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Gabrb3 gene deficient mice exhibit increased risk assessment behavior, hypotonia and expansion of the plexus of Locus coeruleus dendrites

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Abstract

Gabrb3 gene deficient (gabrb3^{-/-}) mice, control littermates (gabrb3^{+/+}) and their progenitor strains, C57Bl/6J and 129/SvJ were assessed for changes in the morphology of the main noradrenergic nuclei, the locus coeruleus (LC) and LC-associated behaviors including anxiety and muscle tone. While the area defined by the cell bodies of the LC was found not to differ between gabrb3^{-/-} mice and controls, the pericoerulear dendritic zone of the LC was found to be significantly enlarged in gabrb3^{-/-} mice. Relative to controls, gabrb3^{-/-} mice were also found to be hypotonic, as was indicated by poor performance on the wire hanging task. Gabrb3^{-/-} mice also exhibited a significant increase in stretch-attend posturing, a form of risk assessment behavior associated with anxiety. However, in the plus maze, a commonly used behavioral test for assessing anxiety, no significant difference was observed between gabrb3^{-/-} and control mice. Lastly, relative to controls, gabrb3^{-/-} mice exhibited significantly less marble burying behavior, a method commonly used to assess obsessive-compulsive behavior. However, the poor marble burying performance of the gabrb3^{-/-} mice could be associated with the hypotonic condition exhibited by these mice. In conclusion, the results of this study indicate that the gabrb3 gene contributes to LC noradrenergic dendrite development with the disruption of this gene in mice resulting in an enlarged plexus of LC dendrites with a concurrent reduction in muscle tone and marble burying behavior, an increase in risk assessment behavior but no change in the plus maze parameters that are commonly used for assessing anxiety.

Section: Disease-related Neuroscience

Keywords

Locus coeruleus; Hypotonia; gabrb3 gene; Risk assessment

1. Introduction

Disruption of the gabrb3 gene (encoding the β_3 subunit of the γ -amino butyric acid_A (GABA_A) receptor) in mouse leads to numerous behavioral abnormalities including learning

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and memory deficits, epilepsy, poor motor skills, repetitive behavior, hyperactivity, abnormal sleep architecture, changes in odor discrimination and altered nociception (DeLorey, 2005; DeLorey et al., 1998; Nusser et al., 2001). These behaviors are modulated, in part, by the noradrenergic nuclei, the locus coeruleus (LC), which regulates a broad spectrum of physiological and behavioral processes that include anxiety, attention, muscle tone, arousal, neophobia, emotionality and stress (Aston-Jones and Cohen, 2005; Berridge and Waterhouse, 2003; Jiang et al., 1996; Kiyashchenko et al., 2001; Priolo et al., 1991). Consequently, deficits in LC function have been implicated in several neurological disorders such as attention deficit hyperactivity disorder, depression and epilepsy (Berridge and Waterhouse, 2003).

The neurotransmitter GABA is present during embryogenesis and influences the development of numerous brain regions including the monoaminergic neurons of the brainstem (Lauder et al., 1986). During this period, a dense plexus of GABA-immunoreactive fibers can be visualized in the rat brainstem as early as embryonic day 13 (Lauder et al., 1986), coinciding temporally and spatially with the appearance and differentiation of tyrosine hydroxylase (TH) containing neurons in the same region (Konig et al., 1988). GABA_A receptors are hetero-oligomeric GABA-gated chloride channels constructed from eight classes of subunits exhibiting varying amino acid sequence homologies (α_{1-6} , β_{1-4} , γ_{1-3} , δ , $\rho_{1,2}$, π , ϵ and θ) and when combined produce multiple isoforms of GABA_A receptors with varying GABA sensitivities and pharmacologies (Bonnert et al., 1999; Olsen and DeLorey 1999). During this developmental period several of the many GABA_A receptor subunit proteins are expressed. In adult rats, GABA is found in about 80% of the small and medium sized neurons uniformly scattered throughout the LC (Iijima, 1993) with only a few of the GABA_A receptor subunits being expressed in this nucleus ($\alpha_3, \beta_3 > \alpha_2, \beta_1 > \gamma_1$) (Luque et al., 1994). The abundance of the β_3 subunit during both brainstem development and in adult LC supports its presumed importance for proper LC development and mature function.

The LC nucleus is a small collection of noradrenergic neurons located posterior to the periaqueductal gray in the dorsorostral pons. The LC-norepinephrine (LC-NE) neuronal system is a brainstem neuromodulatory nuclei that provides the bulk of NE in the brain, giving rise to highly divergent and extensive efferents projecting throughout the neuraxis, including ascending projections to the neocortex, cerebellum and hippocampus (Aston-Jones and Cohen, 2005). The global NE output by the LC is controlled by relatively few sources of excitatory inputs, which include multiple pathways from the glutaminergic nucleus paragigantocellularis (Aston-Jones et al., 1991). A number of inhibitory inputs to the LC arise from the nucleus prepositus hypoglossi and involve GABAergic and adrenergic systems and serve to modulate the gain of the LC's output (Aston-Jones et al., 1991). Consequently, a variety of compounds that facilitate membrane hyperpolarization via the GABA_A receptor chloride channel complex, including barbiturates (Osmanovic and Shefner, 1990), alcohol (Sanna et al., 1990), muscimol (Scotti de Carolis et al., 1969) and benzodiazepines (Tanaka et al., 2000), inhibit LC function, resulting in a reduction in alarm/anxiety and punishment-avoidance responses (Priolo et al., 1991; Rasmussen and Jacobs, 1986). Projections from the LC to brain regions involved in the physiological and behavioral responses to pain and fear are thought to contribute to "anxiety-like" animal behavior (Bremner et al., 1996). Furthermore, evidence arising from a variety of clinical studies support the hypothesis that a dysfunctional LC may be involved in the pathophysiology of some forms of anxiety, principally panic disorders, and that the somatic symptoms, present during panic attacks, may result from LC hyperactivity (Charney et al., 1984; Goddard and Charney, 1997; Salchner et al., 2006). Conversely, microinfusion of the GABA_A receptor antagonists picrotoxin and bicuculline or the benzodiazepine binding site inverse agonists beta-carboline carboxylate ethyl (β CCE) directly into the LC of free-moving rodents excites the LC, increasing alarm/anxiety and punishment-avoidance responses. Furthermore, the injection of these compounds elicit dose-dependent behavioral stimulation involving increases in locomotor activity, stereotyped behavior and an increase in sensitivity

to external stimuli with higher doses culminating in seizures and electrocortical epileptic discharges (Priolo et al., 1991). Interestingly, each of these behavioral traits is likewise observed in *gabrb3*^{-/-} mice, without the need for administering GABAergic antagonists, suggestive of LC involvement.

The current study sought to assess the gross morphology of the LC in *gabrb3*^{-/-} mice, relative to control mice, and to evaluate LC associated behaviors within these mice, including muscle tone and anxiety related behaviors. The findings of this study provide additional insight into the potential role of the *gabrb3* gene in LC development and function. Interestingly, behavioral abnormalities observed in *gabrb3*^{-/-} mice overlap extensively with phenotypic traits often reported for autism spectrum disorder (ASD) (for review see (DeLorey, 2005)). The findings of this study add to an already abundant body of evidence implicating the GABRB3 gene in ASD (Buxbaum et al., 2002; Cook et al., 1998; Shao et al., 2003).

2. Results

2.1. Plus maze

The percentage of open arm entries and percentage of time spent on the open arms are both utilized as measures of anxiety while the number of closed arm entries is typically used to assess motor activity (File, 2001). Lack of gender differences in plus maze performance parameters, and in stretch-attend posturing (SAP), a form of risk-assessment behavior, has been reported in a variety of mouse strains (Ramboz et al., 1998; Rodgers and Cole, 1993). There were no gender differences observed within *gabrb3*^{-/-} and *gabrb3*^{+/+} mice in the plus maze behavioral parameters, therefore, data from both genders were combined for *gabrb3*^{-/-} mice (n=8, 4 males and 4 females) and *gabrb3*^{+/+} (n=16, 8 males and 8 females) for analysis. This was necessary due to the high mortality rate for *gabrb3*^{-/-} mice at birth of around 90%, largely due to cleft palates, thereby limiting the numbers of animals available for study (Homanics et al., 1997). *Gabrb3*^{-/-} mice were found not to differ significantly from *gabrb3*^{+/+}, C57Bl/6J (n=8, male) and 129/SvJ (n=8, male) mice with respect to the number of closed arm entries; % time spent in open arm and % open arm entries (Table 1). Interestingly, *gabrb3*^{-/-} mice exhibited a significantly higher level of SAP behavior relative to that of control mice ($F_{(3,34)}=22.3$, $p<0.01$) (Table 1).

2.2. Marble test

Marble burying behavior in rodents has been proposed as a means to assess obsessive-compulsive behavior (Njung'e and Handley, 1991b). Previous reports have demonstrated an absence of significant gender differences with respect to marble burying behavior or to general digging behavior in mice (Masuda et al., 2000; Njung'e and Handley, 1991b). We also observed no gender differences in the number of marbles buried by male or female mice within *gabrb3*^{-/-} and *gabrb3*^{+/+} mouse groups. Therefore, data from both genders, within each genotype, were combined for analysis. Significant differences in marble burying behavior was observed between *gabrb3*^{-/-} (n=7, 4 females and 3 males) and *gabrb3*^{+/+} (n=14, 8 females and 6 males) mice, with *gabrb3*^{-/-} mice burying on average 1.3 ± 1.3 (mean $\pm$ SEM) marbles during the test period as compared to the *gabrb3*^{+/+} mice who buried 15.4 ± 1.2 marbles ($F_{(3,37)}=14.4$, $p<0.001$) (Fig 1A). *Gabrb3*^{-/-} mice likewise buried significantly fewer marbles than the C57Bl/6J (n=8, male) and 129/SvJ (n=12, male) mice (14.1 ± 2.1 , $p<0.001$ and 10.6 ± 1.5 , $p<0.001$, respectively). Marble burying behavior between *gabrb3*^{+/+}, 129/SvJ (n=12, male) and C57Bl/6J (n=8, male) mice did not differ significantly ($F_{(2,31)}=3$, $p>0.05$). As *gabrb3*^{-/-} mice exhibit hyperactivity, especially after being handled, an extended one-hour period of exposure to the test chamber was also evaluated. Within the extended test period *gabrb3*^{-/-} mice continued to perform poorly despite the allowances given for resolution of handling-induced hyperactivity (Fig 1B), burying no more marbles than in the shorter test period with the surface

of the sawdust revealing little evidence of digging behavior. Moreover, close observation of control mice suggest that the mice randomly dig in the bedding as opposed to deliberately burying the marbles and often continued digging even after the marbles were completely buried. Furthermore, when mice were placed in a divided cage with marbles placed on the sawdust of only one side of an open partition, the control mice exhibited digging behavior to an equal extent on both sides of the partition, regardless of the presence of marbles or not (data not shown). In contrast, *gabrb3*^{-/-} mice exhibited little digging behavior on either side of the partition.

2.3. Wire hanging test

No significant gender differences in wire hanging behavior were observed in either the *gabrb3*^{-/-} (n=12, 6 females and 6 males) or *gabrb3*^{+/+} (n=16, 8 females and 8 males) genotypes therefore, data were grouped within each genotype for analysis. In addition, no significant differences ($F_{(2,37)}=2.2, p>0.05$) in wire hanging performance were observed between the three control mouse genotypes *gabrb3*^{+/+}, C57Bl/6J (n=16, male) and 129/SvJ (n=8, male). However, *gabrb3*^{-/-} mice exhibited a significant deficit in the ability to hang from the wire relative to *gabrb3*^{+/+}, C57Bl/6J and 129/SvJ mice ($F_{(3,48)}=10.9, p<0.001$) (Fig. 2). Furthermore, 75% of the control mice were able to coordinate their efforts well enough to be able to grip the wire with one or both of their hind limbs, with all three control genotypes not differing significantly ($p>0.05$) from one another. In contrast, only 33% of the *gabrb3*^{-/-} mice were able to grasp the wire with one or both of their hind limbs. Subsequently, *gabrb3*^{-/-} mice were found to differ significantly from *gabrb3*^{+/+} mice in regards to their abilities to grasp the wire with their hind limbs ($p<0.05$). Moreover, the longest time that a *gabrb3*^{-/-} mouse was able to hang onto the wire was for 40 sec, which required the use of its hind limbs and tail. In contrast, *gabrb3*^{+/+}, C57Bl/6J and 129/SvJ mice that utilized their hind limbs and tail, were able to hang on to the wire in excess of 120 seconds.

2.4. Immunohistochemistry

The LC has a large defined dendritic area surrounding the compact cell-dense region. The region immediately surrounding the LC's compact cell dense nucleus has been termed the pericoerulear dendritic zone, containing a dense plexus of dendrites (Aston-Jones et al., 2004; Shipley et al., 1996). In this study we measured the surface area containing the cell bodies of the LC as well as the surface area that encompassed the pericoerulear dendritic zone. Between 5-13 separate LC surface area measurements were taken from coronal sections at Bregma -5.40 mm from each mouse genotype, *gabrb3*^{-/-} (n=3, male), *gabrb3*^{+/+} (n=6, male), C57Bl/6J (n=3, male) and 129/SvJ (n=3, male). Therefore, 1-4 images were assessed and averaged per mouse within each genotype and includes images taken from both the left and right LC. Prior to combining right and left brainstem data, we ascertained that there was no significant difference ($p>0.05$) between surface areas of the left and right LC within each individual mouse. Representative examples of the coronally sectioned LC from both *gabrb3*^{+/+} and *gabrb3*^{-/-} mice are presented in Figure 3 A & B, respectively. The cell body area was found to be relatively consistent ($p>0.05$) between all male control mice (*gabrb3*^{+/+}, C57Bl/6J, and 129/SvJ) as well as in comparison to *gabrb3*^{-/-} mice (Table 2). However, the pericoerulear dendritic zone of *gabrb3*^{-/-} mice was found to be significantly larger ($25\% \pm 3.1\%, p<0.05$) than in *gabrb3*^{+/+} mice and significantly larger than observed in the progenitor strains 129/SvJ and C57Bl/6J ($53.8\% \pm 3.8\%, p<0.01$ and $42.9\% \pm 3.6\%, p<0.01$, respectively) (Table 2). The thickness of tissue sections (45 μ m) and consequently the high density of dendrites within the sections prevented the counting of individual neurons within the LC.

3. Discussion

Gabrb3^{-/-} mice exhibit a variety of behavioral abnormalities including repetitive behavior, hyperactivity, tactile hyperresponsivity, altered odor discrimination, enhanced nociception, cognitive impairment, abnormal sleep architecture, epilepsy and reduced benzodiazepine ligand binding in brain (DeLorey, 2005). Many of these behaviors are regulated in part by the LC (Aston-Jones and Cohen, 2005; Berridge and Waterhouse, 2003). We now report that gabrb3^{-/-} mice also exhibit an enlarged pericoerulear dendritic zone of the LC and additional behavioral abnormalities that may likewise be partially attributed to abnormal LC function.

The dendrites that define the pericoerulear region of the LC are extensive, and represent important sites through which afferents may modulate LC neuronal activity. GABAergic interneurons within the pericoerulear zone represent a potentially significant element in the integration of afferent inputs, which could substantially regulate activity within the LC-NE system (Aston-Jones et al., 2004). The LC and the locus subcoeruleus (SC) belong to the descending pathway that regulates nociceptive neurons in the dorsal horn of the spinal cord with the norepinephrine produced in the LC being involved in the suppression of nociceptive neuronal activity (Jones and Gebhart, 1986; Mokha et al., 1985). In addition, increased activity in LC-NE projections to the olfactory bulb results in enhanced detection of relatively weak odors (Jiang et al., 1996). Consequently, the semi-quantitative finding that the pericoerulear dendritic zone of the LC in gabrb3^{-/-} mice is significantly larger than in control mice, may underlie the previously reported enhanced responsiveness to low intensity thermal stimuli in the tail flick and hot-plate test (Ugarte et al., 2000) and to altered odor discrimination (Nusser et al., 2001).

We report here that gabrb3^{-/-} mice also exhibit a significant increase in SAP, associated with risk assessment behavior. SAP is closely related to defensive behaviors in mice (Griebel et al., 1996) and is particularly responsive to an array of anxiolytic drugs, suggesting an association with anxiety related psychopathology (Blanchard et al., 2003). High levels of SAP were also observed in the gabrb3^{-/-} mice in the open field test (data not shown), a paradigm based on exploratory behavior that also gauges general anxiety (Bourin, 1997). Interestingly, risk assessment behavior (SAP) has also been reported to be associated with an increase in Fos expression in the LC (Ribeiro-Barbosa et al., 2005). In contrast, gabrb3^{-/-} mice were found not to differ significantly ($p > 0.05$) from controls in the commonly measured plus-maze behavioral parameters for anxiety. However, we have not been able to fully rule out whether the hyperactive behavior exhibited by most gabrb3^{-/-} mice may have masked the detection of anxiety-like behaviors by this method.

Gabrb3^{-/-} mice, compared to controls, were found to exhibit a significant reduction in the latency time to fall from a wire in which they were placed hanging from their forepaws. In addition, gabrb3^{-/-} mice were also observed to be poor at using their hind limbs or tail to grasp the wire during the test. The inability to use hind limbs in order to grasp a wire in which they are hanging from has been suggested as being indicative of the presence of impaired motor-coordination (Freitag et al., 2003). This finding is supported by previous observations that gabrb3^{-/-} mice exhibit poor motor-coordination as assessed by the rotarod test, in which mice are tested for their ability to walk on a rotating dowel (DeLorey et al., 1998). Both the reduced latency to fall and the inability to coordinate their hind limbs to grasp the wire suggest that the gabrb3^{-/-} mice are hypotonic. One could argue that the observed impairment in wire hanging ability could be attributed to lower muscle strength; however, the expression of the gabrb3 gene is specific, with few exceptions, to the CNS, therefore, ruling out this hypothesis. The observation that gabrb3^{-/-} mice are hypotonic may seem to be at odds with these mice also being hyperactive, however hyperactivity and hypotonia have been reported previously to occur concurrently in mice (Ikegami et al., 2000) as well as in humans (Wolpert et al., 2000).

Whether the enlarged pericoerulear dendritic field of the LC observed in *gabrb3*^{-/-} mice contributes in any way to the poor performance exhibited by the *gabrb3*^{-/-} mice on the wire hanging test is unclear. However, the LC has also been reported as being involved in maintaining muscle tone (Kiyashchenko et al., 2001; Pompeiano, 2001), with a correlation being demonstrated between LC activity and the maintenance of muscle tone during waking (Andre et al., 1995). In contrast, reduction in LC activity, observed during cataplexy and REM sleep, has been demonstrated as being associated with loss of muscle tone (Siegel et al., 1992). Interestingly, REM sleep abnormalities have also been reported in *gabrb3*^{-/-} mice (Wisor et al., 2002). In a separate study, mice disrupted for a different gene, were also found to be hypotonic and exhibit poor feeding skills with little milk found in their stomachs resulting in an unusually high mortality rate (Tsai et al., 1999). *Gabrb3*^{-/-} mice likewise exhibit poor feeding ability shortly after birth, also resulting in a high mortality rate (~90%). This inability to nurse is largely due to a cleft palate, occurring in approximately 57% of the offspring that possess a disrupted *gabrb3* gene. However, another 30%, with intact palates, likewise die within 24 hrs of birth due to the inability to feed (Homanics et al., 1997). One may speculate that the hypotonic condition present in both these gene disrupted mouse lines may be a contributing factor in their poor feeding (suckling) ability.

Marble burying behavior in rodents, thought to be a model of obsessive-compulsive disorder (Njung'e and Handley, 1991b), is sensitive to selective serotonin re-uptake inhibitors (SSRI), the current medication of choice for obsessive compulsive disorder (Njung'e and Handley, 1991a). Several studies have linked burying behavior with 5-HT function (Ichimaru et al., 1995), with elevated 5-HT activity causing a decrease in burying behavior (Abe et al., 1998; Njung'e and Handley, 1991a). Increased 5-HT expression in dorsal raphe nucleus of neuromedin B receptor deficient mice, was also found to be associated with a decrease in marble burying (Yamada et al., 2002). Innervation of the dorsal raphe by the LC has been reported to be involved in the excitatory influenced release of 5-HT in the dorsal raphe (Pudovkina et al., 2002). As the *gabrb3* gene is highly expressed in the LC (Luque et al., 1994), one would expect the *gabrb3* gene product (β_3 subunit of the GABA_A receptor) to have a role in LC mediated 5-HT regulation in the dorsal raphe. It therefore follows that the significant decrease in marble burying behavior exhibited by *gabab3*^{-/-} mice may entail some change in LC regulation of 5-HT neurons. It is noteworthy, that we have previously observed mice deficient in the α_{2A} adrenergic receptor to exhibit LC hyperplasia (Davies et al., 2003) and to bury fewer marbles than controls (Lahdesmaki et al., 2002). However, before any conclusions can be drawn between the lack of marble burying behavior in *gabrb3*^{-/-} mice and obsessive-compulsive behavior, several factors need to be considered in regards to the context of the test itself. Digging behavior is found in mice of every age and in both sexes and can be regarded as natural and instinctive (Masuda et al., 2000). Mice exhibit digging behavior in the presence or absence of glass marbles, therefore the burying of marbles serves as a gauge of the intensity of this digging activity, whether or not the marbles themselves evoke an increase in burying behavior (Gyertyan, 1995). Therefore, the virtual absence of digging behavior exhibited by *gabrb3*^{-/-} mice, and consequent lack of buried marbles, could potentially be attributed to the hypotonia observed in *gabrb3*^{-/-} mice.

In summary, the results of this study suggest the *gabrb3* gene is important to normal LC maturation, with its loss resulting in an enlarged pericoerulear dendritic zone of the LC in *gabrb3*^{-/-} mice, compared to controls. This hypertrophic LC pericoerulear region may partially contribute to several of the behavioral abnormalities observed in *gabrb3*^{-/-} mice. Interestingly, the *GABRB3* gene has been strongly implicated in ASD and the disruption of this gene in mouse results in numerous behaviors that parallel those observed in ASD (DeLorey, 2005). In addition, it has been previously suggested that a decrease in tonic innervations of the LC and the raphe nuclei to the spinal locomotion center may be responsible for the hypotonia and impaired locomotion observed in early infantile autism (Segawa, 1989). Subsequently,

hypotonia is commonly reported in ASD cases that are associated with abnormalities to a specific region of chromosome 15 that contain the GABRB3 gene (Wolpert et al., 2000). Interestingly, while anxious behavior has been reported in ASD (Prater and Zylstra, 2002), *gabrb3*^{-/-} mice were found not to be anxious, relative to controls, as measured by the commonly used plus maze parameters often used to assess anxious behavior in rodents. However, the hyperactive behavior exhibited by *gabrb3*^{-/-} mice may have masked the detection of anxious behavior by this method. Nevertheless, we did observe an increase in SAP behavior in *gabrb3*^{-/-} mice, suggesting an increase in risk assessment behavior, thought to also be associated with anxiety related psychopathology (Blanchard et al., 2003). Interestingly, an increase in SAP behavior in rodents has been correlated with a decrease in novelty seeking behavior (Johansson and Hansen, 2001). Both risk assessment and novel object exploration behaviors are indicators of emotional reactivity in rodents (Roy and Chapillon, 2004). Therefore, the high SAP exhibited by *gabrb3*^{-/-} mice may be reflective of a higher state of emotional reactivity and neophobia. This is of particular interest, as a study examining the genetic association between ASD susceptibility gene candidates and ASD phenotypes found a strong association between the GABRB3 locus and a particular ASD phenotype known as “insistence on sameness”, a trait in which ASD individuals react poorly to novel situations (Shao et al., 2003). The finding that *gabrb3*^{-/-} mice exhibit hypertrophy of the pericoerulear dendritic zone of the LC, a region associated with several of the behaviors that are abnormal in ASD, suggests further assessment of LC morphology in ASD postmortem tissue is warranted. This assessment has begun, to a limited extent, with one report observing that in one out of six autistic postmortem brains, the locus coeruleus appeared disorganized and coarse (Kemper and Bauman, 1993). In a separate report, two out of six autistic postmortem brains exhibited LC abnormalities, with one case exhibiting loosely grouped neurons within the LC and a second case with a widely dispersed LC (Bailey et al., 1998). However, a third report failed to find significant differences in neuronal density, volume or total cell count in the LC's of five postmortem autistic brains examined (Martchek et al., 2006). Nevertheless, as there is a general agreement that a number of genes are likely to play pivotal roles in the etiology of autism, one might expect a strong phenotypic overlap between members of a particular ASD subset with less overlap between members of different ASD subsets. The findings presented here on the *gabrb3*^{-/-} mouse coupled with our current understanding of the genetics of autism, suggests that postmortem autistic brain tissue derived from cases with known abnormalities to the region of chromosome 15 that contains the GABRB3 gene or ASD cases that possess the phenotypic trait referred to as “insistence on sameness”, reported to be linked to the GABRB3 locus, would provide a greater likelihood of exhibiting LC abnormalities.

4. Experimental procedure

4.1. Mice

C57Bl/6J and 129/SvJ mice were obtained from Jackson Laboratory (Davis, CA) at 6-8 weeks of age. The F3 generation of *gabrb3* gene knockout mice (*gabrb3*^{-/-}) and wild-type littermates (*gabrb3*^{+/+}) were supplied by Dr. Gregg Homanics of the University of Pittsburgh and were bred in-house. Techniques used to disrupt the *gabrb3* gene in mouse have been previously described (Homanics et al., 1997). All mice were evaluated between 28-52 weeks of age and were within the weight range of 22-35 gr. Due to the high mortality rates at birth exhibited by *gabrb3*^{-/-} mice (Homanics et al., 1997), male and female *gabrb3*^{-/-} and *gabrb3*^{+/+} mice were grouped within genotype to increase the number of test subjects available for evaluations. This was only done in circumstances where there was evidence that a particular behavior was not influenced by gender. Mice were housed in groups of 4-8 on a 12/12-h light/dark cycle under controlled temperature with food and water provided *ad libitum*. All animal protocols conform to guidelines determined by the National Institutes of Health (USA) Office for Protection from

Research Risks and were approved by the Animal Care and Use Committee of the Veterans Affairs Palo Alto Health Care System.

4.2. Plus Maze

The elevated plus maze, a widely used method for assessing anxiety (File, 2001), consists of two closed arms and two open arms (each arm measured 30×5 cm) that are elevated 30 cm above the floor. The arms are arranged in the form of a “plus” sign with a square center platform (5×5 cm) at the intersection of the arms. Mice were acclimated to the testing room for one hour prior to testing. Each mouse was placed individually onto the central square at the intersection of the four arms, facing an open arm and allowed to explore the maze for five minutes. A mouse was considered to be in either an open or closed arm when all four paws were inside the arm (Brunner et al., 1999). To reduce lingering olfactory cues, the apparatus was thoroughly wiped with a clean damp cloth between trials. All sessions were videotaped for review. The following behaviors were measured: 1) The number of entries a mouse made onto either the closed or open arms from the central square. 2) The percentage of time a mouse spent on the open arms. 3) Stretch attend posturing (SAP): the number of times a mouse stretched forward and then return to its original position without moving its hind paws (File, 2001). Collected data was used to calculate percentage of open arms entries [open arms entries/(close arm entries + open arms entries) $\times 100$], percentage of time on open arms [time on open arms/(time on close arms + time on open arms) $\times 100$], closed arm entries, and SAPs that occurred within the entire plus maze. Data were analyzed by one-way ANOVA with Bonferroni posthoc test.

4.3. Marble burying test

The marble burying procedure, which assesses obsessive-compulsive tendencies, was adapted, with minor modifications, from previous studies (Skalisz et al., 2004; Woods-Kettelberger et al., 1997). Briefly, mice were acclimated to the testing room for 1 hr prior to test chamber adaptation (Day 1) and prior to testing (Day 2). During adaptation, each animal was individually acclimated for 30 min to the clear polypropylene testing chamber ($13 \text{ cm} \times 20 \text{ cm} \times 30 \text{ cm}$) containing a 5 cm deep layer of sawdust bedding. On day 2, the mice were individually placed in the test chamber, which then contained 24 clear glass marbles (one cm in diameter) placed in six rows of four, on top of a 5 cm deep layer of sawdust. The number of marbles buried in 30 min was recorded and data analyzed using a one-way ANOVA with a Bonferroni posthoc-test. In addition, a 1 hr test assessment was also conducted for both the *gabrb3*^{-/-} and *gabrb3*^{+/+} mice to assure that any handling-induced hyperactivity exhibited by the *gabrb3*^{-/-} did not directly confound the assessment.

4.4. Wire hanging test

The wire hanging test is a simple approach used to measure neuromuscular ability (muscle tone and grip strength) of a rodent by assessing the animals ability to hang suspended by its forepaws from a two mm wire 30 cm above a sawdust covered surface for a maximum time of one min (Insel, 2001; Ogura et al., 2001). This method likewise evaluates motor-coordination, by noting the ability of the animal to recruit its hind limbs and tail in order to grip the wire (Freitag et al., 2003). Latency to fall was measured from the time a mouse was placed hanging by its forepaws on the wire until it fell. The test was performed twice for each mouse with results from each mouse being averaged and data analyzed by one-way ANOVA with a Bonferroni posthoc test. In addition, whether a mouse was able to grip the wire with its hind limbs was also noted with data analyzed by Fisher and chi-square tests.

4.5. Immunohistochemistry

Mice were sacrificed by CO₂ inhalation and immediately perfused with 50 ml of 0.9 % saline solution at room temperature into the left cardiac ventricle and exiting through a small cut made in the right ascending aorta. Saline perfusion was followed by perfusing the mouse with 50 ml of fixing solution (4% paraformaldehyde in 0.1M phosphate buffer (PB, pH 7.6). Brains were removed from the skull and immediately immersed in fixing solution at 4°C for 16 hr. After fixation, the brains were prepared for cryopreservation by washing with 0.1 M PBS then transferred to 15% sucrose in 0.1 M PB kept at 4°C. After the brains sank to the bottom they were transferred to a 30% sucrose solution overnight at 4°C. The brains were then embedded in tissue freezing medium (Triangle Biomedical Sciences, Durham, N.C.) and stored at -80°C until needed. Frozen brains were cut in serial coronal sections of 45-µm thicknesses on a cryostat microtome (Leica CM 1850, Huston TX) and free floated in 0.1 M PBS on 24 well plates. Free-floating sections were treated with 0.6 % H₂O₂ in 0.1M PBS with 0.3% Triton X-100 (PBST) for 20 min. Nonspecific antibody binding was blocked with 3% normal goat serum (Vector Labs, Burlingame, CA) in PBST for 3 hours. Sections were then incubated in PBST containing a polyclonal antibody against tyrosine hydroxylase (TH) (1:600, Chemicon, Temecula, CA) for 48 hr at 4°C. After incubation, sections were washed three times in PBST and then treated with biotinylated goat anti-rabbit IgG in PBST (1:250, Vector Labs, Burlingame, CA) for 1 hr followed by incubation in avidin-biotin complex (ABC kit, Vector Labs, Burlingame, CA). 3, 3' diaminobenzidine (DAB, Vector Labs, Burlingame, CA) was used as the chromogen for visualization. This reaction was terminated by washing with water, followed by transfer of the sections to glass Superfrost plus microscope slides (Fisher Scientific, Pittsburgh, PA). Tissues on slides were dried at room temperature and coverslipped using Permount (Fisher Scientific, Pittsburgh, PA).

4.6. Locus coeruleus measurements

Images of the LC from each section were taken using a SPOT RT slider camera (software v4.0.9, Diagnostic Instrument Inc, Sterling Heights, MI) mounted on an Olympus BHTU-BH2 microscope (Scientific Instrument Company, Sunnyvale, CA). Images were superimposed onto the corresponding diagram of the coronal section of mouse brainstem taken from Paxinos and Franklin (2001) at the level of the LC (Bregma -5.40 mm). The cross section areas of the LC (including either the TH stained cell bodies alone or the pericoerulear dendritic zone encompassing the dense plexus of LC dendrites) were measured at 4 × magnifications by two independent observers that were blinded to the mouse genotype. The dispersed neurons of the subcoeruleus nucleus and extreme outliers LC neurons were excluded in cell bodies area measurements. The borders of the pericoerulear dendritic field were delineated by specific TH staining associated with the dense plexus of LC dendrites and did not include scattered dendrites and/or weak TH signal (see inset for pericoerulear border region measured on Fig. 3 A & B). The parameters for delineating the borders of LC was consistent for all sections used in this study. Statistical significance was tested using one-way ANOVA with Bonferroni posthoc-test (Prism 4, GraphPad, San Diego CA).

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Abbreviations

ASD, autism spectrum disorder; GABA, γ -amino butyric acid; gabrb3, GABA_A receptor, subunit β_3 gene; LC, locus coeruleus; NE, norepinephrine; SAP, stretch-attend posturing; β CCE, Beta-carboline carboxylate ethyl; 5-HT, serotonin; TH, tyrosine hydroxylase.

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Fig 1.

A) Marble burying behavior of $\text{gabrb3}^{-/-}$ and control mice measured after a 30 min exposure time. **B)** Marble burying was likewise assessed after a one hour period to assure handling induced hyperactivity in the $\text{gabrb3}^{-/-}$ mice had abated prior to assessment. Data is presented as the mean $\pm$ SEM, *** $p < 0.001$ relative to $\text{gabrb3}^{-/-}$ mice.

Fig 2. Wire hanging test. Comparison of the latency to fall for *gabrb3*^{-/-} and control mice. Data presented as the mean ± SEM, ****p*<0.001 relative to *gabrb3*^{-/-} mice.

Fig 3. Representative examples of the coronally sectioned LC taken at Bregma -5.40 mm and stained with a tyrosine hydroxylase antibody, from **A)** a *gabrb3*^{+/+} mouse, and **B)** a *gabrb3*^{-/-} mouse. Insets display the border region of the pericoerulear dendritic region measured.

Table 1

Plus maze behaviors.

Behavioral parameters	gabrb3 ^{-/-}	gabrb3 ^{+/+}	C57Bl/6J	129/SvJ
Close arm entries	15.1 ± 2.6	11.4 ± 0.7	12.0 ± 1.3	12.9 ± 1.7
% Open arm entries	14.6 ± 5.2	16.0 ± 2.4	17.3 ± 2.5	30.4 ± 9.4
% Open arm time	21.6 ± 9.1	10.4 ± 2.3	10.0 ± 2.9	33.9 ± 11.5
Number of SAP events	37.4 ± 5.1	11.5 ± 1.2***	18.5 ± 1.8***	24.0 ± 1.5**

Data presented as mean ± SEM.

**
 $p < 0.01$;***
 $p < 0.001$ relative to gabrb3^{-/-} mice.

Table 2
Cross section measurements of the surface area of the LC.

	gabrb3^{-/-}	gabrb3^{+/+}	C57Bl/6J	129/SvJ
Pericoerulear zone (mm ²)	0.40 ± 0.01	0.32 ± 0.01 *	0.28 ± 0.04 **	0.26 ± 0.04 **
Cell bodies area (mm ²)	0.13 ± 0.01	0.11 ± 0.01	0.10 ± 0.01	0.10 ± 0.02

The surface area of the LC was measured from coronal sections taken from Bregma - 5.40 mm in all genotypes. The pericoerulear zone included both the cell bodies and the dense plexus of dendrites stained by TH (see insets Fig. 3). Data is presented as the mean ± SEM,

* $p < 0.05$;

** $p < 0.01$ relative to gabrb3^{-/-} mice.