Published in final edited form as:

Alcohol Clin Exp Res. 2014 July ; 38(7): 1894–1901. doi:10.1111/acer.12414.

Alcoholics have more Tryptophan Hydroxylase 2 mRNA and Protein in the Dorsal and Median Raphe Nuclei

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Abstract

Background—Chronic alcohol use depletes brain serotonin (5-HT), yet we previously found more tryptophan hydroxylase 2 (TPH2), the rate-limiting biosynthetic enzyme for 5-HT, in the dorsal raphe nucleus (DRN) of alcoholics. We sought to determine whether the increase in amount of TPH2 enzyme is associated with more TPH2 mRNA gene expression in the DRN of a new cohort of alcoholics and controls.

Methods—TPH2 mRNA and protein were measured by *in situ* hybridization and immunohistochemistry, respectively, in the DRN and median raphe nucleus (MRN) of age and sex-matched pairs (n=16) of alcoholics and non-psychiatric controls. Alcohol use disorder (AUD) diagnosis and medical, psychiatric and family histories were obtained by psychological autopsy. Age and sex were covariates in the analyses.

Results—TPH2 mRNA in alcoholics was greater in the DRN and MRN compared to controls (DRN: Controls: 3.6±1.6, Alcoholics: 4.8±1.8 nCi/mg of tissue, F= 4.106, *p*=0.02; MRN: Controls: 2.6±1.2, Alcoholics: 3.5±1.1 nCi/mg of tissue, F=3.96, *p*=0.024). The difference in TPH2 mRNA was present in all DRN subnuclei (DRd: 135%, DRv: 135%, DRvl: 136% percent of control *p*<0.05) except the caudal subnucleus. Alcoholics also had more TPH2 protein in the DRN and MRN than controls (DRN: Controls: 265±47, Alcoholics: 318±47 μCi/g, F=8.72, *p*=0.001; MRN: Controls: 253±33, Alcoholics: 345±39 μCi/g, F=7.78, *p*=0.001). There is a positive correlation between TPH2 protein and mRNA expression in the DRN (*r*=0.815,

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Financial Disclosures: Dr. Mann has received past unrelated grants from Novartis and GSK, and receives royalties from the Research Foundation for Mental Health for commercial use of the C-SSRS. None of the other authors have any financial disclosures or conflicts of interest.

$p < 0.001$), suggesting that the higher amount of TPH2 protein is due to an increase in TPH2 gene expression.

Conclusions—These findings suggest that greater TPH2 gene expression is the basis for more TPH2 protein in the DRN and MRN in alcoholics.

Keywords

5-HT; serotonin; human; postmortem; raphe; family history

Introduction

Alcohol directly and indirectly alters brain serotonin (5-hydroxytryptamine, 5-HT) through effects on 5-HT synthesis, release and metabolism (reviewed in (Sari et al. 2011)). Acutely, alcohol elicits 5-HT release in multiple brain regions involved in mediating reward-related behaviors, notably the nucleus accumbens (Szumlinski et al. 2007; Yoshimoto et al. 1992), ventral tegmental area (Yan et al. 1996), amygdala (McBride 2002; Yoshimoto et al. 2000) and hippocampus (Thielen et al. 2002). Chronically, alcohol diminishes 5-HT release as evidenced for example by lower cerebrospinal fluid (CSF) 5-hydroxyindoleacetic acid (5-HIAA) in alcoholics (reviewed in (Mukherjee et al. 2008)) indicating lower serotonergic neurotransmission (Thielen et al. 2002). Alcoholics also have low serotonin transporter (SERT) binding in cortical and subcortical regions (Mantere et al. 2002; Storvik et al. 2006; Storvik et al. 2008). Chronic alcohol exposure may alter expression and function of serotonergic pre- and post-synaptic neurotransmitter receptors as part of the mechanism underlying the reinforcing effects of alcohol and the maintenance of alcohol dependence in rodents (Sari et al. 2011). Alternatively, serotonergic deficits may exist prior to alcohol exposure and predispose to alcohol abuse (Buydens Branchey et al. 1989). Taken together, the evidence suggests possible bidirectional relationships between alcohol exposure and 5-HT neurotransmission, resulting in both acute and chronic changes in 5-HT release involving 5-HT reuptake and other receptor and cellular alterations.

In brain, 5-HT is synthesized by neurons in the raphe nuclei. Of the seven raphe nuclei, the dorsal and median raphe nuclei (DRN and MRN, respectively) provide serotonin innervation to the forebrain including both cortical and subcortical brain regions (Törk & Hornung 1990). Neuronal tryptophan hydroxylase2 (TPH2) is the rate-limiting enzyme in the biosynthesis of 5-HT, converting tryptophan to 5-hydroxytryptophan (5-HTP) before subsequent decarboxylation into 5-hydroxytryptamine (see (Mockus & Vrana 1998) for review). We (Underwood et al. 2007) but not others (Bonkale et al. 2006) found more TPH2 protein across all DRN subnuclei neurons in alcoholics compared to controls.

In the present study, we sought to confirm our previous observation of increased TPH2, using a different method, in a different cohort of alcoholics and extend those observations by determining whether the changes in TPH2 protein are associated with comparable changes in TPH2 mRNA gene expression. To determine the relationship between the amount of protein and the amount of gene expression, we measured TPH2 protein and TPH2 mRNA expression in the same DRN and MRN regions of the same cases. Since serotonergic deficiency is hypothesized as a contributor to the etiology of Major Depressive Disorder

(MDD) and also to suicide, we excluded cases with diagnosed MDD and or suicide as cause of death from our study sample.

Materials and Methods

Subjects

Brain samples were obtained from the Division of Molecular Imaging and Neuropathology in the New York State Psychiatric Institute. Cases used in this study were collected in the United States and the Republic of Macedonia. Procedures for collection and use of brain tissue were approved by the Institutional Review Board for Human Use Considerations of the New York State Psychiatric Institute or the University of Pittsburgh.

This study included 16 pairs (32 cases in total) of sex- (28 males: 4 females) and age- (± 8 years; Controls 39 ± 15 years; Alcoholics 41 ± 14 years; $t = -0.470$, $p = 0.642$) matched controls and alcoholics. Alcoholics met DSM-IV criteria for alcohol use disorder (AUD) as determined by the responses to the Structured Clinical Interview for DSM (SCID)-I administered as part of the psychological autopsy from next-of-kin (Kelly & Mann 1996). Control cases also underwent the complete psychological autopsy procedure. Information on family history, age of onset and duration of alcohol use disorder was determined by questionnaire. Positive family history was defined as presence of AUD in a first-degree relative (mother, father, brother, or sister). All cases have postmortem intervals (PMI) of 24 hours or less (Controls 14 ± 4 hours; Alcoholics 13 ± 6 hours; $t = 0.654$, $p = 0.518$). No suicides were included in this study. Cases with diagnosed Major Depressive Disorder were excluded; there was one alcoholic case with a comorbid diagnosis of bipolar disorder. Demographics are in Table 1.

Brainstems

Brains were collected at autopsy. All subjects were free of gross neuropathology and had negative toxicology screens for psychoactive and neurotoxic drugs with the exception of alcohol in the alcoholic group. Other neurological diagnoses were ruled out clinically by psychological autopsy, and by neuropathological examination, including evaluations of Alzheimer-type pathology by thioflavine S or immunohistochemical stains for all cases aged 45 or greater. The only underlying neuropathological findings (i.e. findings other than acute changes related to the terminal event) were hypertensive vascular changes in a woman aged 66, hypertensive vascular changes and a lacunar infarct of the caudate nucleus in a 51-year-old man who died suddenly from a hypertensive cerebral hemorrhage, and an old contusion of the occipital lobe in a 48-year-old man.

The brainstem was isolated by a transverse cut along the rostral border of the superior colliculus. The block was approximately 3 cm in length and, encompassed the midbrain and rostral pons containing the DRN and MRN. The block was frozen in liquid Freon (-20°C) and stored at -80°C until sectioning. Tissue sections were cut in a cryostat (20 μm thickness), mounted on gelatin-subbed glass slides and, stored at -80°C until assayed. pH was measured in cerebellar tissue to assess the integrity of the RNA (Harrison et al. 1995). While direct examination of mRNA integrity is not possible on slide-mounted tissue, only

cases with resulting *in situ* hybridization films that demonstrated strong and specific TPH2 signal were quantified and included in the study. Tissue sections for *in situ* hybridization and immunoautoradiography (using a phenylalanine hydroxylase [PH8] antibody that cross-reacts with TPH2, a gift from R.G.H. Cotton, Royal Melbourne Hospital, Melbourne, Australia) were collected every millimeter throughout the rostrocaudal extent of the DRN/MRN, corresponding to 16-20 sections per case. Sets of intercalated adjacent sections from each case were stained for Nissl to help identify boundaries of the raphe nuclei for the analysis of TPH2 mRNA in *in situ* hybridization autoradiograms.

***In situ* Hybridization Histochemistry**

In situ hybridization experiments were carried out on sections spanning the rostrocaudal extent of the DRN and MRN from each matched pair allowing for comparison of the distribution of TPH2 mRNA in the DRN and MRN between groups. Matched pairs of alcoholics and controls were assayed together with the same solutions to reduce experimental variability within the pair. Riboprobes were designed specifically for human TPH2 and *in situ* hybridization experiments were carried out as previously described (Bach-Mizrachi et al. 2006). Briefly, Sections were fixed (4% paraformaldehyde in 0.1 M PBS, pH 7.4) for 15 minutes, rinsed (PBS) for 5 minutes, and acetylated (0.25% acetic anhydride in 0.1 M triethanolamine, pH 8.0) for 10 minutes. Then sections were dehydrated (increasing concentrations of ethanol) and delipidated (chloroform). Sections were incubated in hybridization solution (50% formamide, 10mM EDTA, 20mM Pipes, 0.75 M NaCl, 10% dextran sulfate, 5X Denhardts, 250 µg/ml tRNA) and denatured radiolabeled probe (3 ng, 2×10^6 counts per 100 µl) overnight at 55°C. The sections were then washed in a series of high stringency washes to reduce background. Sections were dried and exposed to autoradiography film (Biomax MR, Kodak) with two slides of ^{14}C standards (ARC-146, 146A, American Radiolabeled Chemicals, Inc.) for 3 days. Films were developed according to manufacturer's directions.

Immunoautoradiography

The immunoautoradiography assays were performed using an intercalated set of sections (every millimeter) with PH8 as the primary antibody and an anti-mouse IgG secondary antibody labeled with ^3H -biotin as previously described by our laboratory (Boldrini et al. 2005). Tissue was exposed to film (Biomax MS, Kodak) alongside ^3H -standards (ART 123 and ART 123A), for 7 days and developed.

Imaging Analysis and Densitometry

The methods for semi-quantification of *in situ* hybridization autoradiograms have been previously described (Bach-Mizrachi et al. 2006) and are only summarized. Autoradiograms were digitized using an image analysis system (MCID Elite; Imaging Research Inc., St. Catharines, Ontario, Canada). Two measurement strategies were employed. First, optical density-based measurements of the DRN were taken using a circle of fixed area (80 mm²). A separate circle of the same size was used to measure the MRN. The target area within the circle had a threshold applied so that only pixels above tissue background levels were included in the measurement. The measures included: the area of the circle, the area of the

“target” within the circle, and the calibrated mean optical density of pixels above threshold within the circle. The measurements of TPH2 mRNA reported here therefore reflect the mean concentration of TPH2 mRNA within the target in the circle so the measured amount is not diluted or negatively biased by unlabeled areas. Measurements from film background and non-raphe tissue section background with the same fixed area circle determined levels of non-specific labeling. The second approach was to manually draw contours of the DRN and MRN and also of the DRN subnuclei. A schematic of the sampled DRN, the DRN subnuclei and MRN is shown in Figure 1. Nissl stains and PH8 immunoreacted sections of the DRN and MRN were used as a guide to help delineate the subnuclei in the TPH2 autoradiograms. The subnuclei of the DRN sampled were the dorsal (DRd), ventral (DRv), ventrolateral (DRvl), interfascicular (DRif) and caudal (DRc) subdivisions.

Optical density measurements were calibrated using the ^{14}C standard slides that were co-exposed with the ^{35}S -labeled tissue sections on each film. The linear relationship between ^{14}C and ^{35}S (Miller 1991) allowed for the measurement of the amount of radioactivity as a semi-quantitative measurement of the amount of ^{35}S labeled mRNA; relative optical density measurements of the ^{14}C standards were obtained and plotted against the tissue radioactivity equivalents (nCi/mg of tissue). Tissue radioactivity equivalents were adjusted for 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. In a separate single experiment, 2 sections from each case were taken, labeled and exposed to film together so that all 32 cases in the study underwent the same experimental conditions. This allowed for between group comparisons independent of potential probe batch labeling differences and other inter-experimental confounds.

Immunoautoradiograms were also quantified using MCID. Sampling of autoradiograms was performed by taking contours of the DRN (Boldrini et al. 2005), as well as the DRN subnuclei and the MRN as described (Bach-Mizrachi et al. 2006).

Statistical Analyses

The 2 main outcome variables were TPH2 mRNA and TPH2 protein expression in controls and alcoholics and were tested using a two-way analysis of variance with the fixed variables group and neuroanatomical level; the covariates were age and sex. Anatomical localization of expression differences along the anteroposterior axis between groups was considered an exploratory hypothesis; expression differences within the DRN were examined *post hoc* by measuring individual DRN subnuclei. Relationships between TPH2 mRNA and TPH2 protein were determined using Pearson correlations and regression analysis using a general linear model. The effects of age, age of onset of AUD, duration of AUD, PMI and tissue pH on the TPH2 mRNA and protein measures were also determined using Pearson correlations; Bonferroni correction was performed to adjust for the multiple comparisons.

Results

TPH2 mRNA

TPH2 mRNA labeling was localized in both controls and alcoholics with the majority of labeling corresponding to areas within the boundaries of the DRN and MRN (Figure 1A and 1B). TPH2 mRNA labeling was also observed outside the anatomical boundaries of the DRN and MRN, largely reflecting autoradiographic signal arising from neurons of the adjacent serotonergic B9 cell group. The heterogeneous distribution of autoradiographic label was consistent with the distribution of serotonergic neurons within the DRN (Figure 1A and 1B) and its subnuclei (outlines of subnuclei are shown in Figure 1C and 1D).

The density (nCi/mg of tissue) of TPH2 mRNA in the DRN was 36% greater than in the MRN, and the expression in both the DRN and MRN was 32-34% higher in alcoholics compared to controls (DRN: Controls: 3.6 ± 1.6 nCi/mg of tissue *vs.* Alcoholics: 4.8 ± 1.8 nCi/mg of tissue, $F=4.106$, $p=0.02$; MRN: Controls: 2.6 ± 1.2 nCi/mg of tissue *vs.* Alcoholics: 3.5 ± 1.1 nCi/mg of tissue, MRN, $F=3.96$, $p=0.02$).

The size of the DRN, as defined by the cross-sectional area of TPH2 mRNA expressed in the fixed area circle (or the proportional area expressed as a percent of the total area), was not different between controls and alcoholics in the DRN. Likewise, the sum of the cross-sectional areas measured of the DRN determined by contours delineating the DRN boundaries provided an index of volume and was not different between groups (Controls: 8.9 ± 4.1 mm² *vs.* Alcoholics: 8.0 ± 8.2 mm², $p > 0.05$). There was an increase in the volume of the MRN in alcoholics, but this difference did not reach statistical significance at any neuroanatomical level.

The greater amount of TPH2 mRNA in alcoholics compared to controls was distributed through the middle and rostral DRN (group*neuroanatomical level, $F=4.57$, $p=0.03$). The difference in TPH2 mRNA between controls and alcoholics was found in all the subnuclei of the DRN except in the caudal subnucleus (Figure 2: 35% higher in the DRd ($p=0.05$), 39% higher in the DRif ($p=0.03$), 35% higher in the DRv ($p=0.01$) and 36% higher in the DRvl ($p=0.004$).

In the MRN, alcoholics had 51% more mean TPH2 mRNA expression than controls (Controls: 2.2 ± 1.3 nCi/mg of tissue *vs.* Alcoholics: 4.3 ± 2.3 nCi/mg of tissue, $F=8.29$, $p=0.04$).

TPH2 Immunoautoradiography

TPH2 protein immunoreactivity (TPH2-IR) had the same anatomical distribution as TPH2 mRNA expression (See Figure 1C and 1D). TPH2-IR was greater in the DRN and MRN of alcoholics than controls when co-varying for age and sex (DRN: Controls: 265 ± 47 *vs.* Alcoholics: 318 ± 47 $\mu\text{C/g}$ of tissue, $F=8.72$, $p=0.001$, MRN: Controls: 250 ± 33 *vs.* Alcoholics: 345 ± 39 $\mu\text{C/g}$ of tissue, $F=7.78$, $p=0.001$).

The amount of TPH2-IR correlated with the amount of TPH2 mRNA in the DRN across all cases ($r^2=0.815$, $p<0.001$), but only the slope of the alcoholics was significant (alcoholics:

$r=0.606$, $p=0.028$; controls: $r=.312$, $p=0.323$). Similarly, the cross-sectional area of TPH2-IR correlated with TPH2 mRNA proportional area (measured within fixed area circles) in the DRN of all cases (DRN: $r^2=0.33$, $p<0.0001$). In alcoholics, the area occupied with TPH2-IR was larger in DRN ($F=2.3$, $p=0.013$) and the density of TPH2-IR was greater in the DRN ($t=1.97$, $p=0.05$,) compared to controls. However, the differences in the amount of TPH2-IR in individual DRN subnuclei between groups did not reach statistical significance, suggesting the difference in the DRN in alcoholics as a group is distributed throughout the nucleus and is not anatomically localized.

Age, Duration of Alcohol Use Disorder, Age of Onset and Family History

The amount of TPH2 mRNA correlated with duration of alcohol use disorder ($F=2.73$, Bonferroni corrected p value $=0.035$), but not age at death or age at onset (Bonferroni adjusted p values >0.05). TPH2 mRNA correlated with blood alcohol level (DRN: $r=0.5$, Bonferroni adjusted $p=0.03$; MRN: $r=0.5$, Bonferroni adjusted $p=0.05$). The correlation between TPH2-IR sampled area with duration of alcohol use disorder was not significant (total area: $r=0.574$, Bonferroni corrected $p=0.2$).

Cases with a positive family history for AUD had more TPH2 protein in the DRN compared with cases without a family history of AUD (negative family history: 237.2 ± 121.8 $\mu\text{C/g}$ of tissue vs. positive family history: 275.8 ± 189.4 $\mu\text{C/g}$ of tissue, $F=3.314$, $p=0.05$). Cases with a positive family history for AUD also had a greater duration of AUD than cases without a family history ($F=4.168$, $p=0.05$). However, after controlling for family history, the correlation of TPH2 mRNA with duration of alcoholism was not significant ($F=2.33$, $p=0.096$).

TPH2 mRNA did not correlate with PMI or pH ($p>0.05$, data not shown). TPH2 protein had a positive correlation in the DRN and MRN with age independent of diagnosis (Figure 3, DRN: $r=0.405$, $p=0.02$, MRN: $r=0.383$, $p=0.03$). However, no correlation was found between TPH2 protein or TPH2 mRNA and age of onset of alcohol use disorder, regardless of family history ($p>0.05$, data not shown).

Discussion

The observation of higher TPH2 protein in the DRN of alcoholics by immunoautoradiography in this new cohort confirms our previous finding of higher TPH2 by immunohistochemistry (Underwood et al. 2007), and the novel finding here of more TPH2 gene expression provides an explanation. The positive correlation between TPH2 protein and mRNA expression suggests that the higher amount of TPH2 protein is due to an increase in TPH2 gene expression. The difference in TPH2 mRNA and TPH2-IR in the DRN of alcoholics compared to controls was present in all the DRN subnuclei with the exception of the caudal subnucleus. This suggests that there is more TPH2 mRNA and TPH2 enzyme in the majority of 5-HT synthesizing neurons in the DRN and MRN in alcoholics. However, it is not known whether there is more 5-HT or 5-HIAA associated with the TPH2 mRNA and protein in the brainstem of alcoholics.

TPH2 protein correlated with duration of alcohol use disorder, but not age, suggesting that TPH2 protein increases with the duration of exposure to alcohol, or alternatively, that TPH2 degradation is less in alcoholics than controls. Either of these novel mechanisms could conceivably contribute to the effects of chronic alcohol exposure by increasing the availability of 5-HT to brain reward circuits that could, in turn, explain the reinforcing effects of alcohol (LeMarquand et al. 1994b).

Upregulation of TPH2 transcription is reported in the literature and can occur through at least two potential mechanisms. The TPH2 gene promoter has a novel bipartite neuron restrictive silencing element (NRSE) that represses transcription (Patel et al. 2007) and ethanol has been shown to decrease the activity of this silencing transcriptional element thereby increasing gene expression (Qiang et al. 2005). Alcohol has also been shown to stimulate the transcription regulating complex activator protein-1 (AP-1, (Pignataro et al. 2009) that regulates the TPH2 promoter (Lenicov et al. 2007).

As the rate-limiting enzyme in 5-HT synthesis, the finding of more TPH2 suggests an increase in 5-HT synthesis. However, evidence from other studies suggests that alcohol use disorder is associated with a 5-HT deficit (Cloninger 1987; Hammoumi et al. 1999; LeMarquand et al. 1994a), and that the serotonergic alterations are more pronounced in Cloninger Type II alcoholics (Leggio & Addolorato 2008). Our finding of increased TPH2 in alcoholics could represent an increased capacity for 5-HT synthesis or alternatively, a deficit in 5-HT transport to, or release from, innervated target regions. We recently found (Bach et al. 2013) elevated TPH2 in the DRN and MRN in suicides compared to controls that was associated with elevated 5-HT and its metabolite 5-HIAA. If the elevated TPH2 enzyme is accompanied with an elevation in 5-HT in alcoholics as we observed in suicides, this would support the hypothesis that there is adequate or even increased synthesis of 5-HT and decreased transport or release in innervated regions. Decreased serotonin transporter density in alcoholics is reported in the anterior cingulate cortex (Mantere et al. 2002), caudate (Storvik et al. 2006) and dorsal amygdala (Storvik et al. 2007). Determining 5-HT synthesis, transport and release in alcoholics is critically important to understanding 5-HT neurotransmission, but these measures are beyond the scope of the present study.

The 5-HT system plays a key role in the regulation of alcohol intake, reward, preference and dependence (LeMarquand et al. 1994b; McBride & Li 1998; Roy et al. 1987; Sellers et al. 1992) presumably through projections originating in the raphe nuclei and innervating many of the brain regions involved in the rewarding effects of alcohol (Johnson 2004; Steinbusch 1981). The widespread changes throughout the DRN, including all of its component subnuclei (with the exception of the caudal subnucleus), and the MRN, regions with different efferent projections would be consistent with multiple and complex effects and consequences of alcohol exposure on behavior, cognition and mood.

Alcohol use disorder has a strong genetic and familial component that is consistent with our finding that the upregulation of TPH2 expression is greater in those individuals with a positive family history for alcohol abuse. Specifically, the highly heritable Type II subcategory of alcohol use disorders has been linked to deficient serotonergic neurotransmission (Sari et al. 2011). In our sample there are two nonalcoholic control cases

that have a positive family history for alcohol use disorders. These two cases had more TPH2 mRNA expression compared to controls with no family history of alcohol use disorders. Such a finding in a larger sample would suggest that family history of alcohol use disorders may predispose individuals to the addictive and other epigenetic effects of alcohol through increased TPH2 expression. Some other biology or epigenetic effect in this subpopulation could conceivably confer resilience to the effects of alcohol exposure. While there is a functional single nucleotide polymorphism (SNP) in TPH2 described in rodents that also exists in humans, the SNP is rare and has been reported not to contribute to alcohol use disorder (Zill et al. 2007). Agrawal and colleagues (2012) reported an association of several SNPs in the TPH2 gene and in the dopa-decarboxylase gene (involved in dopamine synthesis) with alcohol consumption in young adult females (Agrawal et al. 2011). We have not evaluated the genotype of these particular SNPs in the cases studied here because the small sample size does not permit a meaningful association study.

A limitation of this study is the inability to distinguish changes in TPH2 that are causally contributing to alcohol use disorders from those that are a consequence. Alcoholics are not homogeneous and the co-existence of family history, early onset, and violent death could well be related to the serotonergic defects. Hence it is possible that the causal relationship between serotonergic alterations and alcohol use may go in both directions; alcohol exposure may lead to serotonergic deficit or alternatively, serotonergic deficit may predispose to the reinforcing effects of alcohol. Buydens-Branchey et al. (1989) found an association between age of onset of alcohol abuse and tryptophan availability and suggested that a serotonin deficit may precede alcohol abuse. We did not find correlations between age of onset of alcohol abuse and TPH2 mRNA or TPH2 protein, but we may well be statistically underpowered to detect such a relationship. Another limitation is that we do not know whether the increase in TPH2 is associated with an increase in the synthesis of 5-HT. Such studies are beyond the scope of this work, however, as noted above, we have found that the increase in the DRN of suicides is associated with an increase in 5-HT in the DRN (Bach et al. 2013). There is also inadequate power to determine the contribution of gene variants in serotonergic genes to alcohol use disorders. Additional studies are needed to explore the genetic and molecular mechanisms of the involvement of the serotonergic system and TPH2 in the etiology and maintenance of alcohol use disorders. Brain tissue in general, and mRNA in particular, is subject to postmortem degradation (Birdsill et al. 2011; Stan et al. 2006). However, pH is not likely to explain the differences in TPH2 mRNA between controls and alcoholics since the pH did not differ between groups, there was no correlation between pH and the amount of TPH2 mRNA expression, and the PMI was less than 24 hours. Importantly, TPH2 mRNA did not correlate with PMI or brain pH in a postmortem study with a similar, but somewhat longer, PMI than in our study (De Luca et al. 2005). It is also reported that cases from the Medical Examiner's office, such as those in our collection, and cases with PMI <24hours typically have high tissue quality (Stan et al. 2006). RNA integrity number (RIN) is reported to be a superior indicator for tissue quality and RIN has a good correlation with pH (Birdsill et al. 2011; Stan et al. 2006), however, RIN was not available in our study.

The findings in the present study are not associated with suicide as a cause of death, as suicides were excluded from the present study. In suicides, we found a similar paradox of

more TPH2 protein and mRNA in the DRN than in normal controls (Bach-Mizrachi et al. 2006; Bach-Mizrachi et al. 2008; Boldrini et al. 2005) and the increased amount of TPH2 was associated with an increase in the amount of 5-HT and 5-HIAA in the brainstem (Bach et al. 2013). Interestingly, both suicide and alcohol use disorder are hypothesized to represent conditions of reduced 5-HT neurotransmission in the prefrontal cortex, and perhaps additional brain areas. More studies are needed to determine the respective underlying regulators of TPH2 expression.

Acknowledgments

This work was supported by grants from the National Institute of Alcohol Abuse and Alcoholism (AA11293) and National Institute of Mental Health (MH62185). We thank Mihran J. Bakalian and Virginia Lynn Johnson for their work with image analysis, sampling and quantification. Some of the brain samples and their psychiatric characterization were funded by MH40210, AA09004, MH64168.

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Figure 1.

Photomicrographs of TPH2 immunocytochemistry at rostral (A) and caudal (B) anatomical levels in a representative control. The brain tissue was fixed in formaldehyde and TPH2 was labeled using a PH8 antibody (Underwood et al., 2007). TPH2 mRNA measured by *in situ* hybridization (panels C and D). Panels E and F show photomicrographs of TPH2 protein assayed by immunohistochemistry at comparable rostral and caudal levels. Anatomical boundary contours are drawn of the DRN, DRN subnuclei and MRN (DRd: dorsal, DRv: ventral, DRvl: ventrolateral, DRif: interfascicular, DRc: caudal).

Figure 2.

Bar graph showing mean mRNA density measurements (mean $\pm$ S.E.M.) of the DRN subnuclei and the MRN in controls (white) and alcoholics (black). Density measurements were taken within outlined contours of each DRN subnucleus. Note that alcoholics have more TPH2 mRNA in the dorsal (DRd), interfascicular (DRif), ventral (DRv) and ventrolateral (DRvl) DRN subnuclei and in the MRN. No significant change was found in the caudal subnucleus (DRc).

Figure 3.

Relationship between age and tryptophan hydroxylase (TPH2) protein. TPH2 protein was measured by immunautoradiography to PH8, an antibody labeling TPH2. Note that TPH2 increased with age independent of diagnosis. The relationship was not statistically significant in controls (circles) or alcoholics (stars) alone, and was not dependent on family history of alcoholism.

Table 1

Case Demographics

All demographic information was made available by the medical examiner and psychological autopsy interviews with the next-of-kin. Age, in years; Race: B: Black, AS: Asian, W: White. AUD: Alcohol Use Disorder. MVA, Motor vehicle accident; PMI: postmortem interval, in hours; Duration of AUD in months. NA: Not available. Positive family history represents AUD in first-degree relatives.

Pair #	Age	Sex	Race	PMI	pH	Brain Toxicology	Axis I	Cause of Death	Duration of AUD	Family History of Alcoholism	Blood Alcohol
1	53	Male	B	9	NA	Clear	None	Industrial Accident	None	Negative	0
1a	48	Male	B	24	6.74	Clear	AUD	Natural Causes	216	Negative	0.34
2	43	Male	B	21	6.76	Clear	None	Cardiovascular	None	Negative	0
2a	48	Male	W	18	6.36	Clear	AUD	Accident (Fall)	372	Negative	0.54
3	44	Male	AS	21	6.86	Clear	None	Accident (Electrocution)	None	Negative	0
3a	51	Male	B	9.5	6.50	Clear	AUD	Hemorrhage	144	Positive	0
4	43	Male	B	19	6.49	Clear	None	Cardiovascular	None	Negative	0
4a	51	Male	B	19.5	6.95	Caffeine	AUD	Natural Causes	276	Positive	0.02
5	24	Male	W	12	NA	Clear	None	Hemorrhage	None	Negative	0
5a	28	Male	W	14.5	6.43	Clear	AUD	MVA; Driver	108	Positive	0.31
6	37	Male	B	15	6.75	Clear	None	Myocardial Infarction	None	Positive	0
6a	45	Male	W	5	6.85	Caffeine	AUD	Cardiovascular	108	Negative	0.03
7	51	Male	W	9	6.35	Lidocaine	None	Cardiovascular	None	Negative	0
7a	48	Male	W	7.5	6.87	Clear	AUD	Cardiovascular	264	Positive	0.16
8	18	Male	W	16	6.80	Clear	None	MVA; Pedestrian	None	Negative	0
8a	17	Male	W	12	6.88	Clear	AUD	MVA; Driver	36	Negative	0.23
9	19	Male	B	13	6.84	Clear	None	Cardiovascular	None	Positive	0
9a	20	Male	W	12.5	6.43	Lidocaine	AUD	MVA; Driver	48	Positive	0.14
10	39	Male	W	14	6.79	Clear	None	Cardiovascular	None	Negative	0
10a	33	Male	B	15	6.80	Caffeine	AUD	Homicide	120	Negative	0.22
11	34	Male	W	12	6.38	Lidocaine	None	Cardiovascular	None	Negative	0
11a	32	Male	W	9.75	6.36	Anxiolytics, Caffeine, Nicotine	AUD	Homicide	NA	Negative	0.23
12	22	Male	W	21	6.68	Caffeine	None	Homicide	None	Negative	0
12a	23	Male	W	6	6.82	Clear	AUD	MVA; Driver	NA	Negative	0.03

Pair #	Age	Sex	Race	PMI	pH	Brain Toxicology	Axis I	Cause of Death	Duration of AUD	Family History of Alcoholism	Blood Alcohol
13	35	Male	W	10	6.64	Caffeine	None	MVA; Driver	None	Negative	0.13
13a	35	Male	W	15	6.60	Lidocaine	AUD	MVA; Pedestrian	NA	Negative	0.16
14	54	Male	W	9	6.31	Clear	None	Cardiovascular	None	Negative	0
14a	53	Male	W	10	6.50	Clear	AUD	Cardiovascular	NA	Positive	0.06
15	51	Female	W	12.25	6.30	Lidocaine, Caffeine	None	Cardiovascular	None	Negative	0
15a	50	Female	B	14.5	6.84	Lidocaine, Caffeine	AUD, Bipolar	Respiratory Failure	72	Positive	0
16	71	Female	W	12.5	6.33	Clear	None	Cardiovascular	None	Negative	0
16a	66	Female	B	24	6.66	Clear	AUD	Accident (Fall)	432	Positive	0.34