Neuropsychopharmacology (2006) 31, 814–824

www.neuropsychopharmacology.org

Neuronal Tryptophan Hydroxylase mRNA Expression in the Human Dorsal and Median Raphe Nuclei: Major Depression and Suicide

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Major depressive disorder (MDD) and suicide are associated with deficient serotonergic neurotransmission. Tryptophan hydroxylase (TPH) is the rate-limiting biosynthetic enzyme for serotonin. Previously, we reported elevated levels of TPH protein in the dorsal raphe nucleus (DRN) of depressed suicides and now examine expression of neuronal TPH2 mRNA in a cohort of matched controls and depressed suicides ($n = 11$ pairs). DRN TPH2 mRNA was measured by densitometric analysis of autoradiograms from *in situ* hybridization histochemistry experiments. TPH2 mRNA is confirmed as the raphe-specific isoform of TPH in human brain, and is expressed in neurons throughout the anteroposterior extent of the DRN and median raphe nucleus (MRN). TPH2 mRNA expression correlates with TPH protein distribution in the DRN, and has a negative correlation with age. In drug-free suicides, TPH2 expression is 33% higher in the DRN and 17% higher in the MRN as compared to matched nonpsychiatric controls. Higher levels of TPH2 mRNA were found throughout the entire extent of the rostrocaudal axis of the DRN, and were not specific to any single subnucleus. Higher TPH2 mRNA expression may explain more TPH protein observed in depressed suicides and reflect a homeostatic response to deficient brain serotonergic transmission.

Neuropsychopharmacology (2006) **31,** 814–824. doi:10.1038/sj.npp.1300897; published online 28 September 2005

Keywords: serotonin; serotonin synthesis; *in situ* hybridization histochemistry; postmortem

INTRODUCTION

Suicide is the 11th leading cause of death in the United States across all age groups, the third leading cause in the 10–24 years of age group and second in people between the ages of 25 and 35 (www.cdc.gov/ncipc/wisqars 2002). Mood disorders are strongly associated with suicidal behavior, with an estimated 60% of completed suicides having a lifetime diagnosis of mood disorder (Mann, 2003; Turecki *et al*, 2001). Extensive evidence implicates the brain serotonergic system in major depression and suicide. More than 20 studies have reported a correlation between low levels of cerebrospinal fluid 5-hydroxyindoleacetic acid (5-HIAA) and suicidal behavior (reviewed by Placidi *et al*, 2001). Postmortem studies of depression and suicide report low levels of serotonin and/or 5-HIAA in the brainstem of suicides with or without major depression (Mann *et al*, 1989). Neuroendocrine studies find blunted serotonin-mediated prolactin release related to aggressive and suicidal behavior (see Dulchin *et al*, 2001; Malone *et al*, 1996; Weiss and Coccaro, 1997). Furthermore, in postmortem serotonin receptor-binding studies, presynaptic alterations are identified in the brainstem (Arango *et al*, 2001) and pre- and postsynaptic alterations are described in the prefrontal cortex (Arango *et al*, 1995; Hrdina *et al*, 1993; Mann *et al*, 2000; Pandey *et al*, 2002).

Most of the serotonin in the forebrain is produced by neurons in the dorsal and median raphe nuclei (DRN and MRN, respectively) in the rostral brainstem (Törk, 1990). In primates, the serotonin-synthesizing neurons of these rostral raphe nuclei send projections throughout the cerebrum, but importantly to the prefrontal cortex (Pierce *et al*, 1976; Wilson and Molliver, 1991a, b). In serotonin neurons, tryptophan hydroxylase (TPH) is the rate-limiting enzyme in the biosynthesis of serotonin. Despite much investigation of the serotonergic system in suicide and

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Received 10 February 2005; revised 5 August 2005; accepted 11 August 2005

Online publication: 18 August 2005 at http://www.acnp.org/citations/Npp081805050086/default.pdf

depression, measurement of the TPH gene or protein expression level in the brain has been rarely reported.

At the transcript level, brain serotonin in rodents is regulated by a neuron-specific isoform of TPH, known as TPH2 (Patel *et al*, 2004; Zhang *et al*, 2004). Until the recent discovery of TPH2 (Walther *et al*, 2003), quantitative studies of TPH gene expression in brain had been hampered by the seemingly almost undetectable level of transcript expression in the raphe (Austin and O'Donnell, 1999; Clark and Russo, 1997). These studies were unknowingly measuring peripheral TPH (TPH1), the non-neuronal isoform of the enzyme (Walther *et al*, 2003).

At the protein level, we reported greater density and number of TPH-immunoreactive (TPH-IR) neurons in the DRN of suicides relative to normal controls (Underwood *et al*, 1999), suggesting that the serotonergic deficiency in suicide is not due to fewer serotonin-synthesizing neurons in the DRN or due to less TPH protein. Other reports have found fewer number of neurons in the DRvl subnucleus of patients with mood disorders (Baumann *et al*, 2002) and no change in TPH immunoreactivity in any of the DRN subnuclei of depressed suicides (Bonkale *et al*, 2004). However, these studies did not quantify systematically the entirety of the DRN, or lacked specificity by not limiting counts to serotonergic neurons. Furthermore, we have confirmed our finding of higher TPH immunoreactivity in a second cohort of suicides and controls using immunoautoradiography (Boldrini *et al*, 2005). Further evidence of disordered function in DRN neurons of suicide subjects includes altered 5-HT_{1A} autoreceptor binding (Arango *et al*, 2001; Stockmeier *et al*, 1998), and both less serotonin transporter binding and expression (Arango *et al*, 2001). The combination of postmortem studies reporting low serotonin/5-HIAA midbrain levels in suicide, and *in vivo* studies reporting low CSF 5-HIAA in suicide attempters (Placidi *et al*, 2000), combined with postmortem findings of greater DRN TPH-IR neuron density, more TPH protein, and fewer transporter sites, suggests upregulation of biosynthetic capacity and putative transporter internalization in response to less serotonin.

To determine whether more TPH protein in depressed suicides is associated with increased TPH2 mRNA gene expression, we measured the expression of TPH2 mRNA in the DRN and MRN of depressed suicides compared to matched psychiatrically normal controls. We analyzed DRN subnuclei to determine the anatomical distribution of TPH2 message in depressed suicide cases.

MATERIALS AND METHODS

Subjects

Tissue was provided by the Allegheny County Coroner and the NYC Medical Examiner's Office, with protocols approved by all applicable Institutional Review Boards. The next of kin consented to tissue collection, review of relevant records, and a psychological autopsy (see Mann *et al*, 2000) for description of psychological autopsy procedures). All cases died suddenly. Subject demographics are presented in Table 1. Control cases died of causes other than suicide and had no Axis I or Axis II psychiatric diagnosis based on a structured clinical interview (SCID I and II; First *et al*, 1996; Kelly and Mann, 1996; Spitzer *et al*, 1992; Williams *et al*, 1992). In all, 10 suicides had an Axis I diagnosis of major depression, one of which had a diagnosis of major depression as part of a schizoaffective disorder. Comorbidities included one case with an eating disorder and one with obsessive–compulsive disorder. One case with schizoid personality disorder was suspected to have major depression, but failed to meet DSM criteria. Brain collection, neuropathology, toxicology, and tissue-sectioning protocols are described elsewhere (Arango *et al*, 2001). Postmortem interval, defined as the time of death to time of freezing the brain tissue, averaged 16.6 h. Brain pH was measured from cerebellar tissue and was 6.44 ± 0.12 in controls and 6.63 ± 0.08 in suicides ($p > 0.05$). Studies of normal TPH2 mRNA distribution were performed on sections from a total of 13 control cases. Experiments designed for comparing depressed suicides and controls were performed in 11 matched case–control pairs. Cases were matched for age ± 5 years (C: 56 ± 5 *vs* S: 57 ± 5 year), sex (C: nine males : two females *vs* S: nine males : two females), race (C: seven Caucasian, one Hispanic, three African American *vs* S: six Caucasian, five Hispanic), and PMI ± 5 h (C: 13 ± 1.5 *vs* S: 19 ± 1.6). Manners of suicide were hanging ($n = 6$), fall from height ($n = 3$), gun shot ($n = 1$), and drowning ($n = 1$). The causes of death in the controls were: acute myocardial infarction ($n = 9$), motor vehicle accident (pedestrian, $n = 2$), motor vehicle accident (passenger, $n = 1$), and industrial accident ($n = 1$). Four depressed subjects were reported to have received prescriptions for psychotropic medication within the three months before death (SSRIs, $n = 2$; mood stabilizers, $n = 1$; tricyclic antidepressants, $n = 1$). However, all cases had clear peripheral and brain toxicological screens at the time of death.

Brainstems

Brains were collected at autopsy. The brainstem was isolated by a transverse cut along the rostral border of the superior colliculus and a second cut through the middle cerebellar peduncle. The block was approximately 3 cm in length, encompassed the midbrain and rostral pons, and contained the DRN and MRN. The block was flash-frozen in Freon (-20°C) and stored at -80°C until sectioning. Transverse sections were cut in a cryostat at 20 μm thickness, mounted on subbed glass slides, and stored at -80°C until assayed. pH was measured in cerebellar tissue (Harrison *et al*, 1995) to assess integrity of the RNA. Tissue sections for *in situ* hybridization assay were collected approximately every millimeter throughout the rostrocaudal extent of the DRN, corresponding to 16–20 sections per case. Sets of adjacent sections from each case, assayed for TPH protein with biotinylated-PH8 (Boldrini *et al*, 2005) and [^{3}H]8-OH-DPAT (a 5-HT_{1A} receptor agonist (Arango *et al*, 2001), were used to identify boundaries of the raphe nuclei and to further delineate DRN subnuclei for the analysis of TPH2 mRNA in *in situ* hybridization autoradiograms.

Riboprobe Preparation

Riboprobes specific for TPH2 were generated by RT-PCR from human RNA. Human total RNA was extracted from

Table 1 Case Demographics

Pair #	Age	Sex	Race	PMI	pH	Storage time	Brain toxicology	Axis I	Cause of death	Antemortem prescriptions
1	66	Male	White	19	6.1	3514	None	None	Heart attack	None
1a	63	Male	Hispanic	17	6.74	2706	None	None	Hanging (S)	None
2	30	Female	African-American	8	6.73	2755	None	None	Heart attack	None
2a	26	Female	Hispanic	18	6.88	2799	None	Schizoaffective, depressed type	Hanging (S)	Mood stabilizers
3	79	Male	White	9.75	5.957	4870	Lidocaine/antiarrythmic	None	Heart attack	None
3a	77	Male	White	18	6.396	4909	None	MDD	Hanging (S)	None
4	27	Female	White	15	6.717	4314	None	None	MVA	None
4a	28	Female	White	19	6.32	4547	None	Eating disorder, MDD	Fall from height (S)	TCA
5	51	Male	Hispanic	7.5	NA	4867	None	None	Heart attack	None
5a	66	Male	White	11	NA	4846	Caffeine	None	Gun shot (S)	None
6	53	Male	White	18.5	NA	3898	None	None	Heart attack	None
6a	50	Male	Hispanic	30	6.82	3089	None	MDD	Fall from height (S)	None
7	37	Male	African-American	15	6.75	2715	None	None	Heart attack	None
7a	40	Male	White	20	6.77	2607	Analgesics	MDD	Hanging (S)	SSRI
8	85	Male	White	7	NA	4200	None	None	Heart attack	None
8a	74	Male	White	21	NA	4168	Opiates, analgesics, caffeine	MDD	Fall from height (S)	None
9	53	Male	African-American	9	NA	1905	None	None	Industrial accident	None
9a	59	Male	Hispanic	24.5	6.77	2546	None	MDD	Hanging (S)	None
10	58	Male	White	22	6.44	2188	Anesthetics	None	Heart attack	None
10a	62	Male	White	21	6.37	1950	None	MDD	Drowning (S)	SSRI
11	56	Male	White	14	6.41	1463	Caffeine	None	Heart attack	None
11a	56	Male	Hispanic	21	6.56	335	None	MDD	Hanging (S)	None
12	36	Male	White	14	NA	4257	CO	None	MVA	None
13	40	Female	White	26	NA	2426	None	None	MVA	None
Mean	53			16.9	6.55	3245				
SEM	4			1.2	0.07	254				

All demographic information was made available by the medical examiner and psychological autopsy provided by next of kin.
Age, in years; CO: carbon monoxide; MDD: major depression; MVA: motor vehicle accident; PMI: postmortem interval, in hours; SSRI: serotonin-specific reuptake inhibitor; storage time, days in freezer; S: suicide; TCA: tricyclic antidepressants.

frozen sections using Trizol reagent (Invitrogen, Carlsbad, CA). These sections were cut at 60 μm and stored in Eppendorf tubes at −80°C for later mRNA isolation. mRNA was reverse transcribed into cDNA using SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA), which was subsequently used for PCR. TPH2-specific primers for PCR were generated from the published human cDNA sequence (Walther *et al*, 2003) available on Genbank (accession number: AY098914). The forward primer sequence was 5′-CTCTCCAAACTCTATCCCACT-3′ and the reverse primer sequence was 5′-AGGCATCAAATCCCC AGA-3′. These primers were designed from a region of the transcript that is conserved across species (human, rat, and mouse).

PCR cycling conditions were as follows: 90°C denaturing temperature for 30 s, ramping down to 50°C annealing temperature for 15 s, and then 72°C for 15 s. These cycling parameters were repeated for 30 cycles. The resulting 800 base pair product was purified and cloned into the PCRII-TOPO vector (Invitrogen, Carlsbad, CA). The TPH2-containing plasmids were transformed into *Escherichia coli*, grown overnight, extracted, and purified. The resulting construct was linearized with *Hind*III and transcribed with SP6 RNA polymerase (Promega Madison, WI) to generate a specific TPH2 antisense probe. The control sense probe was created by linearizing the same construct with *Xba*I, and transcribing with T7 RNA polymerase. The sequences of both probes were verified. Riboprobes were labeled with [^{35}S]rCTP (Perkin-Elmer, Boston, MA) by adding the radiolabeled nucleotide to the *in vitro* transcription reaction. The probe was then passed through a spin column (RNeasy Kit from Qiagen, Valencia, CA) to remove all unincorporated nucleotides. Probe-specific activity was determined by measuring the counts per minute (cpm) in an aliquot of the purified probe using a scintillation counter. Incorporation of radioactive nucleotide was determined by taking scintillation counts of labeled reaction before and after removal of unincorporated nucleotides.

In Situ Hybridization Histochemistry

In situ hybridization experiments were carried out in two phases on tissue sections of brainstems from 11 pairs of matched suicide and control cases. First, in order to compare the expression of TPH2 mRNA in all subjects, two tissue sections from a midcaudal level of the DRN (see Figure 1) from each case were assayed together in a single experiment. The use of the same solutions and probe preparation and exposure to the same autoradiographic film in a single cassette controlled for all possible interassay variables. The midcaudal level of the DRN was selected because it has a relatively homogeneous composition of the DRN (ie without representation of multiple subnuclei within a single section), and to be comparable and available between all subjects. In the second phase, sections spanning the rostrocaudal extent of the DRN and MRN from each matched pair were assayed together with the same solutions to reduce experimental variability within the pair. The assay throughout the anatomical extent of the raphe allows for the determination of the distribution and localization of TPH2 mRNA in the DRN between groups.

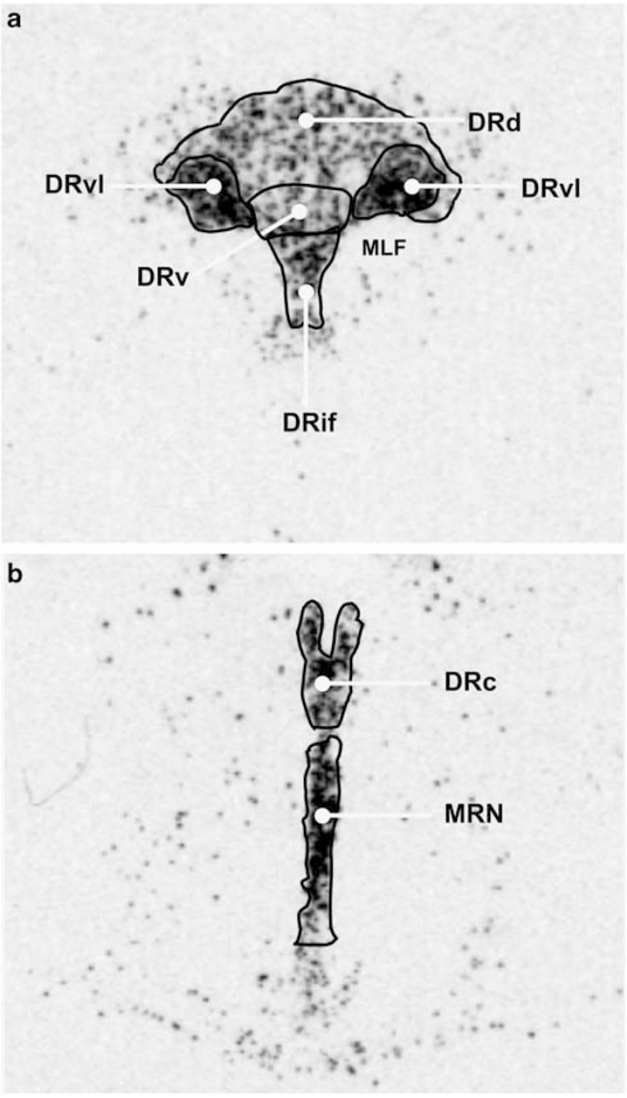

Figure 1 (a) Schematic of DRN subnuclei. The sampled subnuclei are outlined in black and include the dorsal (DRd), ventral (DRv), ventrolateral (DRvl), and interfascicular (DRif) subnuclei. In (b), the caudal subnucleus (DRc) is outlined in a more caudal section of the DRN. The medial longitudinal fasciculus (MLF) is labeled in the rostral section.

Sections were brought to room temperature and dried under vacuum. Sections were fixed in 4% paraformaldehyde in 0.1 M PBS (pH 7.4) for 15 min, rinsed in PBS for 5 min, and acetylated in 0.25% acetic anhydride in 0.1 M triethanolamine, pH 8.0 for 10 min. Then sections were dehydrated through increasing concentrations of ethanol, delipidated in chloroform for 10 min, washed in 100 and 95% ethanol for 1 min each, and covered with 100 μl of hybridization solution (50% formamide, 10 mM EDTA, 20 mM Pipes, 0.75 M NaCl, 10% dextran sulfate, 5 × Denhardts, 250 μl/ml tRNA, and denatured radiolabeled probe (3 ng, 2×10^6 counts per 100 μl)). After coverslipping, sections were placed in an incubation chamber that was sealed with tape and parafilm and incubated overnight at 55°C. For each case, two sections were treated with the sense probe to measure nonspecific binding. Next day, coverslips were removed and slides were washed in a bath of

4 × saline-sodium citrate (SSC), pH 7.2 (1 × SSC = 0.15 M sodium chloride, 0.015 M SSC, pH 7.2) with 0.2% β-mercaptoethanol for 15 min and again in 4 × SSC for 15 min. Sections were then washed in formamide buffer containing a 1 : 1 ratio of formamide to formamide buffer (0.6 M NaCl, 0.04 M tris base, 2 mM EDTA, 0.02 N HCl, pH 7.7) for 20 min at 55°C. The sections were washed in 2 × SSC and then incubated in RnaseA solution (10 mg/ml RNaseA in 0.5 M NaCl/10 mM Tris Base/1 mM EDTA) for 30 min at 37°C to reduce the background due to nonhybridized probes. The sections were then passed through the following washes, each for 5 min at room temperature: 2 × SSC, 1 × SSC, and 0.5 × SSC. One high-stringency wash was performed in 0.1 × SSC for 30 min at 55°C and cooled for 5 min in the same buffer. Sections were dipped briefly in 60% ethanol/0.33% ammonium acetate, dried, and exposed to autoradiography film (Biomax MR, Kodak) with ^{14}C calibration standard slides (ARC-146, 146A, American Radiolabeled Chemicals, Inc.) for 3 days. After development of films, sections were dipped in photographic emulsion (Kodak NTB-2) to develop silver grains.

Imaging and Densitometry of *In Situ* Hybridization Autoradiograms

Methods for semiquantification of *in situ* hybridization autoradiograms have been previously described (Arango *et al*, 2001) and are summarized here. Briefly, autoradiograms of each section were digitized using an image analysis system (MCID Elite; Imaging Research Inc., St Catherine's, Ontario, Canada). Two analytical approaches were taken. First, densitometric measurements of the DRN and MRN were taken from sections by outlining the raphe nuclei. Contours of the DRN and MRN were constructed from images of adjacent [^{3}H]OH-DPAT receptor autoradiograms (Arango *et al*, 2001). These contours were then digitally overlaid on TPH2 *in situ* hybridization images for densitometric quantitation. A schematic of the sampled subnuclei is shown in Figure 1. Anatomical descriptions based on Nissl stains of the DRN (Baker *et al*, 1990) were used as a guide to delineate the subnuclei in the images of [^{3}H]OH-DPAT autoradiograms. The subnuclei of the DRN include the dorsal (DRd), ventral (DRv), ventrolateral (DRvl), interfascicular (DRif), and caudal (DRc) subdivisions. The MRN was identified at its most rostral point by the presence of the decussation of the superior cerebellar peduncle in adjacent Nissl-stained sections (Baker *et al*, 1991a; Törk and Hornung, 1990).

Second, DRN measurements were made using a circle of fixed area (80 mm^2) as shown in Figure 3a. A separate circle of the same size was used to measure the MRN. 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, the concentration of label within the circle, and the concentration of label within the target. The amount of TPH2 mRNA reported here reflects the mean concentration of TPH2 within the target in the circle and is therefore not diluted or biased by unlabeled areas. Rather, this definition of DRN results in a more restricted 'contour' of the DRN than that is derived from the distribution of the 5-HT$_{1A}$ autoreceptor. Measurements from film background and non-raphe tissue section background with the same fixed area circle determined levels of nonspecific labeling.

Optical density measurements were calibrated using ^{14}C standard slides (American Radiolabeled Chemicals, Inc., ARC-146 and ARC-146A) that were coexposed with the ^{35}S-labeled tissue sections on each film. The linear relationship between ^{14}C and ^{35}S (Miller, 1991) allowed the use of these standards for the semiquantitative analysis 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/g 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. 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 circle.

Statistical Analyses

The difference in TPH2 mRNA expression between controls and suicides was calculated using percent of control for each pair and then testing the significance with a one-sample *t*-test. Expression along the anteroposterior axis was determined by averaging the densities across approximately 2 mm intervals for each case and then calculating the mean percent of control within each interval. The TPH2 mRNA expression in DRN subnuclei was examined using an ANOVA.

RESULTS

TPH2 mRNA Expression in the DRN and MRN

Specific, robust expression of TPH2 mRNA was observed in the DRN and MRN throughout the rostrocaudal extent of these nuclei (shown in Figure 2). TPH2 mRNA expression was also observed in the adjacent supralemniscal nuclei (B9) known to have serotonin-synthesizing neurons (Baker *et al*, 1991b). At the microscopic level, emulsion-dipped slides revealed that TPH2 is expressed within raphe neurons (Figure 3c). Sense sections generated a homogeneous gray film background (Figure 3b) and emulsion-dipped sense sections had few silver grains in the background and no indication of specific labeling (Figure 3d). TPH2 mRNA density levels correlated with TPH protein in a subset of cases that had previously been reacted for PH8 immunoautoradiography (Boldrini *et al*, 2005). In a mixed model with TPH protein as the dependent variable, TPH2 as the independent, and pair, probe, and subject as random effects, the estimate of the TPH2 effect was 0.23 with standard error = 0.058 ($p = 0.01$).

Densitometry readings obtained from DRN contours correlated with fixed circle measurements ($r = 0.96$ in controls and $r = 0.94$ in suicides; $p < 0.01$). In controls, the mean TPH2 optical density for anatomical levels within the DRN was rostral > mid-raphe > caudal raphe. A negative

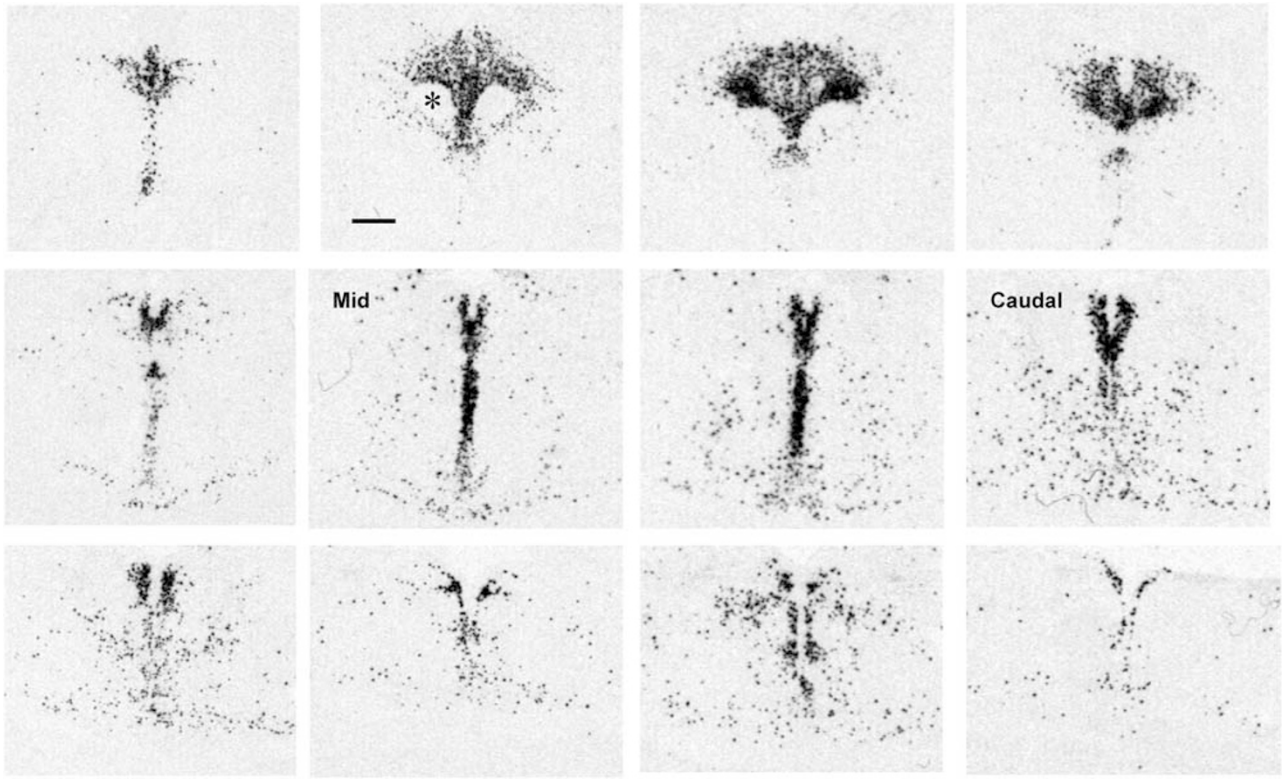

Figure 2 Autoradiograms of TPH2 mRNA distribution by *in situ* hybridizaton of representative brainstem sections along the anteroposterior axis of a single case. Note that the labeling is anatomically restricted and most dense in regions corresponding to the DRN and MRN. Sections spanning the mid and caudal levels of the DRN are representative of those sections selected from all cases for comparison of controls and suicides. The TPH2 in DRN is localized posterior to the medial longitudinal fasciculus (*). Bar = 2 mm.

correlation was found with age such that TPH2 mRNA expression decreases with increasing age ($r = -0.429$, $n = 22$, $p = 0.046$) (Figure 4). TPH2 expression levels in females ($n = 4$) did not differ from males ($n = 18$) (13.5 ± 2.5 *vs* 10.3 ± 0.7 nCi/g; $F = 3.441$, $p = 0.078$). TPH2 expression did not correlate with PMI, brain pH, or freezer storage time ($p > 0.05$, data not shown). TPH2 also showed no correlation with estimated time of death or season of death.

TPH2 mRNA Expression in the DRN and MRN of Controls and Suicides

The area of the DRN, as defined by TPH mRNA expression, at each rostrocaudal level is not different between groups (Figure 5, $p > 0.05$). The volume of the DRN, determined by the summed area of TPH2 expression along the anteroposterior axis, is also not different (C: 46.7 ± 6.7 *vs* S: $51.6 \pm 7.9\,mm^3$; $p > 0.05$). Film densitometric quantification of TPH2 mRNA revealed higher TPH2 expression levels in the DRN of depressed suicides cases compared with matched controls. When two levels of the raphe (labeled mid and caudal, respectively, in Figure 2) from each case of all 11 pairs were assayed together and averaged, 33% more TPH2 mRNA expression was found in suicides compared with matched controls in the DRN ($t = -2.421$, df = 10, $p = 0.03$, C: 9.31 ± 0.9 *vs* S: 12.42 ± 1.1 nCi/g; see Table 2) and 17% more in the MRN ($t = -2.168$, df = 21, $p = 0.04$, C: 6.40 ± 0.6 *vs* S: 7.5 ± 0.4 nCi/g).

To compare TPH2 expression along the entire DRN, each matched pair was assayed individually and the difference expressed as a percent of control every 2.4 mm interval along the rostrocaudal axis. A repeated-measures ANOVA analysis revealed no significant differences in the change in TPH2 expression across the rostrocaudal axis. The highest difference is observed in midcaudal levels (10–14 mm for the DRN and 8–10 mm of the MRN see Figure 6). The mean difference of 30% found at this midcaudal level of the DRN is comparable to the anatomical level and difference observed in the previously described experiment examining the midcaudal level in all subjects simultaneously. Using the measurements from the experiment in which each pair was assayed individually, we further calculated the mean percent difference in the DRN and MRN for each pair when all sections were included. Taking the average of the percent difference of each pair resulted in a mean change of 18% ($t = 3.089$, df = 5, $p = 0.03$) in the DRN and 39% in the MRN. However, in the MRN this change was not statistically significant.

To determine whether the increase in TPH2 expression is specific to particular subdivisions of the DRN, density measurements were taken from five subnuclei (DRd, DRvl, DRv, DRif, and DRc). Significant differences were not detected in any individual subnucleus (136% of control in the DRd; 133% in the DRvl; 86% in the DRv; 105% in the

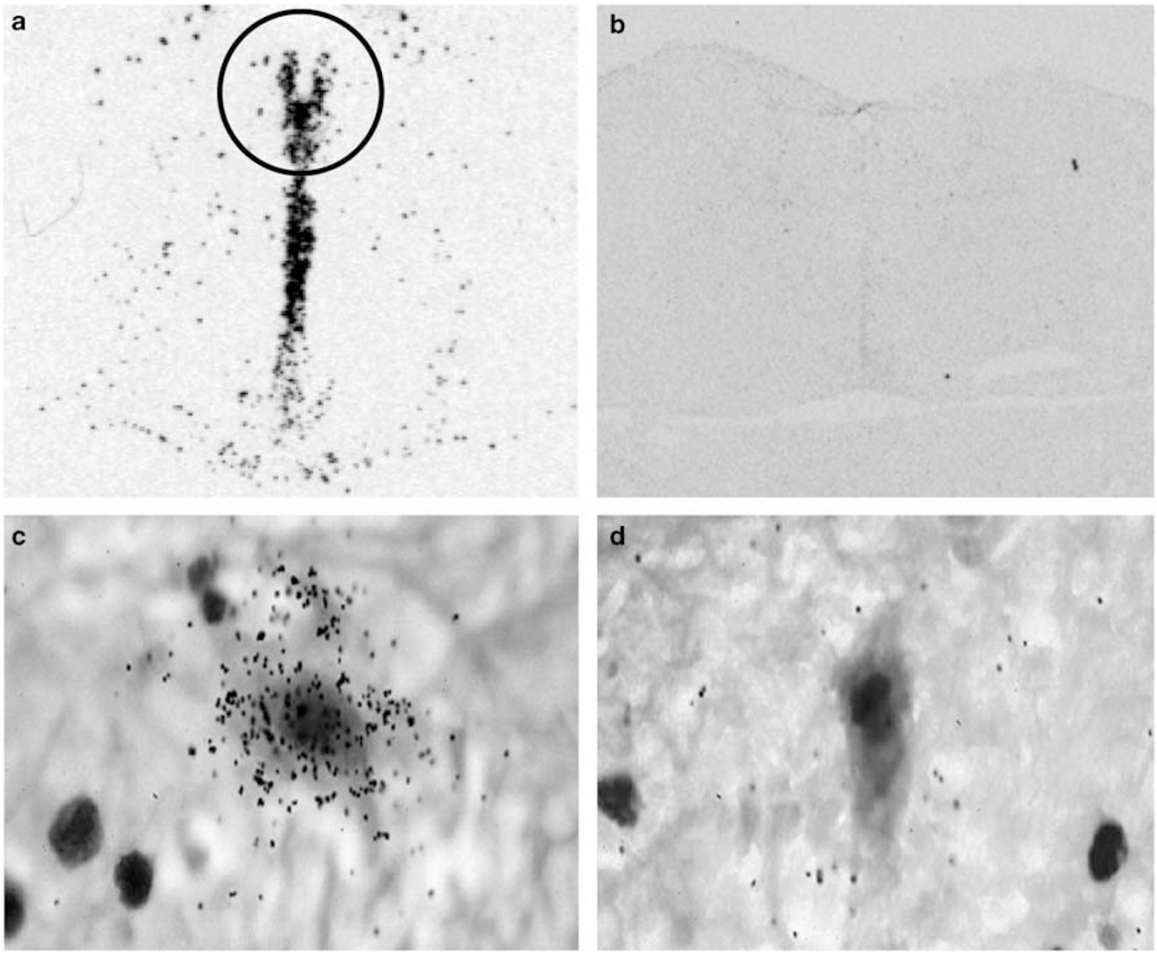

Figure 3 (a) An *in situ* hybridization autoradiogram with the antisense TPH2 probe in a representative human brainstem section. The circle shown encompassing the DRN was used for densitometric analysis of TPH2 expression. The area of this circle (80 mm^2) remained unchanged for all sections measured. (b) An autoradiogram of an adjacent section to (a) that was reacted with the sense TPH2 riboprobe. Note the absence of specific labeling. (c, d) High-power photomicrographs of DRN serotonergic neurons found in TPH2 antisense (c) and sense (d) emulsion-dipped sections. Silver grains are specific to neurons and are absent in neurons in sense sections.

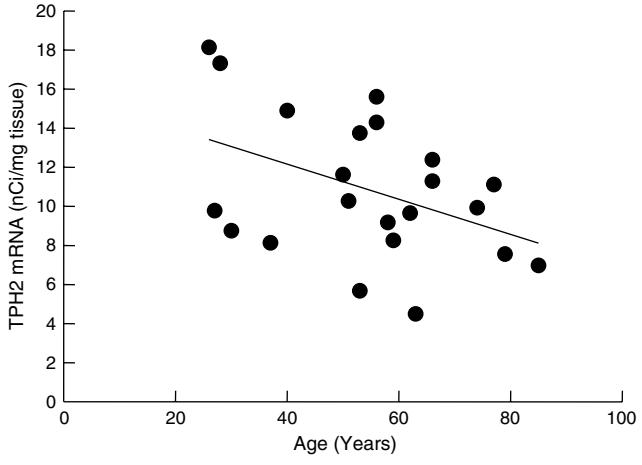

Figure 4 Relationship between TPH2 mRNA density and age. Note that TPH2 mRNA expression decreases with increasing age ($r = -0.049$, $p < 0.05$, $n = 22$).

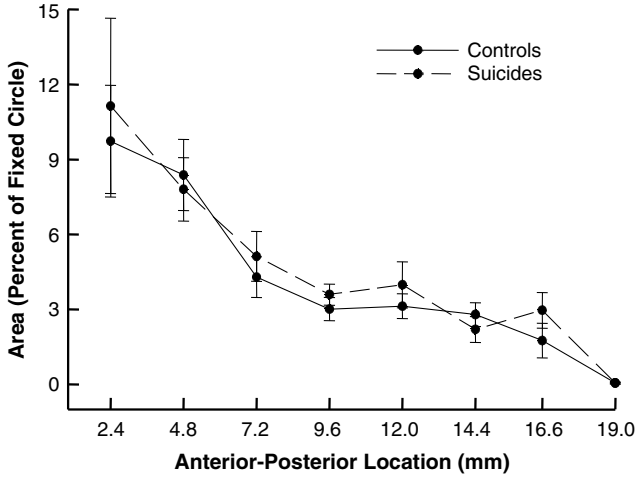

Figure 5 Area of the DRN defined by TPH2 mRNA expression in controls (solid line) and suicides (dashed line) along the rostrocaudal axis. Area is defined as a percentage of the fixed area (80 mm^2) sampling circle.

DRif; and 108% in the DRc), suggesting that the difference between controls and suicides is distributed across the rostrocaudal extent of the DRN involving each subnucleus. The relatively larger 36 and 33% increases in the DRd and the DRvl in suicides may indicate a more pronounced involvement of these subnuclei.

Table 2 Mean TPH2 mRNA Expression in Mid and Caudal Levels of the DRN/MRN of Controls *vs* Suicides

Pair #	Control DRN (nCi/mg)	Suicide DRN (nCi/mg)	Control MRN (nCi/mg)	Suicide MRN (nCi/mg)
1	4.50	12.38	4.05	6.74
2	8.75	18.13	5.70	8.72
3	11.11	7.56	6.78	10.01
4	9.78	17.32	7.27	7.28
5	10.27	11.29	10.38	7.48
6	13.74	11.62	7.22	8.86
7	8.13	14.89	5.26	6.55
8	6.98	9.93	5.51	6.11
9	5.68	8.25	3.88	6.17
10	9.17	9.65	6.45	6.19
11	14.29	15.60	7.87	8.49
Mean	9.31	12.42	6.40	7.51
SEM	0.92	1.09	0.56	0.40

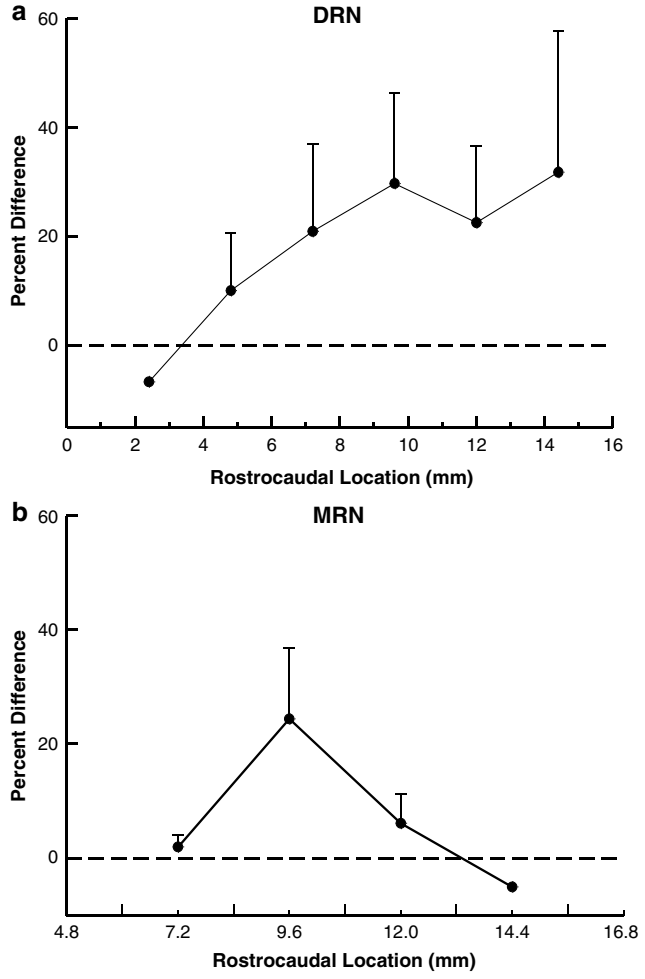

Figure 6 Difference in TPH2 expression between suicides and controls across the rostrocaudal axis of the DRN (a) and MRN (b). Difference in expression is determined as the mean of percent of control of each pair at 2.4 mm intervals across the rostrocaudal axis.

DISCUSSION

We show, for the first time, that expression of TPH2 mRNA in the human DRN and MRN is specific and robust. In the human brainstem, TPH2 mRNA expression was present only in the DRN-, MRN-, and supralemniscal serotonin-synthesizing nuclei and, for example, was not observed in the adjacent locus coeruleus, which contains noradrenergic neurons. These results are consistent with data from rodents (Patel *et al*, 2004; Zhang *et al*, 2004) and suggests that the neuronal TPH transcript (TPH2) is the isoform responsible for the production of the serotonin biosynthetic enzyme TPH in the human brain.

We have confirmed our finding of higher TPH immunoreactivity in a second cohort of suicides and controls using immunoautoradiography (Boldrini *et al*, 2005). In the present study, we report higher gene expression of TPH2 mRNA, the neuronal isoform of TPH, in the DRN of depressed suicides compared with nonpsychiatric controls. In a subset of cases in which both TPH immunoreactivity and TPH2 mRNA were assayed and measured in adjacent sections, we found that TPH2 mRNA and TPH-IR have comparable anatomical distributions within the DRN and MRN, with greater density of expression in the rostral part of the DRN complex. Our subsample, showing a correlation of TPH2 mRNA and TPH-IR that is similar in direction and magnitude across the rostrocaudal axis, suggests a functional relationship between transcript and protein levels where greater amounts of TPH2 gene expression may account for higher levels of DRN TPH-IR in depressed suicides (Boldrini *et al*, 2005; Underwood *et al*, 1999). This is further supported by our previous finding that volume of the DRN, as defined by TPH immunoreactivity, does not differ between suicides and controls (Underwood *et al*, 1999) and also does not differ when defined by TPH2 mRNA expression.

Previously, we reported greater number and density of TPH immunoreactive neurons in the DRN of suicides compared with controls (Underwood *et al*, 1999). In other studies, fewer number of neurons have been found in the DRvl subnucleus of patients with mood disorder (Baumann *et al*, 2002), and no change in TPH protein expression in any DRN subdivision of depressed suicides (Bonkale *et al*, 2004). A confounding factor of the first study is that DRN neurons were identified by a Nissl stain, and therefore included DRN cell types that are not serotonergic. In the second study, a failure to find a change in any particular subdivision of the DRN may represent a cumulative change spread throughout the subnuclei that could only be detected when the DRN is assayed as a whole. Bonkale *et al* (2004) examined only two levels of the DRN, thereby potentially missing changes in TPH across the rostrocaudal axis that may not be specific to DRN subdivisions. In our study, we provide evidence for an increase in TPH2 mRNA expression in suicides that is spread throughout the length of the DRN and MRN and is not restricted to any individual subnucleus, thereby lending support for the previous findings where individual levels were assayed.

Having found that both TPH protein and gene expression are increased in depressed suicides, the question remains as to why. In rats, TPH protein and mRNA are elevated in response to stress (Azmitia *et al*, 1993; Chamas *et al*, 1999,

2004), suggesting that depression and suicide-related stress may account for elevated TPH gene expression and protein. Alternatively, the serotonergic system may be altered as part of a homeostatic response to deficient serotonin levels in depressed suicides. There is an apparent paradox, namely we find more serotonin neurons, higher gene expression, and more TPH-IR (protein), and, yet, previous studies report less serotonin and/or 5-HIAA in the midbrain of suicides (reviewed by Mann *et al*, 1989) and in the CSF of suicide attempters (Placidi *et al*, 2001). The leading hypothesis regarding the pathophysiology of major depression and suicide is a deficiency in serotonergic neurotransmission (Arango *et al*, 2001; Mann, 2003). Greater TPH2 gene expression in the DRN of depressed suicides may be a compensatory mechanism for deficient brain serotonin levels or serotonergic neurotransmission to target areas. Lower levels of serotonin could result from impaired biosynthetic capacity of the TPH enzyme. A variant in the TPH gene has been recently found (Zhang *et al* (2004) in mice and Zhang *et al* (2005) in humans) that results in lower levels of serotonin synthesis *in vitro* although the reported mutation was present in only 10% of the major depressive disorder (MDD) cases studied and these cases were mostly suicide attempters (Zhang *et al*, 2005). Other potential polymorphisms (Zill *et al*, 2004) or enzyme defects affecting TPH2 catalytic activity could explain deficient serotonergic synthesis in other cases of MDD or suicide. Furthermore, the finding that high levels of TPH2 are present throughout the rostrocaudal extent of the DRN and may be specific to particular rostral subnuclei suggests that finer targets within the dorsolateral prefrontal cortex may be implicated in depression and suicide. The prefrontal cortex is found to have alterations in serotonin receptor binding (Arango *et al*, 2001) and is implicated as a site involved in the pathophysiology of MDD. Heterogeneous changes in DRN with more pronounced changes in subnuclei with neurons providing the serotonergic innervation to target regions implicated in the disorder suggest aberrant circuits or inadequate homeostatic compensatory responses that are specific and targeted and not global and divergent.

The analysis of TPH2 expression was examined in two types of experiments; one assayed two anatomical levels from each case in one single large experiment, and the second assayed all levels across the rostrocaudal axis of the DRN. The first approach lacked anatomical precision, but allowed the simultaneous assay of all cases under identical conditions. This experiment removed the possibility of variability that could not be controlled when individually analyzing each pair. Furthermore, since only one sheet of autoradiographic film was used for this experiment, we could directly measure and compare TPH2 expression levels across the 11 pairs. This experiment confirmed the difference in TPH2 expression between controls and suicides in direction and magnitude. In the second experiment, TPH2 mRNA expression was quantified within a fixed area circle, an index of the total amount of TPH2 in the DRN or MRN and a contour, a measure of the concentration of TPH2. DRN subnuclei contours provided anatomical definition that a fixed area circle did not. Results from both the fixed area circle ROI and anatomical contouring of the entire DRN for the densitometric analysis of TPH2 expression were highly correlated. Furthermore, the fixed circle ROI measurements did not underestimate the amount of TPH2, an indication that the partial volume effect seen with *in vivo* positron emission tomography (PET) scanning does not apply to this film-based method.

The change in TPH2 expression was found across the entirety of the anteroposterior axis of the DRN. Analysis of individual DRN subnuclei and the lack of statistically significant differences in any single subnucleus support the notion that TPH2 mRNA expression is distributed throughout the DRN. Studies on cortical innervation of the DRN in macaques demonstrate that there is a rough rostrocaudal topographic organization between dorsal raphe neurons and their cortical terminations. For example, the dorsolateral prefrontal cortex is predominantly innervated by the rostral DRN, while primary somatosensory and primary visual cortices are innervated predominantly by neurons from the caudal DRN (Wilson and Molliver, 1991b). The implication of a widespread increase in TPH2 expression in the DRN is that serotonin alterations in target regions likely extend beyond the prefrontal cortex. More specific DRN alterations such as increased TPH2 expression in particular subnuclei would be suggestive of comparatively greater effects in select targets such as the prefrontal cortex. A caveat is that the homology of projections between non-human and human primates remains unknown.

The finding of greater TPH2 gene expression in the DRN of depressed suicides cannot be explained by differences in demographic variables, brain pH, or storage effects between controls and suicides, since this study used a matched, pairwise case-control design. Furthermore, brain toxicology screens show that all subjects were free from drugs or medications. Although we cannot rule out previous effects of antidepressants that were stopped long enough before death, so as to be no longer detectable, the informants interviewed for the psychological autopsy revealed that the majority of cases did not receive treatment in the 3 months before death. Only four depressed subjects were reported to have received prescriptions for psychotropic medication in the 3 months before death (SSRIs, $n=2$; mood stabilizers, $n=1$, tricyclic antidepressants, $n=1$), but none tested positive for these drugs at death, suggesting that they had discontinued the medication earlier or never taken it. Like the group as a whole, these four depressed cases also had higher levels of TPH2 mRNA compared to controls. Therefore, higher TPH2 mRNA levels in depressed suicides are likely associated with a pre-existing mood disorder and/or suicidal behavior. Further studies are required to determine the effects of antemortem treatments on TPH2 expression.

The incidence of suicide and depression is known to increase in the later decades of life in males (www.cdc.gov/ncipc/wisqars 2002). Serotonergic receptor binding in the cortex, specifically $5HT_{1A}$, $5HT_{1B/D}$, and $5HT_{2A}$, has been found to be decreased postmortem in elderly suicides and some of these findings have been confirmed in depressed elderly patients in PET studies (Meltzer *et al* (1998b) and also reviewed in Meltzer *et al* (1998a)). Therefore, it is likely that changes in serotonergic neurotransmission that are associated with depression and suicide are only exacerbated in the elderly. The observation of decreased TPH2 with age raises the possibility that changes in TPH2 mRNA

expression may also contribute to the risk for depression or suicide in elderly males.

In the study of suicide and mood disorders in postmortem human brain, it is necessary to distinguish the molecular pathophysiology of major depression from that of the diathesis for suicide. The results of this study need to be replicated and extended in a larger series of cases that includes a sufficient number of suicides without major depression to separate the effects of major depression from suicide. Future studies should also determine the expression level per cell by grain-counting of emulsion-dipped slides to determine whether higher levels of expression are a function of more serotonin neurons or whether expression per neuron is greater. Such studies should be combined with assays in which the biosynthetic capacity of TPH can be tested so as to link higher gene expression with a functional outcome which would ultimately lead to deficient levels of brain serotonin. For example, functional mutations in the TPH catalytic site may result in a low-activity enzyme. Alternatively, assays measuring changes in postmortem TPH activity in the presence of limiting cofactors such as oxygen, pteridines, or ferrous iron can be conducted to determine whether all the molecular machinery is in place for the efficient function of TPH. Another possible avenue is measuring the level of endogenous TPH inhibitors that could decrease the efficacy of the enzyme. Taken together, these speculations provide a framework for future directions of studies that may interlace changes in TPH2 gene expression with low serotonin levels in depression and suicide.

ACKNOWLEDGEMENTS

This work was supported by MH40210, MH62185, MH64168 grant, The Diane Goldberg Foundation, and the American Foundation for Suicide Prevention. We thank Jennifer Lau for her work with image analysis, sampling and quantification. We also thank Stephen P Ellis who provided expertise with statistical analyses.

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