indicate that types of stress inducing anxiety can cause contrasting patterns of dendritic remodeling in neurons of the amygdala and hippocampus. Moreover, not all stressors induce anxiety or remodeling of output neurons in the amygdala or DHC.

Given the above considerations, it is timely to ask in more detail if neurons of the BLA and their plasticity are involved in individual differences in lasting response to predator stress. In previous work only the right amygdala was studied and stellate and pyramidal cells were combined in the data analysis. In the present study dendritic morphology of stellate and pyramidal neurons were studied separately. In addition neurons were examined in both right and left amygdalas. Finally spine density was measured, which was not done previously [27]. A similar analysis was done in DHC dentate granule cells. If behavioral changes are dependent on right amygdala excitability, then dendritic morphology differences between predator stress responsive and non responsive rats should be localized to the right amygdala. Finally, unlike previous work, two controls were used. One was a handled control that showed average anxiety-like behavior in the elevated plus maze (EPM) characteristic of a large sample of handled controls used in previous studies (less anxious – LA). A second handled control was spontaneously more (extremely) anxious (MA). Attempts were successful in establishing groups of comparable LA and MA predator stressed rats and handled controls. These four groups were established to tease out dendritic morphology characteristics that reflect anxiety level per se or stress effects on morphology. Two hypotheses can be proposed to explain individual differences. First, in MA animals, stress

causes neural expansion in BLA dendrites related to the enhanced anxiety they experience and/or increased spine density, while stress causes dendritic hypotrophy and/or reduction of dendritic spine density in DHC dentate cells. Second, stress causes amygdala dendritic retraction in LA animals and/or reduced spine density, while stress causes dendritic expansion and/or increased dendritic spine density in DHC dentate cells, and together these plastic changes prevent MA effects of trauma. The handled MA and LA groups of comparable anxiety levels provide a control that tested the predator stress specificity of the EPM anxiety related morphological differences. Given that predator stress has been proposed as an animal model for hyperarousal and anxiety aspects of PTSD [16], findings in this study should shed light on mechanisms of vulnerability to these aspects of PTSD.

2. Methods and materials

2.1. Subjects and groups

A total of 302 adult male Long–Evans hooded rats were used. At arrival from Charles River, Canada, rats were approximately 4 weeks of age and weighed between 76 and 100 g. Rats were housed individually in standard clear polycarbonate cages. The animals were fed and watered ad lib, and were maintained on a 12 h light–dark schedule (lights on at 07:00). Rats were first habituated to their home cage for 1 day, after which they were handled once per day for 1 min over the following 5 days. Finally, rats were randomly assigned to either the predator stressed group (to be exposed to a cat, $n = 56$) or handled control ($n = 246$). Given that the rats were young at time of treatment (5 weeks of age), it is likely that they were in a neuroplastic period of their development.

2.2. Predator stress and handling

One week after arrival, predator stress group animals were exposed to one of two cats. Exposures were unprotected and occurred between the hours of 09:00 and 12:00. All exposures took place in an enclosed room with a floor area of 3.252 m² (1.524 m × 2.134 m) as described elsewhere [21]. Exposures lasted for 10 min and were videotaped to capture the activities of both the rat and the cat. Cat response consisted of watching the rat from a distance, followed by several approaches, pawing, and the occasional mild attack. No rats were injured. Handled animals were handled for 1 min on the day of cat exposure of predator stressed groups. Handled and predator stressed rats were housed in separate rooms and did not come into contact with each other. Time of treatment was counterbalanced among all groups. Following treatment, all rats were returned to their home cages and left unhandled until testing for lasting effects on rodent anxiety and hyperarousal 2 weeks later.

2.2.1. Behavioral measures taken from cat exposures—Behavior of both the rat and cat was analysed from videotape. Cat behaviors were the number of approaches near to the rat, time spent near the rat, number of bites and sniffs of the rat, number of cat vocalizations, and frequency of pawing. The cat was considered near the rat when it moved to within 30.48 cm of the rat determined from 30.48 cm square floor markings.

Rat behavior in response to the cat was also analysed. Activity was measured as number of lines of the 30.48 cm square floor markings crossed. Defensive behavior was categorized as

frequency of active defense (approaches to the cat) or rat flight from the cat as described elsewhere [21].

2.3. Post treatment behavioral testing

Two weeks after handling or predator stress, anxiety-like behavior was examined in all rats using the hole board and EPM tests. Such tests are commonly used to assess rodent exploration, activity, and anxiety [41–43]. Behavior in all tests was video taped remotely for later blind analysis. All tests were 5 min in duration and conducted between 09:00 and 11:00 under normal room lighting. Rats were tested first in the hole board immediately followed by testing in the EPM.

2.3.1. Hole board testing—The hole board test provided independent measures of activity and exploratory tendency [43]. The hole board apparatus was an open top square wooden box measuring 60 cm × 60 cm × 35 cm (length × width × height). In addition four evenly spaced holes were drilled 14 cm from the walls in a floor that was raised 12 cm above the ground. Both floor and walls were painted with grey enamel. Tape marked a square inside the box, separating it into center (containing the 4 holes) and perimeter (near the wall) segments.

Rats were placed in the center of the hole board apparatus and allowed to explore freely for 5 min. Rats were then immediately transferred to the EPM located in the same room for a further 5 min of testing. After each test the box was cleaned with a 5% alcohol solution and wiped dry with paper towels.

Measures of activity and exploratory behavior were taken from video taped records. Activity was recorded as number of rears. Exploratory tendency was scored as the number of head dips (placing the head or snout into one of the four holes drilled in the floor). In addition the amount of time spent near the walls of the box was measured. Rats were considered to be near the wall of the box when all four feet were outside the center square marked by tape. Time in the center of the box was also measured when all four feet were within the center square marked by tape.

2.3.2. Elevated plus maze (EPM)—Immediately following the hole board test, rats were placed in the EPM. The EPM was a wooden four armed platform with arms arranged in the shape of a plus. The platform was painted with grey enamel, and was raised 50 cm above the floor. All arms were 10 cm wide and 50 cm long and joined in the center by a 10 cm on a side square platform. Two arms facing each other were closed arms, the other two were open. Closed arms were surrounded by 40 cm high wooden walls which were open at the top, while open arms were bounded by a 3 cm high edge only.

At the start of each test, rats were placed in the center square facing the same open arm, and were allowed to move freely for 5 min. At the conclusion of each test, rats were returned to their home cage and the maze was cleaned and wiped dry using a 5% alcohol solution.

A number of behavioral measures were taken from videotape. These included standard measures of rodent anxiety: ratio time and ratio entry. Ratio time refers to the total time

spent in the open arms of the maze divided by the total time spent in any arm of the maze. Ratio entry refers to the total entries into the open arms of the maze divided by the total entries into any arm of the maze. Smaller ratios indicate less open arm exploration, or more “anxiety”. A rat was considered to be within an arm of the maze when all four feet were within the arm. A composite anxiety score was calculated from ratio time and entry as: $\text{anxiety} = 1 - (\text{ratio time} + \text{ratio entry})/2$. This score is 1 when ratio time and entry are zero or extreme anxiety (open arm avoidance), and 0 when ratio time and entry are 1 or no anxiety (no open arm avoidance). In addition, entries into the closed arms of the maze were taken as a measure of activity/exploratory tendency in the EPM [44].

2.4. Selection of more anxious (MA – stress responsive) and less anxious (LA –stress non-responsive) predator stressed rats and selection of MA and LA handled controls

On the day of EPM testing, ratio time measures were calculated for each rat. Inclusion into the MA group of predator stressed rats required a ratio time score of 0–.06. LA predator stressed rats were identified as those with ratio time scores falling between .25 and .50. This was based on an extensive data base of handled hooded rat data in the Adamec laboratory and was considered characteristic of the range of handled control EPM response. The same ratio time criteria were applied to handled controls. From the 56 predator stressed rats it was possible to identify and select six LA rats or 10.7% of predator stressed rats (PSLA). From 29 identified MA predator stressed rats (51.8% of predator stressed rats) six predator stressed MA rats (PSMA) were selected. PSLA rats were considered stress non responsive and PSMA rats were considered stress responsive. The work of Cohen and colleagues supports the classification of more anxious rats as stress responsive and less anxious rats as stress non responsive [18]. Cohen and colleagues used EPM criteria similar to ours to classify predator stressed rats as more and less anxious. Less anxious rats behaved in the EPM like handled controls. Moreover they found that more anxious rats had significantly higher plasma corticosterone and ACTH concentrations, increased sympathetic activity, diminished vagal tone, and increased sympathovagal balance compared to handled controls and less anxious stressed rats, which did not differ [18]. From the 246 handled controls, seven MA rats or 2.8% of handled rats were identified and selected (non stressed handled or HCMA). From 198 identified LA handled control rats (80.5% of handled controls), six LA handled rats were selected (non-stressed handled or HCLA). LA and MA rats were selected such that predator stressed and handled LA and MA rats had comparable ratio time and anxiety index scores. LA and MA rats were tested for acoustic startle response on the day after EPM testing.

2.5. Acoustic startle testing

Unconditioned startle response to an acoustic stimulus was measured using a standard startle chamber (San Diego Instruments). The apparatus was fitted with a 20.32 cm plastic cylinder which was used to hold the animal, as well as a speaker for producing sound bursts. A piezoelectric transducer positioned directly below the cylinder was used to record the motion of a rat during each sound burst. Output from the transducer was led to a computer for sampling.

Prior to startle testing, animals were acclimatized to the darkened apparatus for 5 min with a background white noise level of 60 dB. Following acclimatization, rats received a 50 ms burst of 120 dB rising out of the 60 dB background noise once every 30 s for 15 min. The 30 trials were conducted in the dark. A computer attached to the apparatus recorded 30 samples of transducer output. Samples included a 5 ms baseline and 250 ms sample after onset of the noise burst. Average transducer output just prior to the noise burst was saved as a baseline (V_{start}). In addition, the computer determined the peak startle amplitude within each of the samples (V_{max}) and this value was also stored. Peak startle amplitude was expressed as $V_{\text{max}} - V_{\text{start}}$ for analysis.

Predator stress prolongs the habituation of startle response [17], so habituation to startle in the different groups was determined and compared. For this analysis, peak startle amplitudes observed on 30 startle trials were averaged over rats for each group. Average peak startle amplitudes over 30 trials were fitted with the Table Curve 4.0 program (Jandel) to an exponential decline function:

$$y = y_0 + ae^{-t/\tau}$$

In the equation, y and y_0 are peak startle amplitude, a is a constant, e is the base of the natural logarithm, t is trial, and τ (Tau) is the trial constant (number of trials required to decline to 37% of the maximal peak startle amplitude). The trial constant was taken as a measure of rate of habituation, the greater the trial constant, the more delayed the habituation. Data were smoothed to improve fit. The smoothing was done with an FFT function (20% smooth for all fits) provided by the curve fitting program. Care was taken to ensure the smoothing did not distort the data.

At the end of each startle session the rats were returned to their home cages. The apparatus was washed using warm water.

2.6. Morphological methods and analyses

2.6.1. Golgi staining—Animals were sacrificed under deep (sodium pentobarbital, 150 mg/kg) anesthesia and perfused transcardially with 300 ml of saline (25 ml/min) followed by 300 ml (25 ml/min) of 10% buffered formalin (Fisher Scientific Canada) with a peristaltic pump. Perfusions took place 1 day after startle testing and 16 days after predator exposure or handling. The perfused brains were stored in 10% buffered formalin for no more than 2 weeks and then cut and processed for Rapid Golgi staining. Using the anterior commissure as a reference, coronal blocks of BLA tissue including left and right BLA and DHC were taken from approximately 2.80 mm posterior to bregma back to 3.8 mm posterior to bregma (coordinates from the atlas of Paxinos and Watson [45]). This was done to capture that part of the right posterior BLA in which potentiation of ventral hippocampal afferent transmission is produced by predator stress [29,30]. Coronal blocks of tissue incorporating the BLA and DHC were stained using the Rapid Golgi method [46]. In this technique, the tissue blocks were first immersed in a solution containing potassium dichromate and osmium tetroxide. The tissue was kept in the dark, on a rotating platform, for 5–6 days. The tissue blocks were then thoroughly rinsed in a dilute solution of freshly prepared silver

nitrate, and placed in a staining jar with more silver nitrate for 39–42 h. The blocks were then dehydrated through ascending solutions of alcohols and ether alcohol. The dehydrated blocks were then placed in ascending concentrations of low viscosity nitrocellulose. The nitrocellulose was hardened by exposure to chloroform. Tissue blocks were then cut in the coronal plane at 120 μ m thickness. The tissue sections were cleared in alpha-terpineol, thoroughly rinsed in xylene, placed on coded slides for blind analysis and cover slipped under Permount.

2.6.2. Selection of BLA stellate and pyramidal and DHC dentate cells—From the coded slides, neurons of three cell populations were selected for analysis of dendritic branching and dendritic spines. BLA neurons were classified as either stellate or pyramidal. In general, stellate cells had an ovoid soma and branches of largely the same diameter splaying out in all directions from the soma. The BLA pyramidal cells, in contrast, were defined as having a much thicker and dominant main apical branch as well as a basilar tree composed of a number of branches of smaller diameter. Fig. 1 contains photomicrographs of representative examples of Golgi stained stellate (Fig. 1A) and pyramidal (Fig. 1B) neurons in the BLA. Dentate cells were selected from the granule cell layer of the DHC. These cells had dendritic trees extending clearly into the molecular layer. Fig. 2A contains a photomicrograph of a Golgi stained dentate granule cell in the DHC. Fig. 2B contains two examples of dentate dendritic spines from HCLA and HCMA rats.

All selected neurons had to meet certain criteria: they had to be well stained and they had to have branches which were unobscured by other neurons, glia, blood vessels, or precipitate. For the analysis of dendritic branching and length, all the selected neurons had to be located in the middle third of the thickness of the section. This was done to avoid evaluating neurons which – if located too superficially or too deep within the thickness of the section – may have had too many branches foreshortened. For each subject and for each BLA cell type, three neurons were selected from the right hemisphere and three neurons were selected from the left hemisphere for analysis. For the dentate four neurons were selected from each hemisphere for analysis.

In summary, for BLA stellate cells, from each brain, we evaluated three neurons from each hemisphere – a total of 6 stellate cells per brain. Similarly, for the pyramids, we also evaluated 6 neurons per brain. A total of 25 brains were used. Of a possible total of $6 \times 2 \times 25 = 300$ neurons in the study, due to staining issues, three brains were each one neuron short, so a total of 297 BLA neurons were evaluated. The brains missing cells were HCMA right hemisphere missing a pyramidal cell; HCLA left hemisphere missing a pyramidal cell; HCLA right hemisphere missing a stellate cell. For the dentate granule cells we evaluated four neurons from each hemisphere – a total of 8 dentate cells per brain. A total of 20 brains were used (5 rats from each of four groups), these were rats with DHC sections containing granule cells in the middle third of the 120 μ m sections. A total of $8 \times 20 = 160$ dentate granule cells were analysed.

2.6.3. BLA stellate and pyramidal cell and DHC dentate cell analyses—For analysis of dendritic branching and length, camera lucida drawings were first made of the selected neurons. The neurons were identified and drawn using a Zeiss brightfield research

microscope at a magnification of 500 $\times$. After the camera lucida drawings were made, an appropriate plastic template of 34 concentric circles separated by 10 μm equivalent was placed over the drawing centered on the cell body to generate data for the Sholl analysis. This template covered an area out to 340 μm from the soma of the cell. The Sholl analysis is the primary means of statistically assessing the amount and distribution of the dendritic arbor [47]. This involved counting the number of concentric circle crossings of each dendrite of a given cell. The total number of crossings for a given dendrite were used to estimate ($\pm 2\%$) total dendritic length in μm . This was done using a correction factor established previously by measuring both dendritic length directly from camera lucida drawings with a digital tablet and the number of concentric circle crossings. Then a linear relationship was calculated between crossings (x) dendritic length (y). The slope of this relationship is 12.54 and this was multiplied by total dendritic crossings to estimate total dendritic length in μm . In addition to the Sholl analysis we also measured the soma size at 1008 $\times$ magnification by tracing the soma on a digital tablet and the tablet software calculated soma area in μm^2 .

We also evaluated the complexity of the dendritic arbor using a branch point analysis method. In this analysis, we assessed both the numbers of branch points (e.g., the bifurcations of the dendritic branches) and also defined where in the dendritic arbor the bifurcation was occurring. Thus, a primary branch (or first order branch) which emanated from the soma splits into two second order branches at a 1st order branch point. The second order branch in turn splits into third order branches at a 2nd order branch point, ..., and so forth. Essentially, the greater the number of branch points, the more complex is the dendritic arbor. And the branch point order helps to define whether the branch points are occurring close to the cell body in the proximal part of the tree, or more distally.

2.6.4. Spine density analyses—Spine density was also evaluated in the BLA pyramidal and stellate cells and in the DHC dentate granule cells (see Fig. 2B) at 2000 $\times$. From each right and left hemisphere, three pyramidal and three stellate neurons were examined for dendritic spine density. Four terminal tip dendritic segments from each stellate neuron were evaluated. For pyramidal cells, two terminal tip segments were selected from the apical tree and two segments from the basilar tree. For the DHC, three segments from each cell were selected. These granule cell segments were located in the middle third of the molecular layer. In all cases, the segment length was variable (but at least 30 μm in length, BLA pyramidal cells mean length $38.5 \pm 2.2 \mu\text{m}$, BLA stellate cell mean length $35.6 \pm 2.3 \mu\text{m}$, DHC dentate granule cell mean length $71.0 \pm 1.3 \mu\text{m}$). The segment was largely planar to the field of focus and unobscured. Along the dendritic segment all visible flanking spines were counted blind by a single observer (to minimize subjective variability). The segment length was then measured using a digital tablet to provide a divisor for spine density.

2.7. Statistical analysis

Values are reported as mean $\pm$ SEM or median $\pm$ SEMd (standard error of the median). One way analysis of variance (ANOVA) tested behavioral differences between all handled controls and predator stressed rats. EPM and hole board behavior of MA and LA rats were analysed with two way ANOVA with factors of Stress (HC – handled control; PS –predator stressed – cat exposed), and Anxiety Level (MA, LA). Post hoc t tests ($p < .05$) were used

for planned comparison mean contrasts. An exception to this analysis was the peak startle amplitude. Because of serious deviation from normality, MA and LA HC and PS rats were compared with non parametric one way Kruskal–Wallis ANOVA of median peak startle amplitudes, and median contrasts were done using the Kruskal–Wallis multiple Z test ($p < .05$).

Statistical analysis of morphological data varied with the data being examined. For total dendritic length, repeated measures ANOVA assessed groups (HCLA, HCMA, PSMA, PSMA) with repeated measures on hemisphere. For Sholl analysis data, repeated measures ANOVA assessed groups with repeated measures on hemisphere and concentric circle number (1–25 for the amygdala and 1–34 for the dentate). Twenty five concentric circles were used in the BLA analysis as dendritic arbors did not extend beyond 250 μm from the soma, whereas for the dentate the arbor extended to 340 μm from the soma. For the branch point analysis repeated measures ANOVA assessed groups with repeated measures on hemisphere and branch point number (1–6). For spine density analysis in BLA pyramidal cells, repeated measures ANOVA assessed groups with repeated measures on hemisphere and dendrite type (basilar or apical). BLA stellate cell spine density was examined with a repeated measures ANOVA assessing group with repeated measures on hemisphere. For all ANOVA planned mean contrasts were done using t tests ($p < .05$). Due to non normality of the data, DHC spine density was analysed comparing group medians with Kruskal–Wallis non parametric analysis of medians. Median contrasts were done using the Kruskal–Wallis multiple Z test ($p < .05$). Separate analyses of the right and left hemisphere yielded the same result so what is presented is the analysis combining hemispheres.

2.8. Ethical approval

All procedures involving animals in this study adhered to the guidelines of the Canadian Council on Animal care, and were approved by the Institutional Animal Care committee of Memorial University. All efforts were made to minimize pain, stress, and the number of animals used.

3. Results

3.1. Predator stressed animals are more anxious in the EPM than Handled Controls 2 weeks after treatment

Though MA and LA rats were selected based on ratio time criteria, it was important to confirm the overall effects of predator stress on EPM anxiety in the population from which the MA and LA rats were drawn. Moreover, it was necessary to compare groups on measures of activity and exploration in order to ensure that differences in ratio time could be interpreted as differences in anxiety (open arm avoidance due to fear) and not differences in activity/exploration.

As expected, one way ANOVA confirmed that predator stressed rats exhibited reduced open arm exploration (reduced ratio time and entries) and elevated anxiety scores relative to the handled controls (Fig. 3A, all $F(1,300) = 13.15$ all $p < .001$). Similar group differences were observed in closed arm entries (Fig. 3C, $F(1,300) = 22.91$, $p < .001$), suggesting reduced

locomotor activity/exploration in the EPM in stressed rats. To assess if locomotor activity/exploration contributed to group differences in anxiety (i.e., reduced open arm exploration), closed arm entries were used as a covariate in a reanalysis of ratio time, ratio entry and anxiety scores. Reduced locomotor activity in the EPM did not contribute to reduced open-arm exploration, as the original pattern of group differences was preserved in the analysis of covariance (Fig. 3B, all $F(1,299) = 23.67$ all $p < .001$). Consistent with this analysis, there were no group differences in the hole board measures of activity/exploration (rears or head dips, Fig. 4A). However, stressed rats spent more time near the wall and less time in the center of the hole board (Fig. 4 B, all $F(1,300) = 5.86$, $p < .02$). This is consistent with the anxiety data in the EPM. Taken together these data support the conclusion that PS rats as a group were selectively more anxious in the EPM than handled controls.

3.2. LA – PS and HC, and MA – PS and HC animals show equivalent levels of anxiety in the EPM 2 weeks after treatment

Behavior of rats selected for Golgi analysis was analysed using two way ANOVA for Stress (HC and PS) and Anxiety Level (LA, MA). There was a main Anxiety Level effect for anxiety scores in the EPM (Fig. 5A, $F(1,21) = 233.71$, $p < .0001$). There was no Stress or Stress by Anxiety Level interaction (all $F(1,21) = .05$, $p > .80$). So PSMA and HCMA rats displayed equal anxiety scores which were greater than the anxiety scores of PSMA and HCLA rats, which did not differ. There was a Stress effect for closed arm entries in which PS rats entered the closed arms less frequently than did HC rats (Fig. 5B, $F(1,21) = 10.70$, $p < .001$). There was no Anxiety Level (LA, MA) effect or interaction (all $F(1,21) = 1.59$, $p > .22$; Fig. 5B) suggesting reduced locomotor activity/exploration in the EPM in stressed rats. To assess if locomotor activity/exploration contributed to Anxiety Level differences in anxiety scores, closed arm entries were used as a covariate in a reanalysis of anxiety scores. Reduced locomotor activity/exploration in the EPM did not contribute to anxiety score differences, as the original pattern of Anxiety Level differences was preserved in the analysis of covariance (Fig. 5C, Anxiety Level effect, $F(1,20) = 205.54$ $p < .0001$; Stress effects and interaction all $F(1,20) = .17$, $p > .69$). There were no effects in the analysis of behaviors in the hole board test ($F(1,21) = 0.79$, $p > .05$). Together these data support the conclusion that MA rats as a group (PS + HC) were selectively and equally more anxious in the EPM than LA rats as a group (PS + HC), which were selectively and equally less anxious.

3.3. LA, MA – HC rats show a different pattern of startle response than LA, MA PS rats 2 weeks after treatment

Peak startle amplitudes were not normally distributed (Omnibus test 694.62, $p < .001$). So LA and MA HC rats and LA and MA PS rats were compared in a one way non parametric Kruskal–Wallis ANOVA on median peak startle amplitudes. Groups differed ($\chi^2(3) = 35.43$, $p > .01$; Fig. 6A) such that HCMA rats and PSMA rats showed greater peak startle amplitudes than HCLA rats. Moreover HCMA rats equaled PSMA rats, which did not differ (Kruskal–Wallis multiple Z test, $p < .01$).

Rate of habituation (Tau – the trial constant) was calculated as described in the methods section. All fits to the exponential decline function were good (all degrees of freedom

adjusted $r^2 > .505 < .945$, all multiple R , $F(2,27) > 13.78$, $p < .001$, all $\tau > 0$, $p < .01$). The estimate of Tau included a standard error of estimate. These standard errors were used to calculate t -statistics between the trial constants of the different groups. Planned comparisons of the Tau estimates between groups were done using two-tailed t tests. Like peak startle amplitudes, HCMA rats and PSMA rats showed greater Tau values (more prolonged habituation) than HCLA rats. Moreover HCMA rats equaled PSMA rats which did not differ (Fig. 6B, two tailed t tests, all $t(58) \geq 2.66$, $p < .01$).

3.4. The predator stress experience

LA and MA predator stressed rats were compared with respect to cat response to them and their responses to the cat. There were no group differences (all $F(1,5) \leq .75$, $p > .43$). Therefore the predator stress experience, as measured, did not differ between LA and MA predator stressed rats.

3.5. Dendritic morphology of BLA neurons – total dendritic length

BLA stellate and pyramidal principle output neurons of the groups differed in total dendritic length (all $F(3,21) \geq 3.63$, $p < .05$). HCMA rats displayed greater total dendritic length of stellate and pyramidal cells than the remaining three groups, which did not differ (Fig. 7A and B, all $t(21) \geq 2.08$, $p < .05$).

3.6. Dendritic morphology of BLA neurons – Sholl analysis

The Sholl analyses were consistent with the total dendritic length analyses. There were significant Group (HCLA, HCMA, PSMA, PSMA) $\times$ shell interactions for stellate and pyramidal cells (all $F(72,504) \geq 1.98$, $p < .001$, Figs. 8 and 9). Plotted in the figures are mean number of shell crossings at distances of 10 to 250 μM from the soma as mean $\pm$ SEM (Figs. 8A and 9A) and mean $\pm$ 95% confidence intervals (Figs. 8B and 9B). The 95% confidence plot was used to find means with no overlap of 95% confidence intervals to identify the distances from the soma where groups differed. Stellate cells of HCMA rats showed more shell crossings than all other groups which did not differ from 50 to 130 μM from the soma (Fig. 8B). Pyramidal cells of HCMA rats showed more shell crossings than all other groups which did not differ from 40 to 90 μM from the soma (Fig. 9B).

3.7. Dendritic morphology of BLA neurons – branch point analysis

There were group effects of mean total branches but no group by branch order interactions for stellate and pyramidal cells (Fig. 10, $F(3,21) \geq 3.08$, $p < .05$). Stellate cells of HCMA rats had more mean total branches than all other groups which did not differ (Fig. 10A, $t(21) = 2.59$, $p < .02$). The same can be said of pyramidal cells (Fig. 10B, $F(3,21) = 7.22$, $p < .004$, $t(21) \geq 2.15$, $p < .044$).

3.8. Dendritic morphology of BLA neurons – spine density analysis

There were no group effects or interactions for spine density of BLA stellate cells. In contrast, spine densities of BLA pyramidal cells of the groups differed (Fig. 11A, $F(3,21) = 3.08$, $p < .05$). HCMA rats showed elevated spine densities on pyramidal cell dendrites (basilar and apical combined, there being no effects of type of dendritic segment). Moreover,

HCMA rat spine density was greater than all other groups, which did not differ (Fig. 11A, all $t(21) = 2.08, p < .05$).

3.9. Dendritic morphology of BLA neurons – soma size

There were no group differences or interactions in soma sizes of stellate and pyramidal cells. However, pyramidal cells were larger than stellate cells ($F(1,21) = 6.44, p < .02$; means: $44.5 \pm .87$ vs $40.1 \pm .80 \mu\text{m}^2$ pyramidal vs stellate).

3.10. Dendritic morphology of BLA neurons – total dendritic length reanalysis

Recent work in this lab examined total dendritic length in the right posterior BLA (over the same coordinates examined here) in stellate and pyramidal cells combined [27]. In that study there was no hypertrophy of dendrites in PSMA rats, as found here. However, in the same study there was hypotrophy in PSLA rats compared to a handled control (equivalent to HCLA in the present study). It was of interest, therefore to examine total dendritic length in stellate plus pyramidal cells in the right BLA in the present study. There was a main group effect ($F(3,21) = 4.48, p < .015$) and HCMA rats had longer total dendritic lengths than all other groups, which did not differ (all $t(21) = 2.08, p < .05$, Fig. 11B). Therefore the hypotrophy seen in the previous study in PSLA rats was not replicated here. Of additional interest is the fact that the range of total dendritic lengths of PSMA and HCLA in the previous study was 2000–2500 μm matching the range seen in the present study (see Fig. 11B).

3.11. Dendritic morphology – correlations with behavior

The anxiety index (anxiety = $1 - (\text{ratio time} + \text{ratio entry})/2$) and peak startle amplitude (averaged over 30 trials) were correlated with dendritic parameters that differentiated the various groups. Dendritic data were collapsed across hemisphere for each rat and correlated with behavior separately for stellate and pyramidal neurons. Correlations were done over HC rats (HCMA and HCLA) and PS rats (PSMA and PSLA).

3.11.1. BLA total dendritic length correlations with behavior—Over HC rats, anxiety correlated significantly and positively with total dendritic length for pyramidal cells (Pearson product moment $r = .622, p < .024$) and stellate cells ($r = .628, p < .022$). There were no correlations of dendritic length with peak startle amplitude (all $r = .18, p > .54$). Therefore in HC rats longer dendrites predicted greater anxiety. There were no correlations of dendritic length with anxiety or peak startle amplitude in PS rats (all $r = .47, p > .10$).

3.11.2. BLA mean number of dendritic branches correlations with behavior—There were no correlations with behavior (anxiety or peak startle amplitude) of branches in pyramidal or stellate cells of HC or PS rats (all $r = .47, p > .10$). So dendritic complexity in the BLA did not predict behaviors measured here.

3.11.3. BLA spine density correlations with behavior—Spine density did correlate with anxiety but not startle. The correlation was between anxiety and spine density on pyramidal apical dendrites of HCMA and HCLA rats ($r = .51, p < .04$). The positive correlation indicates that greater spine density predicts higher levels of anxiety. There were

no correlations of spine density with behavior on basilar dendrites of HCMA and HCLA rats, nor were there correlations with behavior of apical and basilar dendritic spine densities of PSMA and PSLA rats (all $r \leq .36$, $p > .24$).

3.12. Dendritic morphology of DHC dentate neurons – total dendritic length

There were no group effects or interactions with hemisphere for total dendritic length of dentate granule cells. Averaged over all rats, the dendritic length equaled $2076.3 \pm 37.5 \mu\text{M}$.

3.13. Dendritic morphology of DHC dentate neurons – Sholl analysis

The Sholl analyses were consistent with the total dendritic length analyses. There were no Group or Group (HCLA, HCMA, PSMA, PSLA) $\times$ shell interactions ($F(3,16) = .72$, $p > .55$; $F(99,528) = .83$, $p > .87$).

3.14. Dendritic morphology of DHC dentate neurons – branch point analysis

There were no group effects of mean total branches ($F(3,16) = 0.97$, $p > .42$) or group by branch order interactions ($F(3,15) = 1.41$, $p > .26$; $F(15,75) = .92$, $p > .54$) for dentate granule cells.

3.15. Dendritic morphology of DHC Dentate neurons – spine density analysis

Due to non normality of the data (Omnibus test = 34.58, $p < .001$), DHC spine density was analysed comparing group medians with Kruskal–Wallis non parametric analysis of medians. Median contrasts were done using the Kruskal–Wallis multiple Z test ($p < .05$). Separate analyses of the right and left hemisphere yielded the same result so what is presented is the analysis combining hemispheres. Groups differed ($\chi^2(3) = 20.73$, $p < .001$). Median contrasts revealed that HCMA and PSMA rats had equal spine densities that were less than the spine densities of HCLA and PSLA rats which did not differ (Fig. 12, Multiple Z test $p < .05$, see Fig. 2B).

3.16. Dendritic morphology of DHC Dentate neurons – soma size

There were no group differences or interactions in soma sizes of dentate granule cells (average size $43.8 \pm .84 \mu\text{M}^2$).

3.17. Dendritic morphology of DHC dentate neurons –correlations with behavior

The anxiety index (anxiety = $1 - (\text{ratio time} + \text{ratio entry})/2$) and peak startle amplitude (averaged over 30 trials) were correlated with dendritic parameters that differentiated the various groups. Dendritic data were collapsed across hemisphere for each rat and correlated with behavior. Correlations were done over HC rats (HCMA and HCLA) and PS rats (PSMA and PSLA) separately or over all rats as appropriate.

3.17.1. Dentate total dendritic length correlations with behavior—No correlations were done as this parameter did not differ between groups.

3.17.2. Dentate mean number of dendritic branches correlations with behavior—No correlations were done as this parameter did not differ between groups.

3.17.3. Dentate spine density correlations with behavior—Like the amygdala, spine density correlated with anxiety but not startle. The correlation was over all rats (as spine densities differed between LA and MA rats in HC and PS groups). Anxiety and spine density correlated negatively ($r = -.52$, $p < .021$) indicating that fewer DHC dentate cell spines were associated with greater anxiety.

3.18. Dendritic morphology of BLA and DHC multiple correlations with anxiety

Multiple correlation was used to pick which combination of measures of dendritic morphology best predicted anxiety among HCMA and HCLA rats. Total dendritic length of BLA pyramidal and stellate cells correlated positively with anxiety. The dendritic lengths of these cells were summed to create a single BLA dendritic length variable. A second variable entered into the multiple correlation of anxiety with dendritic morphology was dentate cells spine densities, which correlated negatively with anxiety. A third variable was BLA pyramidal cell apical dendritic spine densities which correlated positively with anxiety. Because some of the data were not normally distributed, a robust multiple regression (Andrew's sine) was used to correlate independent variables (dendritic morphology) with anxiety. The criteria for a successful model included a significant multiple R , and all coefficients of independent variables being significantly different from zero. The correlation was done on HC rats (across HCMA and HCLA). The first model tested included all three independent variables. Two of three coefficients of independent variables were significantly different from zero ($-2.54 = t(7) = 2.54$, $p < .04$) and multiple R was significantly different from zero ($F(3,7) = 62.3$, $p < .001$, $R = .977$). One variable (BLA pyramidal cell apical dendritic spine densities) was removed from the regression since its coefficient was not different from zero. The multiple correlation of dentate cells spine densities and dendritic lengths of BLA stellate and pyramidal cells combined yielded a satisfactory model of the following form: Anxiety = $-1.19 \times$ dentate cells spine densities + $2.12 \times$ BLA pyramidal and stellate cells total dendritic lengths (summed). Coefficients are standardized and differ from zero ($-3.49 = t(7) = 6.24$, $p < .001$) and multiple R was significantly different from zero ($F(2,8) = 113.133$, $p < .001$, $R = .981$). These two variables accounted for 96.2% of the variance of anxiety (df adjusted $R^2 = .962$). For PSMA and PSLA a robust correlation was done using hippocampal spine densities alone. The correlation of dentate cells spine densities yielded a satisfactory model of the following form: Anxiety = $-0.86 \times$ dentate cells spine densities. The coefficient is standardized and differs from zero ($t(7) = -4.47$, $p < .003$) and R was significantly different from zero ($F(1,7) = 20.0$, $p < .003$, $R = .839$). This variable accounted for 70.4% of the variance of anxiety (df adjusted $R^2 = .704$).

4. Discussion

4.1. Behavioral findings

Exposure to a cat induces long-lasting increases in anxiety and hyperarousal in rats [20,21,29,31,48,49]. This was replicated here over all rats (Figs. 3 and 4), where predator stressed rats showed increased anxiety and hyperarousal 16 days after cat exposure. Yet, not all animals showed increased anxiety. A subset referred to here as less anxious (LA), remained unaffected by the cat exposure, showing low levels of EPM anxiety. In contrast another subset we referred to here as more (or extremely) anxious (MA) showed enhanced

anxiety in the EPM (Fig. 5). Thus, the same stress experience evoked different degrees of behavioral response. This replicates previous findings in this and in Cohen's lab [26,27]. This variability in response to stress in rats is mirrored in humans as traumatic experience leads to post traumatic stress disorder (PTSD) in some people, while others are less affected [1–3].

In addition, stress produced hyperarousal expressed as increased peak acoustic startle amplitude. Of interest was the fact that unlike in the EPM, LA and MA stressed rats both showed equal elevation of startle amplitude and delayed habituation (elevated trial constants (tau)) (Fig. 6). This dissociation of startle and EPM anxiety is consistent with studies showing startle and EPM anxiety loading on independent factors of factor analyses assessing the relationships between various tests of anxiety in predator stressed rats [48,49].

It was possible to identify LA and MA rats based on EPM anxiety from among handled controls equivalent to predator stressed rats (Fig. 5). In contrast to predator stressed rats, peak startle amplitudes of handled LA rats (HCLA) were less than peak startle amplitudes of MA handled rats (HCMA), which did not differ from predator stressed rats. Since EPM anxiety levels and acoustic startle levels are heritable [50] [51–54], it is likely that the behavioral differences between HCMA and HCLA rats are phenotypic expressions of underlying genetic influences on EPM anxiety and startle. Clearly predator stress does not create an equivalent behavioral profile to HCMA and HCLA rats when considering EPM and startle responses together. This conclusion suggests differences in the underlying substrates of behavioral effects of stress in PS rats, and spontaneously occurring differences in anxiety in HC rats.

4.2. Golgi findings – basolateral amygdala

Present Golgi data identify several substrate differences between HC and PS rats. We found that HCMA and HCLA animals differ in dendritic architecture of BLA neurons, which form part of the neural circuitry mediating stress-induced anxiety [29,31,32,34]. In contrast, LA and MA stressed rats (PSLA and PSMA) did not differ in BLA dendritic architecture. The lack of hypertrophy in stressed rats' BLA neurons replicates past findings [27]. However the lack of dendritic hypotrophy in PSLA rats differs from previous work, which identified shorter dendrites in BLA neurons of PSLA rats [27]. That study suggested that dendritic hypotrophy was a resilience marker against anxiogenic effects of predator stress. It must be noted that previous work examined BLA over the same AP plane range studied here, but in the right BLA only and averaged over stellate and pyramidal cells. As seen in the results section, reanalysis of the total dendritic length data to match the previous work (Fig. 11B) yielded the same pattern of differences as the bilateral analyses of stellate and pyramidal cells separately. HCMA rats displayed longer dendrites than all other groups which did not differ. It is likely that the inconsistency across studies is due to the different number of animals per group. In the previous study, 4 rats in each of three groups (PSMA, PSLA, and HCLA) with a total of 94 BLA stellate and pyramidal neurons were examined [27]. In the present study 6–7 rats in each of four groups (PSMA, PSLA, HCMA, and HCLA) were studied with a total of 297 BLA stellate and pyramidal neurons examined. The greater

sampling in the present study suggests that present findings in stressed rats are likely more robust.

The lack of BLA morphological changes in response to predator stress suggests that neuroplastic changes in BLA induced by stress are not mediated by dendritic remodeling. Predator stress induces lasting potentiation of excitatory neural transmission from the right ventral hippocampus to the right posterior BLA [31]. Present findings support the view that the potentiation in the BLA is mediated presynaptically, as has been suggested by us and Maren and Fanselow [31,55].

In contrast, anxiety in handled control animals appears to be mediated in part by dendritic remodeling. Handled control MA animals exhibit hypertrophy of total dendritic length of dendrites of stellate and pyramidal cells of the BLA in both hemispheres (Fig. 7). In addition Sholl analyses point to more dendritic material focused on a range of 50–130 μM from the soma in stellate cells (Fig. 8) and on a range of 40–90 μM from the soma in BLA pyramidal cells of HCMA rats (Fig. 9). This might suggest enhanced dendritic branching in target areas for excitatory synapses at least on BLA pyramidal cells [56]. Of interest in this regard was the greater spine densities on apical and basilar dendrites of BLA pyramidal cells of HCMA rats (Fig. 11A). In addition, branch point analysis was consistent with the Sholl analyses revealing the HCMA rats had more total branches than HCLA and PSMA and PSLA rats (Fig. 10).

Thus the BLAs of more anxious handled rats are characterized by dendritic hypertrophy, increased dendritic length and complexity and greater spine density on pyramidal neurons. All of these characteristics would increase the excitability of the principle output neurons of the BLA, and could mediate increased anxiety in HCMA rats. Indeed, local infusion of GABAergic anxiolytics which reduce BLA excitability are anxiolytic in the EPM [57]. Moreover, correlation analysis linked BLA morphology directly with EPM anxiety but not startle in HC rats. These correlations were specific to HC rats, with no correlations between BLA dendritic morphology and behavior in PS rats. In HC rats, anxiety correlated positively with total dendritic length of pyramidal and stellate cells. Therefore in HC rats longer dendrites predict greater anxiety. In contrast there were no correlations with behavior (anxiety or peak startle amplitude) of branches in pyramidal or stellate cells of HC rats. So dendritic complexity did not predict behaviors measured here. On the other hand, spine density on pyramidal apical dendrites of HCMA and HCLA rats correlated positively with anxiety but not startle amplitudes. Thus greater excitatory spine density [56] predicts higher levels of anxiety. In contrast, there were no correlations of spine density with behavior on basilar dendrites of HCMA and HCLA rats.

4.3. Golgi findings – dorsal hippocampus dentate granule cells

There were no group differences between HC and PS rats in total dendritic length or branching of dentate granule cells. However, more anxious PS and HC rats displayed equally reduced spine density on granule cell dendrites relative to less anxious PS and HC rats (Fig. 12). Moreover, across PS and HC rats, spine density correlated negatively with anxiety, but not startle, suggesting fewer dendritic spines predict more anxiety.

Functionally, reduced spines would be associated with less excitatory activity in the trisynaptic circuit of the dorsal hippocampus. This might make sense in view of dorsal hippocampus projections to the medial prefrontal cortex [58]. This circuit when excited could exert an inhibitory influence on BLA and central amygdala (CeA) excitability via medial prefrontal cortex excitatory projections onto inhibitory intercalated nucleus neurons in the amygdala [59]. If this surmise is true, one would predict that in LA PS rats there would be more mPFC output cellular activation during predator stress, and conversely in MA PS rats there would be less mPFC cellular activation during predator stress. This is in fact the case (Adamec et al., accepted).

Since HC rats display these spine differences, they likely reflect a spontaneously occurring and perhaps genetically determined morphological feature. In view of this possibility, the same might be said of the PS rats. If true, then a bias toward more dorsal hippocampal activation in PSLA rats would suppress BLA and CeA excitability and reduce BLA plasticity in response to stress. The opposite would be the case for PSMA rats, where reduced dorsal hippocampal activation would relieve medial prefrontal cortical suppression of the amygdala and promote more BLA plasticity in response to stress. This is an attractive hypothesis from a translation perspective, where smaller human hippocampal volume is a vulnerability marker for PTSD [38].

4.4. Robust correlation and multiple correlation analyses of BLA and hippocampal morphology with behavior

Multiple correlation of BLA and DHC morphology variables distinguishing the groups was done on HC rats (across HCMA and HCLA). The multiple correlation of dentate cells spine densities and dendritic lengths of BLA stellate and pyramidal cells combined yielded a satisfactory model of the following form: Anxiety = $-1.19 \times$ dentate cells spine densities + $2.12 \times$ BLA pyramidal and stellate cells total dendritic lengths (summed). Coefficients were standardized and differed from zero and the multiple R was significantly different from zero. These two variables accounted for 96.2% of the variance of anxiety (df adjusted $R^2 = .962$). The model indicates that lower DHC dendritic spine densities predict more anxiety while greater BLA dendritic lengths predict more anxiety. It is striking how much of the variance of anxiety is accounted for by these two variables, which together account for much of the anxiety seen in HCMA and HCLA rats. As suggested above, the HCMA phenotype likely reflects an underlying genotype, and present data strongly implicate BLA dendritic hypertrophy and dentate spine density reduction as the neural expression of the phenotype. The model suggests dendritic hypertrophy in BLA principle output neurons increases BLA excitability. Moreover reduced excitability in the trisynaptic circuit of the DHC likely adds to this excitability by reducing hippocampal driving of a negative feedback from hippocampal to medial prefrontal cortex to amygdala projections (see above). Together this increase in BLA excitability produces more anxiety in HCMA rats. Conversely reduced BLA dendritic lengths and increased DHC dentate spine density produces less anxiety in HCLA rats.

For PSMA and PSLA a robust correlation was done using hippocampal spine densities alone. The correlation of dentate cells spine densities yielded a satisfactory model of the

following form: Anxiety = $-0.86 \times$ dentate cells spine densities. The coefficient is standardized and differs from zero and R was significantly different from zero. This variable accounted for 70.4% of the variance of anxiety (df adjusted $R^2 = .704$). If spine density is decreased by stress, then the reduced driving of the dorsal hippocampus may serve to prolong anxiety by increasing BLA response to subsequent stress. If spine density is a phenotypic marker of vulnerability to stress, its presence alone does not alter anxiety but by relieving negative cortical feedback during stress enhances BLA excitability changes in response to predator stress.

4.5. Conclusions

Our findings implicate variation in dendritic arbor of amygdala neurons as a candidate mechanism for variation in anxiety but not startle in spontaneously more and less anxious rats. Correlation analysis suggests that dendritic length is particularly relevant to anxiety levels. It is surprising that the same cannot be said of variation in anxiety of stressed rats. The BLA can undergo structural reorganization in response to stressors as diverse as immobilization, maternal stress and external application of the stress hormone, corticosterone [32,34,60,61]. A prominent feature of such structural reorganization is dendritic expansion (hypertrophy) of excitatory neurons of the BLA. Once evoked, BLA hypertrophy is as lasting as long-lasting anxiety [33]. Conversely, dendritic retraction achieved by viral-mediated over expression of inhibitory SK2 potassium channels in BLA results in reduced anxiety [35]. Clearly predator stress is not a stressor which induces dendritic hypertrophy. Therefore, dendritic remodeling of BLA by stress seems to be stressor specific. This is consistent with earlier work of Vyas and colleagues [32]. This raises a caution regarding translation of findings in animal models of stress vulnerability/resilience. While morphological characteristics of amygdala cells are a putative substrate for individual differences in response to stress, there are other possibilities. So effects of a variety of stressors on brain and behavior should be examined to categorize candidate mechanisms. It is an open question if different stressors impact limbic circuits in humans differently.

In contrast, spine density of DHC dentate granule cells is a negative predictor of anxiety in both handled (HCMA and HCLA) and predator stressed (PSMA and PSLA) rats. This either means that predator stress reduces spine density on DHC dentate granule cells, or given the spontaneous reduction of spine densities in HCMA rats, represents a preexisting phenotype. If the latter, reduced DHC spine densities represents a vulnerability factor in anxious response to predator stress. From our present data, it is difficult to determine if morphimetric differences between MA and LA stressed animals were stress induced, or were preexisting differences. It is not possible to achieve paired measurements before and after stress, because of the post-mortem nature of Golgi staining. Nor is it yet possible to reliably predict MA or LA responses to predator stress. So test of whether reduction in dendritic spine density of dentate granule cells is induced by stress or is preexisting is difficult at this time. The fact that dentate dendritic spine density behavior correlations hold for both stressed and handled rats is consistent with a preexisting characteristic of dentate spines in both handled and stressed rats.

Morphimetric differences in the present study were related to EPM anxiety but not to startle response. Clearly some other substrate or mechanism controls startle differences between HCMA and HCLA rats and the lack of startle differences between PSMA and PSLA rats. A likely starting point is the dorsolateral amygdala [62] where NMDA receptor block before predator stress prevents increases in startle, but is without effect on EPM anxiety.

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Abbreviations

BLA	basolateral amygdala
DHC	dorsal hippocampus
EPM	elevated plus maze
HC	handled control
5-HTTLPR	reduced functioning polymorphisms in the serotonin transporter
LA	less anxious
MA	more anxious
mRNA	messenger RNA
PS	predator stressed
PSS	predator scent stressed
PTSD	post traumatic stress disorder

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Fig 1.
(A) A photomicrograph of a Golgi stained stellate cell of the basolateral amygdala (BLA).
(B) A photomicrograph of a Golgi stained pyramidal cell of the basolateral amygdala (BLA).

Fig 2.

(A) A photomicrograph of a Golgi stained dentate granule cell of the dorsal hippocampus.
(B) Photomicrograph of Golgi stained dentate granule cell spines of the dorsal hippocampus of a handled less anxious (HCLA top) rat and of a handled more anxious (HCMA bottom) rat.

Fig 3.

(A) Plotted across all handled controls (HC), and all predator stressed (PS) rats are mean + SEM of behaviors measured in the EPM (ratio time, ratio entry and the anxiety index – anxiety which is $1 - (\text{ratio time} + \text{ratio entry}/2)$). (B) Plotted as in (A) are mean + SEM of behaviors measured in the EPM. Here the influence of closed arm entries has been removed with analysis of covariance. (C) Mean + SEM of closed arm entries in the EPM are plotted for all handled controls (HC) and all predator stressed rats (PS). Within a given behavioral plot in (A–C), means marked differently differ.

Fig 4.

Plotted in (A and B) are mean + SEM of behaviors in the hole board for all handled controls (HC) and all predator stressed rats (PS). (A) shows mean + SEM of measures of exploration (head dips) and activity (rears) in the hole board test. (B) shows mean + SEM of time spent in the center and time spent near the walls of the hole board. Within a given behavioral plot in (A and B), means marked differently differ and unmarked means do not differ.

Fig 5.

(A) Plotted are mean + SEM of the anxiety index of less anxious (LA – less EPM anxiety) and more anxious (MA – more EPM anxiety) rats selected for Golgi analysis in the Handled Controls and Predator Stressed groups. Means marked similarly do not differ while means marked differently differ. (B) Plotted are mean + SEM of closed arm entries in the EPM of less anxious (LA – less EPM Anxiety) and more anxious (MA – more EPM anxiety) rats selected for Golgi analysis in the Handled Controls and Predator Stressed groups. In this plot means marked differently differ, means marked the same do not differ. (C). Plotted are mean + SEM of the anxiety index of less anxious (LA – less EPM anxiety) and more anxious (MA – more EPM anxiety) rats selected for Golgi analysis in the Handled Controls and

Predator Stressed groups. Means are those observed after the influence of closed arm entries is removed with analysis of covariance. Means marked similarly do not differ while means marked differently differ.

Fig 6.

(A) Plotted are median + SEMd of peak startle amplitude (in volts) of less anxious (LA – less EPM anxiety) and more anxious (MA – more EPM anxiety) rats selected for Golgi analysis in the Handled Controls and Predator Stressed groups. Medians marked similarly do not differ while medians marked differently differ. (B) Plotted are Tau + SE of trials to decline to 37% of the maximum of peak startle amplitude of less anxious (LA – less EPM anxiety) and more anxious (MA – more EPM anxiety) rats selected for Golgi analysis in the Handled Controls and Predator Stressed groups. Tau values marked similarly do not differ while Tau values marked differently differ.

Fig 7.

Plotted are mean + SEM of total dendritic length (μm) collapsed across hemispheres. Plotted separately are Handled Controls which are less anxious (HCLA) or more anxious (HCMA) and Predator Stressed rats which are less anxious (PSLA) or more anxious (PSMA). (A) shows data from basolateral amygdala stellate cells while data from pyramidal cells appear in (B). For a given plot (A or B) means marked similarly do not differ, while means marked differently differ.

Fig 8.

Plotted are mean shell crossings vs distance from the soma of the crossings from the Sholl analysis of basolateral amygdala stellate cells collapsed across hemispheres. Plotted separately are Handled Controls which are less anxious (HCLA) or more anxious (HCMA) and Predator Stressed rats which are less anxious (PSLA) or more anxious (PSMA). (A) shows mean $\pm$ SEM, while (B) shows mean $\pm$ 95% confidence intervals of shell crossings. In (B), HCMA means marked with an “*” differ from the remaining groups which do not differ. This occurs over a range from the soma of 50–130 μ M.

Fig 9.

Plotted are mean shell crossings vs distance from the soma of the crossings from the Sholl analysis of basolateral amygdala pyramidal cells collapsed across hemispheres. Plotted separately are Handled Controls which are less anxious (HCLA) or more anxious (HCMA) and Predator Stressed rats which are less anxious (PSLA) or more anxious (PSMA). (A) shows mean $\pm$ SEM, while (B) shows mean $\pm$ 95% confidence intervals of shell crossings. In (B), HCMA means marked with an “*” differ from the remaining groups which do not differ. This occurs over a range from the soma of 40–90 μ M.

Fig 10.

Plotted are mean + SEM of number of branches in the branch point analysis collapsed across hemispheres and branch order. Plotted separately are Handled Controls which are less anxious (HCLA) or more anxious (HCMA) and Predator Stressed rats which are less anxious (PSLA) or more anxious (PSMA). (A) shows data from basolateral amygdala stellate cells while data from pyramidal cells appear in (B). For a given figure (A or B) means marked similarly do not differ, while means marked differently differ.

Fig 11.

(A) Plotted are mean + SEM of spine densities (spines/ μM) of basolateral amygdala pyramidal cells collapsed across hemispheres and basilar and apical dendrites. Plotted separately are Handled Controls which are less anxious (HCLA) or more anxious (HCMA) and Predator Stressed rats which are less anxious (PSLA) or more anxious (PSMA). Means marked similarly do not differ, while means marked differently differ.

(B) Plotted are mean + SEM of total dendritic length (μM) of basolateral amygdala stellate and pyramidal cells combined from the right hemisphere. Plotted separately are Handled Controls which are less anxious (HCLA) or more anxious (HCMA) and Predator Stressed rats which are less anxious (PSLA) or more anxious (PSMA). Means marked similarly do not differ, while means marked differently differ.

Fig 12.

Plotted are median + SEMd of spine densities (spines/mM) of dentate granule cells of the dorsal hippocampus collapsed across hemispheres. Plotted separately are Handled Controls which are less anxious (HCLA) or more anxious (HCMA) and Predator Stressed rats which are less anxious (PSLA) or more anxious (PSMA). Medians marked similarly do not differ, while medians marked differently differ.