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Neuronal Tryptophan Hydroxylase Expression in BALB/cJ and C57Bl/6J Mice

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Abstract

BALB/c is an inbred stress-sensitive mouse strain exhibiting low brain serotonin (5-HT) content and a 5-HT biosynthetic enzyme tryptophan hydroxylase (*Tph2*) variant reported to have lower catalytic activity compared to other inbred base strains. To evaluate other mechanisms that may explain low 5-HT, we compared BALB/cJ mice and a control inbred strain C57Bl/6J mice, for expression of *Tph2* mRNA, TPH2 protein and regional levels of 5-HT and its metabolite 5-hydroxyindoleacetic acid (5-HIAA). *Tph2* mRNA and TPH2 protein in brainstem dorsal raphe nuclei (DRN) was assayed by *in situ* hybridization and immunocytochemistry respectively. 5-HT and 5-HIAA were determined by high pressure liquid chromatography (HPLC). BALB/cJ mice had 20% less *Tph2* mRNA and 28% fewer TPH2 immunolabeled neurons than C57Bl/6J mice ($t = -2.59$, $p = 0.02$). The largest difference in *Tph2* transcript expression was in rostral DRN ($t = 2.731$, $p = 0.008$). 5-HT was 15% lower in the midbrain of BALB/cJ compared to C57Bl/6J mice ($p < 0.05$). The behavioral differences in BALB/cJ mice relative to the C57Bl/6J strain may be due in part, to fewer 5-HT neurons and lower *Tph2* gene expression resulting in less 5-HT neurotransmission. Future studies quantifying expression per neuron are needed to determine whether less expression is explained by fewer neurons or also less expression per neuron, or both.

Keywords

Tph2; 5-HT; dorsal raphe nucleus; depression; immunocytochemistry

Introduction

BALB/c mice, an inbred mouse strain that displays anxious behavior and higher passivity in the forced swim test compared to other standard laboratory inbred strains, have been utilized as an animal model for elucidating molecular mechanisms underlying stress-related depressive phenotypes and for screening antidepressant drugs (Dulawa *et al.* 2004; Lucki *et al.* 2001; Cervo *et al.* 2005). The BALB/cJ strain has lower levels of forebrain 5-hydroxytryptamine (5-HT) compared to other inbred strains of mice that may explain their

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Statement of Interest: None

stress response and behavioral differences (Zhang *et al.* 2005). A single nucleotide polymorphism has been identified in BALB/cJ (SNP; C1473G) in the gene encoding for the 5-HT rate-limiting biosynthetic enzyme neuronal tryptophan hydroxylase (*Tph2*, (Zhang *et al.* 2004). BALB/cJ mice carrying the C allele have 50% less brain 5-HT in comparison with the G allele (Winge *et al.* 2006; Zhang *et al.* 2004). Further, C57Bl/6J mice that were back-crossed with BALB/cJ mice displayed increased aggressive behaviors and immobility times in the forced swim test but no change in locomotor activity, suggesting that the BALB/cJ SNP is linked to 5-HT-related behaviors (Osipova *et al.* 2009). Taken together, these studies suggest that the BALB/cJ mouse may represent a stress-sensitive model of a trait 5-HT deficit which is postulated to underlie Major Depressive Disorder (MDD). Brain 5-HT is produced in the brainstem raphe nuclei. The dorsal and median raphe nuclei (DRN and MRN, respectively) provide the majority of 5-HT to the forebrain (Brodal *et al.* 1960; Pierce *et al.* 1976; Hornung 2003), including the prefrontal cortex, a brain region central to regulation of mood and control of behavior. In postmortem human studies, we found more 5-HT neurons (Underwood *et al.* 1999) and greater *TPH2* gene (Bach-Mizrachi *et al.* 2006; Bach-Mizrachi *et al.* 2008) and protein (Boldrini *et al.* 2005) in the DRN and MRN of depressed suicides compared to matched non-psychiatric controls (Bach-Mizrachi *et al.* 2008; Bach-Mizrachi *et al.* 2006; Boldrini *et al.* 2005; Underwood *et al.* 1999). This is a paradoxical finding given reports of low brainstem 5-HT and/or 5-HIAA postmortem in suicides (Träskman *et al.* 1981; Mann *et al.* 2006) and the heuristic hypothesis of a serotonergic deficit contributing to the etiology of depression and suicide. In light of the low brain 5-HT in the BALB/cJ mouse, we sought to determine whether there are alterations in the 5-HT synthesizing *Tph2* gene and TPH2 protein within the DRN of BALB/cJ mice compared to the C57Bl/6J mouse strain which has comparably higher brain 5-HT, and compare the findings to the *Tph2* expression profile seen in the raphe nuclei in the human major depressive disorder.

Method

Dissection and Preparation of Mouse Tissue

All mice were obtained from Jackson Laboratories (Bar Harbor, Maine). All experiments were approved by the New York State Psychiatric Institute Institutional Animal Care and Use Committee. Adult (8 weeks old, weighing approximately 25 grams) male C57Bl/6J (n=22) and BALB/cJ (n=22) mice were used for this study. For *in situ* hybridization experiments (n=9 mice of each strain) and for HPLC (n=5 mice of each strain), mice were sacrificed by cervical dislocation.

The brain was quickly removed from the skull and frozen in Freon (R-12) either whole (for *in situ* hybridization) or was first dissected into regions of interest for HPLC analysis. Brain regions for HPLC analysis included: frontal cortex, thalamus, striatum, hippocampus, midbrain, pons/medulla and cerebellum. Brainstems were sectioned in a cryostat at 20 micron thickness and mounted on subbed glass slides, in such a way that each slide had 10 sections. Adjacent sections on each slide were 200 microns apart. This protocol yielded 10 slides from each animal. Each slide included 5-6 sections containing DRN that spanned the full anteroposterior extent of the raphe.

For immunocytochemistry (n=8 mice of each strain), mice were anesthetized with pentobarbital (50mg/kg, i.p.) and then underwent transcardial perfusion with 4% paraformaldehyde. The brain was removed and placed in a 10% sucrose solution overnight. The next day the brain was placed in a 20% sucrose solution for two hours followed by 30% sucrose solution. The brain was subsequently sectioned on a microtome at 40 μ m thickness. The sections through the brainstem were collected in a cell culture plate containing cryoprotectant (30% glycerol, 30% ethylene glycol, 40mM phosphate buffer). Serial sections were collected and placed individually into each of 10 wells. This sectioning protocol resulted in 10 series of sections (in each well) through the brainstem that were 400 μ m apart (40 μ m $\times$ 10). Each well had 3-4 sections containing the DRN. This sectioning protocol provided a full representation of the anterior-posterior extent of the DRN which is approximately 1.3 mm long in the mouse. Two wells from each animal were assayed for TPH2 protein expression and quantified using unbiased stereology described below.

***Tph2 In situ* Hybridization Assay**

³⁵S-labeled riboprobes specific for *Tph2* were generated by *in vitro* transcription of a plasmid containing the cloned human *TPH2* coding region (800 base pairs, GenBank accession number: AY098914) and used for *in situ* hybridization as previously described (Bach-Mizrachi *et al.* 2006). Briefly, 2 slides (each containing 10-sections) from each animal (n=9 mice of each strain) were fixed in 4% paraformaldehyde, rinsed in PBS, and acetylated in 0.25% acetic anhydride in 0.1 M triethanolamine, pH 8.0. Sections were then dehydrated through increasing concentrations of ethanol, delipidated in chloroform and hybridized with denatured radiolabeled *Tph2* riboprobe (3 ng, 2×10^6 counts per 100 μ l) overnight at 55°C. Slides were then washed in $4 \times$ saline-sodium citrate (SSC), with 0.2% β -mercaptoethanol followed by a series of washes in formamide buffer, $2 \times$ SSC, RNaseA solution (10 mg/ml RNaseA in 0.5 M NaCl/10mM Tris Base/1 mM EDTA). Sections were then dried and exposed to film (Biomax MR, Kodak) with ¹⁴C calibration standard slides (ARC-146, 146A, American Radiolabeled Chemicals, Inc.) for 3 days.

Imaging and Densitometry of *In situ* Hybridization Autoradiograms

Images were digitized (MCID M4 Image Analysis System; Imaging Research Inc., St. Catherine's, Ontario, Canada). and densitometric analysis was carried out as described elsewhere (Arango *et al.* 2001; Bach-Mizrachi *et al.* 2006). Optical density measurements were calibrated using ¹⁴C standard slides that were co-exposed with the ³⁵S-labeled tissue sections on each film. The linear relationship between ¹⁴C and ³⁵S (Miller 1991), allowed the use of these standards for the semi-quantitative measurement of the amount of ³⁵S labeled mRNA. Relative optical density measurements of the ¹⁴C-standards were obtained and plotted against the tissue radioactivity equivalents (nCi/g of tissue). Tissue radioactivity equivalents were adjusted for the efficiency of the incorporation of radioactivity into the batch of riboprobe that was labeled for each experiment as an estimate of the specific activity of the labeled probe. A threshold level for each film was set so that only pixels above background and below saturation levels were included in the final measurement within the sampled area defined.

DRN sampling was made using a circle of fixed area. For all measurements taken with fixed area circles, the target area within the circle was defined so that pixels at or below background levels and pixels at or above saturation levels were excluded from the sample measurement. The measurements taken included: the area of the circle, the area of the target within the circle and the optical density of label within the circle. The amount of *Tph2* mRNA reported here reflects the mean density of *Tph2* within the target in the circle and is therefore not biased by dilution and is, in effect, a more restricted “contour” of the DRN than a subjective hand-drawn contour of the DRN. Measurements from film background and non-raphe tissue section background with the same fixed area circle determined levels of non-specific labeling.

Immunostaining

Immunocytochemistry (n=8 mice of each strain) was carried out using an antibody to TPH2 (Calbiochem, San Diego, California). Standard protocols for immunocytochemistry of fixed tissue were followed using a 1:5,000 dilution of the primary antibody. The tissue was incubated in the primary antibody overnight. The next day, the tissue was washed and incubated in biotinylated secondary antibody for 1 hour. The tissue was subsequently washed and reacted with the Vector ABC kit and DAB for colorimetric tagging of immunopositive neurons.

Stereological Counts of TPH2 Immunoreactivity (IR)

Stereological analysis of TPH2-IR neurons in the DRN were conducted using a light microscope (Leica, model Diaplan; Wetzlar, Germany), a motorized stage (Ludl Electronic Products, Hawthorne, NY), and design-based stereology software (StereoInvestigator; MicroBrightfield, Williston, VT). An Optical Fractionator design was used to estimate the total neuronal cell population (N). This was calculated from the equation $N = 1/ssf \times 1/asf \times 1/hsf \times \Sigma Q-$ (ssf - section sampling fraction, asf- area sampling fraction, hsf – height sampling fraction). A total of approximately 200 labeled neurons were counted per case to achieve a coefficient of error less than 0.10 for the population estimate.

High Pressure Liquid Chromatography (HPLC) Analysis of 5-HT and 5-HIAA

Five mice of each strain were used for HPLC. HPLC was carried out on frontal cortex, midbrain, striatum, hippocampus, cerebellum and pons-medulla. Concentrations of 5-HT and 5-HIAA were measured using reversed-phase high-performance liquid chromatography (HPLC) with electrochemical detection. The dissected brain samples were homogenized in 0.5 ml ice-cold 0.4M perchloric acid with a sonicator probe. An aliquot of the homogenate was saved for protein quantitation. The homogenate was centrifuged for 5 minutes at 14,000g and a 50 µl aliquot of the supernatant was injected over the HPLC system. The HPLC system, equipped with a computer and the Waters Millennium software, consists of a Waters 515 pump, a Waters 717 Autosampler, a Varian Microsorb 100-5 C18 reverse-phase column and an ESA Coulochem electrochemical detector (Model 5100A) with a guard cell and a dual analytical cell (Model 5011A). The EC detector was set at potential of +0.05 for the 1st cell and +0.5V for the 2nd cell. The mobile phase contained 0.75 mM sodium phosphate (pH 3.1), 1.4 mM OSA, 10 M EDTA and 8% acetonitrile. The flow rate was 1.0

ml/min. A calibrated external standard curve was generated with external standards at different concentrations. Values are calculated based on peak area and compared to the standard calibration. The inter- and intra-assay coefficients of variation of the assay were each less than 5%. The sensitivity of the assay was less than 0.5 pmol/injection.

Statistical Analyses

For each assay conducted (*in situ* hybridization or immunocytochemistry) brain tissue from mice of each strain were processed together with the same solutions and under the same conditions to reduce experimental variability. The results between strains were compared using student *t*-tests. ANOVA was used to test for significance of differences in *Tph2* expression in anatomical levels across the anteroposterior axis of the DRN. ANOVA was also used to test significance of changes in 5-HT and 5-HIAA content between brain regions (HPLC).

Results

Tph2 mRNA *in situ* hybridization of BALB/cJ and C57Bl/6J mice

In both BALB/cJ and C57Bl/6J mice, *Tph2* mRNA expression is seen in the dorsal and median raphe nuclei in the brainstem (Fig. 1). Measurement of *Tph2* mRNA expression in the DRN by densitometry showed that BALB/cJ have approximately 20% less *Tph2* expression compared with C57Bl/6J (Fig. 2A, $0.82 \pm .37$ nCi/mg in BALB/cJ vs. $1.04 \pm .63$ nCi/mg in C57Bl/6J, $t = -2.02$, $p = 0.04$,). To determine whether the difference in *Tph2* in BALB/cJ mice is present throughout the rostrocaudal extent of the DRN, the density measurements were binned every 200 μ m according to each section's distance from the interaural line (Franklin and Paxinos, 1997). Although the difference in *Tph2* expression in BALB/cJ mice is evident throughout the rostrocaudal extent of the DRN (ANOVA, $f = 2.638$, $p = 0.016$), it is more pronounced at middle and rostral levels. The greatest difference in *Tph2* expression (approximately 30%) is seen 0.8-1.0 mm caudal to the interaural line ($t = 2.731$, $p = 0.008$ Fig. 2B) which is a relatively rostral neuroanatomical level of the DRN. This level includes the larger and more anatomically complex part of the DRN that contains several subnuclei, including those with serotonergic neurons that project to the frontal cortex.

TPH2-Immunoreactivity (TPH2-IR) in BALB/cJ and C57Bl/6J Mice

Strong TPH2-IR was seen in the cytoplasm of large multipolar neurons in the DRN of both strains of mice. TPH2-IR was seen extending from the caudal raphe nucleus rostrally, and continuing caudally to the medullary raphe nuclei. A visually apparent difference in TPH2-IR between strains was observed throughout the DRN (and MRN) at low power (Fig. 3A, 3B). At higher magnification, the intensity of TPH2-IR staining in the DRN appeared to be less in the neurons of BALB/cJ than C57Bl/6J and markedly less in the neuropil processes (Figs. 3C and 3D). Stereological quantitation of TPH2-IR neurons revealed that BALB/cJ mice have 28% fewer neurons in the DRN compared to C57Bl/6J mice (BALB/cJ: $22,385 \pm 2,821$ vs. C57Bl/6J: $30,925 \pm 1,284$, $t = -2.59$, $p = 0.02$, Fig. 4).

Measurement of 5-HT and 5-HIAA in BALB/cJ and C57Bl/6J mice

We confirmed previous findings (Zhang *et al.* 2004) of lower 5-HT and 5-HIAA in cerebral cortex in BALB/cJ compared to C57Bl/6J mice (5-HT: $f = 18.04$, $p < 0.001$, 5-HIAA: $f = 42.10$, $p < 0.001$). In both strains, 5-HT concentration is regionally different: cerebellum < striatum < frontal cortex < hippocampus < pons/medulla < midbrain. Concentrations of 5-HIAA in the hippocampus of BALB/cJ mice ($n=5$) is lower compared to C57Bl/6J mice (BALB/cJ: 1945 ± 208 pmol/mg protein vs. C57Bl/6J: 2641 ± 417 , $t = -3.335$, $p = 0.016$). In the midbrain, BALB/cJ mice have 15% lower concentrations of 5-HT (BALB/cJ: $2,818 \pm 208$ pmol/mg protein vs. C57Bl/6J: $3,296 \pm 404$ pmol/mg protein, $t = -2.357$, $p = 0.04$) and 5-HIAA (BALB/cJ: $3,578 \pm 270$ pmol/mg protein vs. C57Bl/6J: 4126 ± 254 pmol/mg protein, $t = -2.357$, $p < 0.03$) compared to C57Bl/6J mice. An index of 5-HT turnover was calculated by taking the ratio of 5-HIAA to 5-HT within each brain region of each strain. 5-HT turnover was not significantly different between the strains in any region tested.

Discussion

We found less *Tph2* transcript and fewer TPH2-IR neurons in the DRN of BALB/cJ mice compared to C57Bl/6J mice. The amount of immunocytochemical labeling of TPH2 protein was observed to be lower in BALB/cJ compared with C57Bl/6J mice. The difference in the amount of immunocytochemical staining was consistent with resulting lower levels of 5-HT in BALB/cJ mice. The estimated total number of TPH2-IR neurons that we measured in these strains is consistent with the number of serotonergic neurons in the DRN reported for other inbred mouse strains (Lira *et al.* 2003). The fewer 5-HT synthesizing cells and less *Tph2* mRNA transcript in BALB/cJ mice are alterations that would result in lower levels of 5-HT regardless of the reported deficiency in TPH2 catalytic activity (Zhang *et al.* 2004) and are possible alternative or additional causes of lower brain 5-HT in this strain. These alterations in 5-HT neurons raise the possibility that other processes, such as those determining cell fate and neurogenesis during development, differ between BALB/cJ and C57Bl/6J mouse strains.

Fewer 5-HT synthesizing neurons in BALB/cJ mice can compound the effects of a TPH2 enzyme variant that has lower catalytic activity (Zhang *et al.* 2004). BALB/cJ mice may differ in their brain development such that fewer neurons in the raphe are fated to become serotonergic. While TPH2 is predominantly expressed postnatally in the brain, TPH1, the non-neuronal isoform is expressed in late developmental stages prenatally in the serotonergic precursors within rodent brain and may be critical for the regulation of 5-HT levels at these time points (Nakamura *et al.* 2006). Dysregulation of TPH1 prenatally may affect serotonergic cell fate perhaps resulting in fewer serotonergic neurons in the adult. Consequently, lower number of serotonergic neurons in BALB/cJ mice may confer a neurodevelopmentally determined “**stress sensitivity**” on this strain. Conversely, the higher numbers of serotonergic neurons seen in the C57Bl/6J mice may represent a “**stress resilient**” advantage. This phenomenon is consistent with studies in female macaques where, in response to environmental stress, a subpopulation of macaques had reduced reproductive capabilities and were found to have fewer serotonergic neurons compared to macaques that did not exhibit any changes in reproductive processes (Lima *et al.* 2009;

Bethea *et al.* 2008). We and others have shown that the serotonergic system is stress-sensitive, and stress increases levels of *Tph2* expression in adult rodents (Chamas *et al.* 2004; Lima *et al.* 2009; Briones-Aranda *et al.* 2005). SSRI administration also normalizes the stress-stimulated elevation in TPH2 in rats (Abumaria *et al.* 2007). The molecular mechanisms underlying low 5-HT that are associated with stress-induced behaviors may be analogous to the relationship between 5-HT deficiency and depressive behaviors in MDD.

While changes in TPH2 expression between BALB/cJ and C57Bl/6J mice were easily detectable, we found only a modest decrease in 5-HT content in cortical targets. This is consistent with a study in which the *Tph2* SNP in BALB/cJ was backcrossed into a C57Bl/6 strain background and resulted in only small changes in frontal cortex 5-HT tissue content and no change in 5-HT content in other brain regions tested compared to other inbred strains (Siesser *et al.* 2010). This suggests that only small changes in cortical 5-HT are necessary to generate stress induced 5-HT-related behaviors in mice. Interestingly, BALB/c mice exposed to repeated restraint stress, do not exhibit a change in TPH2 catalytic activity but do show increased 5-HT turnover in the striatum and in the hippocampus (Browne *et al.* 2011) suggesting that in BALB/c, regulation of brain 5-HT may involve mechanisms different from the compensatory TPH2 upregulation seen in humans. While we did not find a change in 5-HT turnover at baseline in BALB/c mice, an increase in turnover under stress demonstrates a dynamic process of 5-HT regulation where stress induced 5-HT synthesis may be counterbalanced by increased turnover resulting in increased 5-HT degradation.

The BALB/cJ inbred strain may be useful as an animal model system for examining changes in the serotonergic system in response to pharmacologic modulation and relating these molecular changes to a behavioral and biologic phenotype. The *Tph2* functional polymorphism in the BALB/cJ mouse has not yet been shown to have a homolog in humans with MDD or suicide. A functional polymorphism that would affect the catalytic capacity of the Tph2 enzyme could contribute to the observed deficit in brainstem 5-HT/5-HIAA of suicides with major depression and of CSF 5-HIAA in depressed suicide attempters. However, in depressed suicides we find increased expression of TPH2 transcript and more TPH2-IR neurons (Bach-Mizrachi *et al.* 2006; Underwood *et al.* 1999), although others have not replicated this observation (Bonkale *et al.* 2004; Goswami *et al.* 2010). Greater TPH2 gene expression in the DRN of depressed suicides (Bach-Mizrachi *et al.* 2006) may represent a compensatory mechanism for deficient brain 5-HT levels or serotonergic neurotransmission in target areas, while lower levels of 5-HT in the BALB/cJ mouse model could result from the mutated enzyme and/or from fewer neurons or less TPH2 expression or dysregulation of TPH2 function. A comparison of findings in humans (Underwood *et al.* 1999; Bach-Mizrachi *et al.* 2006; Träskman *et al.* 1981; Mann *et al.* 2006) and data presented here in mice is shown in Table 1. In both humans and mice, post-translational modifications of TPH2 can also affect function and 5-HT synthesis (Winge *et al.* 2008), and other signal transduction pathways may be involved in regulating TPH2 expression (Beaulieu *et al.* 2008). These mechanisms likely contribute to both the low TPH2 and 5-HT in BALB/cJ mice and the compensatory up-regulation of *TPH2* in depressed suicides. In addition, we have reported that although fewer in number, TPH2 expressing neurons have higher transcriptional capacity in depressed suicides compared to controls. Although,

BALB/cJ mice clearly have less *Tph2* mRNA expression, it remains to be determined whether there is less *Tph2* expression per serotonergic neuron in addition to fewer 5-HT containing neurons.

There is a clear difference between the biological phenotype in the BALB/cJ mouse and in depressed suicides that remains unexplained. The lower number of 5-HT neurons in mice compared with humans does not reflect the fact that the mouse brain has significantly more 5-HT than the human brain when the size of the brain is taken into consideration (Underwood *et al.* 2003). Hence the mouse brain may be above any threshold for the presumably homeostatic response that is seen in depressed suicidal humans. It should also be considered that since the biochemical and behavioral profiles of depression and suicide are complex, behavioral rodent models may only be useful in predicting the response of specific molecules related to these particular behaviors, such as the stress-sensitive TPH2 enzyme described here.

The behavioral phenotype of the BALB/cJ strain is well established (Crawley *et al.* 1997; Holick *et al.* 2007; Dulawa *et al.* 2004) and we did not independently replicate the published behavioral findings, and this may represent a limitation of our study. However, the behavioral measures themselves likely produce effects on the underlying biological measures of the serotonergic system (Bhansali *et al.* 2007) which we sought to avoid. In the behavioral measures of stress in mice and major depression, acute and chronic fluctuations in 5-HT and regulatory control and feedback mechanisms are likely at play. However, 5-HT deficiency in MDD patients is thought to be present between depressive episodes as well as during depressive episodes suggesting that the deficit is likely a trait of MDD rather than a state specific to MDD (Mann *et al.* 2005).

One of the limitations of this study is that the measurements of 5-HT, Tph2 transcript and Tph2 protein level could not be made within the same animal. Although these inbred strains are highly isogenic, we cannot control for inter-animal variability stemming from other variables such as housing, handling or other natural variation, though we did purchase all mice from the same vendor. As a result, direct correlations between our measures are limited.

The present study describes baseline neurobiological differences between a “stress sensitive” and a “stress resilient” mouse strain. Future studies assessing TPH2 expression after exposure to behavioral paradigms that elicit depressive-like responses are necessary to determine whether there is a relationship between the neurodevelopmental differences between strains that confer stress sensitivity and perhaps epigenetic factors that presumably exacerbate the vulnerability to stress.

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References

- Abumaria N, Rygula R, Hiemke C, Fuchs E, Havemann-Reinecke U, Ruther E, Flugge G. Effect of chronic citalopram on serotonin-related and stress-regulated genes in the dorsal raphe nucleus of the rat. *Eur Neuropsychopharmacol.* 2007; 17:417–429. [PubMed: 17182223]
- Arango V, Underwood MD, Boldrini M, Tamir H, Kassir SA, Hsiung S, Chen JJX, Mann JJ. Serotonin 1A receptors, serotonin transporter binding and serotonin transporter mRNA expression in the brainstem of depressed suicide victims. *Neuropsychopharmacology.* 2001; 25:892–903. [PubMed: 11750182]
- Bach-Mizrachi H, Underwood MD, Kassir SA, Bakalian MJ, Sibille E, Tamir H, Mann JJ, Arango V. Neuronal tryptophan hydroxylase mRNA expression in the human dorsal and median raphe nuclei: major depression and suicide. *Neuropsychopharmacology.* 2006; 31:814–824. [PubMed: 16192985]
- Bach-Mizrachi H, Underwood MD, Tin A, Ellis SP, Mann JJ, Arango V. Elevated expression of tryptophan hydroxylase-2 mRNA at the neuronal level in the dorsal and median raphe nuclei of depressed suicides. *Mol Psychiatry.* 2008; 13:507–513. [PubMed: 18180753]
- Beaulieu JM, Zhang X, Rodriguiz RM, Sotnikova TD, Cools MJ, Wetsel WC, Gainetdinov RR, Caron MG. Role of GSK3 β in behavioral abnormalities induced by serotonin deficiency. *Proceedings of the National Academy of Sciences of the United States of America.* 2008; 105:1333–1338. [PubMed: 18212115]
- Bethea CL, Centeno ML, Cameron JL. Neurobiology of stress-induced reproductive dysfunction in female macaques. *Mol Neurobiol.* 2008; 38:199–230. [PubMed: 18931961]
- Bhansali P, Dunning J, Singer SE, David L, Schmauss C. Early life stress alters adult serotonin 2C receptor pre-mRNA editing and expression of the alpha subunit of the heterotrimeric G-protein G $_q$. *J Neurosci.* 2007; 27:1467–1473. [PubMed: 17287521]
- Boldrini M, Underwood MD, Mann JJ, Arango V. More tryptophan hydroxylase in the brainstem dorsal raphe nucleus in depressed suicides. *Brain Res.* 2005; 1041:19–28. [PubMed: 15804496]
- Bonkale WL, Murdock S, Janosky JE, Austin MC. Normal levels of tryptophan hydroxylase immunoreactivity in the dorsal raphe of depressed suicide victims. *J Neurochem.* 2004; 88:958–964. [PubMed: 14756817]
- Briones-Aranda A, Rocha L, Picazo O. Influence of forced swimming stress on 5-HT $_{1A}$ receptors and serotonin levels in mouse brain. *Prog Neuropsychopharmacol Biol Psychiatry.* 2005; 29:275–281. [PubMed: 15694235]
- Brodal A, Taber E, Walberg F. The raphe nuclei of the brain stem of the cat: Efferent projections. *J Comp Neurol.* 1960; 114:239–259.
- Browne CA, Clarke G, Dinan TG, Cryan JF. Differential stress-induced alterations in tryptophan hydroxylase activity and serotonin turnover in two inbred mouse strains. *Neuropharmacology.* 2011; 60:683–691. [PubMed: 21130784]
- Cervo L, Canetta A, Calcagno E, Burbassi S, Sacchetti G, Caccia S, Fracasso C, Albani D, Forloni G, Invernizzi RW. Genotype-dependent activity of tryptophan hydroxylase-2 determines the response to citalopram in a mouse model of depression. *J Neurosci.* 2005; 25:8165–8172. [PubMed: 16148224]
- Chamas FM, Underwood MD, Arango V, Serova L, Kassir SA, Mann JJ, Sabban EL. Immobilization stress elevates tryptophan hydroxylase mRNA and protein in the rat raphe nuclei. *Biol Psychiatry.* 2004; 55:278–283. [PubMed: 14744469]
- Crawley JN, Belknap JK, Collins A, Crabbe JC, Frankel W, Henderson N, Hitzemann RJ, Maxson SC, Miner LL, Silva AJ, Wehner JM, Wynshaw-Boris A, Paylor R. Behavioral phenotypes of inbred mouse strains: implications and recommendations for molecular studies. *Psychopharmacology (Berl).* 1997; 132:107–124. [PubMed: 9266608]
- Dulawa SC, Holick KA, Gundersen B, Hen R. Effects of chronic fluoxetine in animal models of anxiety and depression. *Neuropsychopharmacology.* 2004; 29:1321–1330. [PubMed: 15085085]
- Goswami DB, May WL, Stockmeier CA, Austin MC. Transcriptional expression of serotonergic regulators in laser-captured microdissected dorsal raphe neurons of subjects with major depressive disorder: sex-specific differences. *J Neurochem.* 2010; 112:397–409. [PubMed: 19878438]

- Holick KA, Lee DC, Hen R, Dulawa SC. Behavioral Effects of Chronic Fluoxetine in BALB/cJ Mice Do Not Require Adult Hippocampal Neurogenesis or the Serotonin 1A Receptor. *Neuropsychopharmacology*. 2007; 33:406–417. [PubMed: 17429410]
- Hornung JP. The human raphe nuclei and the serotonergic system. *J Chem Neuroanat*. 2003; 26:331–343. [PubMed: 14729135]
- Lima FB, Centeno ML, Costa ME, Reddy AP, Cameron JL, Bethea CL. Stress sensitive female macaques have decreased fifth Ewing variant (Fev) and serotonin-related gene expression that is not reversed by citalopram. *Neuroscience*. 2009; 164:676–691. [PubMed: 19671441]
- Lira A, Zhou M, Castanon N, Ansorge MS, Gordon JA, Francis JH, Bradley-Moore M, Lira J, Underwood MD, Arango V, Kung HF, Hofer MA, Hen R, Gingrich JA. Altered depression-related behaviors and functional changes in the dorsal raphe nucleus of serotonin transporter-deficient mice. *Biol Psychiatry*. 2003; 54:960–971. [PubMed: 14625138]
- Lucki I, Dalvi A, Mayorga AJ. Sensitivity to the effects of pharmacologically selective antidepressants in different strains of mice. *Psychopharmacology (Berl)*. 2001; 155:315–322. [PubMed: 11432695]
- Mann JJ, Bortinger J, Oquendo MA, Currier D, Li S, Brent DA. Family history of suicidal behavior and mood disorders in probands with mood disorders. *Am J Psychiatry*. 2005; 162:1672–1679. [PubMed: 16135627]
- Mann JJ, Currier D, Stanley B, Oquendo MA, Amsel LV, Ellis SP. Can biological tests assist prediction of suicide in mood disorders? *Int J Neuropsychopharmacol*. 2006; 9:465–474. [PubMed: 15967058]
- Miller JA. The calibration of ^{35}S or ^{32}P with ^{14}C -labeled brain paste or ^{14}C -plastic standards for quantitative autoradiography using LKB Ultrafilm or Amersham Hyperfilm. *Neurosci Lett*. 1991; 121:211–214. [PubMed: 2020377]
- Nakamura K, Sugawara Y, Sawabe K, Ohashi A, Tsurui H, Xiu Y, Ohtsui M, Lin QS, Nishimura H, Hasegawa H, Hirose S. Late developmental stage-specific role of tryptophan hydroxylase 1 in brain serotonin levels. *J Neurosci*. 2006; 26:530–534. [PubMed: 16407550]
- Osipova DV, Kulikov AV, Popova NK. C1473G polymorphism in mouse *tph2* gene is linked to tryptophan hydroxylase-2 activity in the brain, intermale aggression, and depressive-like behavior in the forced swim test. *J Neurosci Res*. 2009; 87:1168–1174. [PubMed: 19006079]
- Pierce ET, Foote WE, Hobson JA. The efferent connection of the nucleus raphe dorsalis. *Brain Res*. 1976; 107:137–144. [PubMed: 1268717]
- Siesser WB, Zhang X, Jacobsen JP, Sotnikova TD, Gainetdinov RR, Caron MG. Tryptophan hydroxylase 2 genotype determines brain serotonin synthesis but not tissue content in C57Bl/6 and BALB/c congenic mice. *Neurosci Lett*. 2010; 481:6–11. [PubMed: 20600620]
- Träskman L, Åsberg M, Bertilsson L, Sjöstrand L. Monoamine metabolites in CSF and suicidal behavior. *Arch Gen Psychiatry*. 1981; 38:631–636. [PubMed: 6166274]
- Underwood MD, Khaibulina AA, Ellis SP, Moran A, Rice PM, Mann JJ, Arango V. Morphometry of the dorsal raphe nucleus serotonergic neurons in suicide victims. *Biol Psychiatry*. 1999; 46:473–483. [PubMed: 10459396]
- Underwood MD, Landau AP, Liu Y, Kassir SA, Francis JH, Lisanby SH, Fairbanks LA, Bakalian MJ, Mann JJ, Arango V. Comparative neuroanatomy of the serotonergic dorsal raphe nucleus in rodents, monkeys, and humans. 2003
- Winge I, McKinney JA, Knappskog PM, Haavik J. Characterization of wild-type and mutant forms of human tryptophan hydroxylase 2. *J Neurochem*. 2006
- Winge I, McKinney JA, Ying M, D'Santos CS, Kleppe R, Knappskog PM, Haavik J. Activation and stabilization of human tryptophan hydroxylase 2 by phosphorylation and 14-3-3 binding. *Biochem J*. 2008; 410:195–204. [PubMed: 17973628]
- Zhang X, Beaulieu JM, Sotnikova TD, Gainetdinov RR, Caron MG. Tryptophan hydroxylase-2 controls brain serotonin synthesis. *Sci*. 2004; 305:217.
- Zhang X, Gainetdinov RR, Beaulieu JM, Sotnikova TD, Burch LH, Williams RB, Schwartz DA, Krishnan KR, Caron MG. Loss-of-function mutation in tryptophan hydroxylase-2 identified in unipolar major depression. *Neuron*. 2005; 45:11–16. [PubMed: 15629698]

Figure 1.

Autoradiograms of Tph2 in situ hybridization at comparable rostral (Figs. 1A and 1B) and caudal (Figs. 1C and 1D) levels of BALB/cJ (Figs. 1A and 1C) and C57Bl/6J (Figs. 1B and 1D) mouse brainstem. Note that Tph2 mRNA expression is restricted to the DRN and MRN of both mouse strains but appears to have much lower expression in BALB/cJ mice compared to C57Bl/6J mice. Bar = 500 microns.

Figure 2.

A: Tph2 density measured in a fixed area circle placed over the DRN in BALB/cJ and C57Bl/6J mice. Note that Tph2 expression is less in the DRN of BALB/cJ mice compared to C57Bl/6J mice. Asterisk represents a significant p value, $p = 0.04$. **Figure 2B:** The anatomical distribution of Tph2 density C57Bl/6J (triangles) compared to BALB/cJ mice (circles). Values are plotted at 200 micron intervals across the anteroposterior axis according to the distance from the interaural line (Franklin and Paxinos, 1997).

Figure 3.

Photomicrographs of immunoreactivity of TPH in the DRN of a representative BALB/cJ (Figs. 3A and 3C) and C57Bl/6J mouse (Figs. 3B and 3D). Figs. 3A and 3B are low power images (10× objective) of a representative rostral section of the DRN (at a position approximately 0.8 mm caudal to the interaural line) of the BALB/cJ and C57Bl/6J, respectively. Scale bar = 100 microns. Figs. 3C and 3D are high power images (40× objective) from the lateral subnucleus of the DRN shown in Figs. 3A and 3B.

Figure 4.

Tph2-IR neurons were counted using unbiased stereology. Note that BALB/cJ mice have fewer Tph2 expressing neurons in the DRN compared to C57Bl/6J mice. The asterisk represents a significance level of $p < 0.05$.

Figure 5.

Bar graph representing measures of serotonin (a) and 5HIAA (b) in BALB/cJ (white bars) and C57Bl/6 (black bars) by HPLC, $n=5$ mice from each strain. Single asterisks represent significance at $p < 0.05$ and double asterisks represent $p < 0.005$. PM, pons medulla; CB, cerebellum; FCX, frontal cortex; MB, midbrain; HP, hippocampus; TH, thalamus; ST, striatum; CCTX, cerebral cortex.

Table 1
Comparison of Tph2 findings in depressed suicides compared with controls and Balb/cJ
compared with C57Bl/6 mice

Biochemical Measure	Depressed Suicides*	Balb/cJ Mice**
5-HT, 5-HIAA ^a	↓	↓
Tph2 mRNA ^b	↑	↓
Tph2 protein ^c	↑	↓
Tph2 cell number / density ^d	↑	↓

* Arrows represent direction of change in depressed suicides as compared to non-psychiatric controls.

^aThese findings are reported in Placidi *et al.*, 2001;

^bBach-Mizrachi *et al.*, 2008;

^cBoldrini *et al.*, 2005,

^dUnderwood *et al.*, 1999).

** Arrows represent direction of change as compared to C57Bl/6 mice.