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ORIGINAL ARTICLE

Elevated expression of tryptophan hydroxylase-2 mRNA at the neuronal level in the dorsal and median raphe nuclei of depressed suicides

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Deficient levels of serotonin are associated with suicide and depression. Paradoxically, in the dorsal raphe nucleus (DRN) there are more serotonin neurons and more neuronal tryptophan hydroxylase-2 (TPH2) expression postmortem in depressed suicides. In this study, we sought to determine whether greater TPH2 expression in depressed suicides was the result of more TPH2 expression per neuron. *In situ* hybridization and computer-assisted image analysis were performed on tissue sections throughout the extent of the raphe nuclei at the level of silver grains per neuron to systematically quantify TPH2 neuronal expression. Depressed suicides have 26.5% more TPH2 grain density per neuron in the DRN compared with matched controls ($P = 0.04$). The difference in grain density is greater at mid- and caudal anatomical levels across the rostrocaudal axis of the DRN. Densitometric analysis of TPH2 expression in the DRN subnuclei showed that higher expression levels were observed at posterior anatomical levels of depressed suicides (121% of control in the caudal subnucleus). Higher TPH2 expression in depressed suicides may explain more TPH2 protein and reflect a homeostatic response to deficient serotonin levels in the brains of depressed suicides. Localized changes in TPH2 expression in specific subnuclei of the DRN suggest that the serotonergic compensatory mechanism in depression and suicide is specifically regulated within the DRN and has implications for regions innervated by this subnucleus.
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Introduction

An impaired brain serotonergic system is hypothesized to contribute to the etiology of major depressive disorder (MDD) and suicide. The findings of low serotonin and/or its major metabolite 5-hydroxyindoleacetic acid in suicides and depressed suicide attempters[1] suggest deficient serotonergic neurotransmission. Furthermore, in suicide attempters, serotonin activity measured by the release of prolactin in response to administration of fenfluramine was found to be blunted.[2–7] Brain serotonin (5-HT) is produced by neurons in the midline of the brainstem, the raphe nuclei. Of the several raphe nuclei, the dorsal (DRN) and median raphe nuclei (MRN) provide the innervation to the forebrain,[8] including the prefrontal cortex, a region that modulates mood and major cognitive processes. The DRN is anatomically subdivided into five subnuclei, that is, the dorsal (DRd), ventrolateral (DRvl), ventral (DRv), interfascicular (DRif), and caudal (DRc). Each of these subnuclei is believed to project to specific parts of the cortex and subcortical regions and consequently may have differential regulation of 5-HT synthesis.

In postmortem studies of suicides, some reported findings in the serotonergic system are not consistent with reduced function and may represent an upregulation of the serotonergic system as part of a homeostatic response to deficient 5-HT neurotransmission. In the brainstem, there are more serotonin neurons, fewer presynaptic serotonin transporter (SERT) binding sites and altered 5-HT_{1A} autoreceptor binding and some, but not all, studies report higher 5-HT_{1A} and 5-HT_{2A} postsynaptic receptor binding in the prefrontal cortex (reviewed by Mann[9]).

Tryptophan hydroxylase (TPH) is the rate-limiting enzyme in the biosynthesis of 5-HT, converting tryptophan to 5-hydroxytryptophan (5-HTP) on route to decarboxylation into 5-hydroxytryptamine (see Mockus and Vrana[10] for review). Alterations in TPH expression or catalytic activity can potentially cause changes in the levels of 5-HT in the brain. At the transcript level, brain 5-HT in rodents is regulated by a neuron-specific isoform of TPH, known as

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TPH2.[11,12] We found specific TPH2 transcript expression in the human DRN and MRN and further discovered that TPH2 mRNA expression is increased in suicides.[13] At the protein level, we found higher density and number of TPH immunoreactive (IR) neurons in the DRN of depressed suicides by immunocytochemistry,[14] a finding replicated in a second cohort using immunoautoradiography.[15] Another group reported higher TPH protein expression restricted to the dorsal subnucleus of the DRN in alcohol-dependent depressed suicides[16] but found no change in non-alcoholic depressed suicides,[17] demonstrating the anatomically restricted contributions of individual DRN subnuclei in regulating serotonin.

In the present study we sought to further analyze the increase in TPH2 gene expression in suicides to determine whether the increase in TPH2 expression was due to more neurons or whether there was an increase in expression at the neuron level. We, therefore, analyzed emulsion-dipped slides of the cases studied in our previous report[13] and quantified expression at a per neuron level in suicides and matched controls. We also examined expression in subnuclei of the DRN.

Materials and methods

Subjects

Study procedures were approved by the applicable Institutional Review Boards. Consent was given by next of kin for tissue collection, review of relevant records and psychological autopsy (see Mann *et al*.[18] for description of psychological autopsy procedures). All cases died suddenly. Controls died of either cardiac arrest ($n=8$), vehicular injury ($n=1$) or industrial accident.[1] Suicides occurred by hanging ($n=5$), fall from height ($n=3$), gunshot ($n=1$) or drowning ($n=1$). Control cases had no psychiatric diagnoses based on a structured clinical interview SCID I and II.[19–22] A total of 9 of the 10 suicides had a diagnosis of Axis I major depression. For one case, the diagnosis of major depression was part of a schizoaffective disorder. Comorbidities included one case with an eating disorder and one with obsessive-compulsive disorder; 1 of the 10 suicide cases met criteria for alcohol abuse in the past and 3 were moderate-to-heavy cigarette smokers.

All procedures involving brain collection, neuropathology, toxicology and tissue sectioning protocols are discussed by Bach-Mizrachi *et al*.,[13] and are briefly summarized here. For all subjects, postmortem interval (PMI) averaged 15.6 h. Brain pH was 6.44 ± 0.12 in controls and 6.63 ± 0.08 in suicides ($P > 0.05$). *In situ* hybridization experiments were performed in 10 matched case–control pairs. Cases are matched first according to sex (eight males, two females), then age within 5 years (controls, 53.9 ± 5 vs suicides, 54.5 ± 5 years), PMI (controls, 13.1 ± 1.7 vs suicides, 20 ± 1.6) and whenever possible race (controls comprise six Caucasian, one Hispanic and three African-American, whereas suicides comprise six Caucasian and four Hispanic). Cause of death is determined by the medical examiner's office. All cases had clear peripheral and brain toxicological screens at the time of death. See Table 1 for summary of subject demographics.

Brainstems

Brains were collected at autopsy (see Arango *et al*.[23]). The brainstem tissue block used for sectioning was approximately 3 cm in length and included the midbrain and rostral pons, which contained the DRN and MRN. The block, standing on a glass slide on its rostral cut surface, was flash-frozen in freon ($-20\,^{\circ}C$) and stored at $-80\,^{\circ}C$. The brainstem was sectioned exhaustively at 20 μm. Sections for *in situ* hybridization experiments were taken every 1200 μm throughout the rostrocaudal extent of the DRN, corresponding to 16–20 sections per case. Sections were mounted and stored at $-80\,^{\circ}C$ until assayed. Brain pH was measured to obtain an indication of the integrity of the RNA.[24]

Grain counting of in situ *hybridization autoradiograms*

Production of riboprobes specific for TPH2 and *in situ* hybridization assays were described by Bach-Mizrachi *et al*.[13] After the completion of the *in situ* hybridization procedure, slides were dried and exposed to autoradiography film (Kodak BioMax MR, Carestream Health, Inc., Rochester, NY, USA) with ^{14}C calibration standard slides (ARC-146, 146A, American Radiolabeled Chemicals Inc., St Louis, MO) for 3 days. After the films were developed, the slides were dipped in photographic emulsion (Kodak NTB-2) and lightly stained with hematoxylin and eosin, allowing for the visualization of cellular structures without obstructing the silver grains produced by the reduction of silver following emulsion dipping, exposure and development.

Bright-field microscope images of the DRN/MRN at $\times 400$ magnification were digitized by a charge-coupled device (Dage-MT1, Michigan City, IN). The images were analyzed for silver grain counts (number of grains per neuron) and grain density (grains per unit area) using computer-assisted image analysis (microcomputer imaging device (MCID); Elite Software, version 7.0). A systematic randomized method of selecting and sampling neurons was employed to measure representative cells for each subject in sufficient numbers ($Q^{-}=150$ neurons per case) so as to reduce measurement error bias and allow for the estimation of the total number of labeled cells in the DRN/MRN per subject. The boundaries of the DRN and MRN were outlined on the slide by matching to the corresponding *in situ* hybridization autoradiogram. These drawn boundaries were used as guidelines and were also used to create graphical coordinates for each neuron sampled in the DRN and MRN to obtain a permanent record of the locations and measurements taken on each slide.

For each slide, the most ventral point of the fourth ventricle was used as the origin. The total DRN or

Table 1 Case demographics

Pair no.	*Age (years)*	*Sex*	*Race*	*PMI*	*pH*	*Brain toxicology*	*Axis I*	*Cause of death*	*Antemortem prescriptions*	*Mean grain counts per neuron*
1	66	Male	White	19	6.1	None	None	Myocardial infarction	None	220
1a[a]	63	Male	Hispanic	17	6.74	None	None	Hanging (S)	None	308
2	30	Female	African-American	8	6.73	None	None	Heart attack	None	466
2a[a,b]	26	Female	Hispanic	18	6.88	None	Schizoaffective, depressed type	Hanging (S)	Mood stabilizers	651
3	79	Male	White	9.75	5.96	Lidocaine/ antiarrhythmic	None	Heart attack	None	341
3a	77	Male	White	18	6.40	None	MDD	Hanging (S)	None	396
4	27	Female	White	15	6.72	None	None	MVA	None	291
4a	28	Female	White	19	6.32	None	Eating disorder, MDD	Fall from height (S)	TCA	456
5	51	Male	Hispanic	7.5	6.43	None	None	Myocardial infarction	None	941
5a	66	Male	White	11	6.27	Caffeine	None	Gun shot (S)	None	664
6	53	Male	White	18.5	NA	None	None	Myocardial infarction	None	900
6a	50	Male	Hispanic	30	6.82	None	MDD	Fall from height (S)	None	1010
7	37	Male	African-American	15	6.75	None	None	Myocardial infarction	None	438
7a	40	Male	White	20	6.77	Analgesics	MDD	Hanging (S)	SSRI	382
8	85	Male	White	7	6.22	None	None	Myocardial infarction	None	555
8a	74	Male	White	21	5.93	Opiates, analgesics, caffeine	MDD	Fall from height (S)	None	1011
9[b]	53	Male	African-American	9	NA	None	None	Industrial accident	None	87
9a	59	Male	Hispanic	24.5	6.77	None	MDD	Hanging (S)	None	148
10	58	Male	White	22	6.44	Lidocaine	None	Myocardial infarction	None	211
10a	62	Male	White	21	6.37	None	MDD	Drowning (S)	SSRI	192
Mean	54			15.9	6.48					
s.e.m.	6			1.9	0.07					

Abbreviations: MDD, major depression; MVA, motor vehicle accident; NA, not available; PMI, postmortem interval, in hours; S, suicide; SSRI, serotonin-specific reuptake inhibitor; TCA, tricyclic antidepressants.
[a]Alcohol abuse.
[b]Cigarette smoker.
All demographic information was made available by the medical examiner and psychological autopsy provided by next of kin.

MRN area of each section was sampled by moving the microscope stage at 500 μm intervals and measuring grains in all neurons in a spatially quantified upper right quadrant (75×50 μm area) of the computer monitor. Exclusion and inclusion lines were drawn as boundaries of the sampling frame. Overlapping cells were not sampled. For each neuron, grain number and grain density was measured.

Grains were measured using a fixed area target circle (3317 μm^2) placed over the cell soma. Group differences were quantified by determining the mean number of grains per neuron in the DRN and MRN using this fixed area circle. Using the image analysis software, the number of grains within the circle was estimated by dividing the total grain area by the predetermined average grain size of 0.3 μm^2. The values for grain densities for all the neurons sampled (including labeled and unlabeled neurons) were divided into fixed ranges. The number of labeled and unlabeled neurons in each range was counted and plotted in a histogram. The plotted densities displayed a bimodal distribution. Neurons were considered positively labeled when their density reading was three times over background, which was determined by extrapolating a normal distribution from the histogram.[25,26] Background grain counts were taken from five regions: adjacent pons, unlabeled neurons in the raphe, a region devoid of neurons, a section hybridized with the sense probe, and slide background without tissue. The mean measurement of the background grain density was calculated and the value was subtracted from neuron measurements of grain density to gain greater specificity and reduce variability. TPH2 silver grain density did not correlate with age, postmortem interval, pH or freezer storage time ($P > 0.05$ for all variables).

Statistical analyses

Expression along the rostrocaudal axis was determined by grouping and averaging the densities across 2.4 mm intervals for each case. Mean grain counts per neuron at each anatomical level across the rostrocaudal axis were plotted for the control group and the suicide group (see Figure 3). These data were analyzed using a linear mixed effect model. The data were log transformed. The fixed effect was the subject type, and the random effect was the pair assignment since the subjects were matched by key demographics and postmortem characteristics. In the primary analysis, the anatomical level was included as a predictor. A similar analysis was carried out with a binary predictor ('rostral DRN' and 'caudal DRN') in place of anatomical level ('rostral DRN' = the most rostral 8 mm of the DRN). The percent change at each anatomical level was also calculated using mean percent of control within each interval and plotted (see Figure 4).

Analysis of covariance and another mixed model analysis were performed on the overall mean grain density to examine the effects of age, sex and PMI on grain density. Again, pair was used as a random effect. Since the overall mean grain counts were used, there was no need to include anatomical level.

Results

Microscopic analysis of TPH2 mRNA in situ *hybridization autoradiograms*

Silver grains representing TPH2 mRNA expression were exclusively clustered over neurons in the DRN and MRN (Figure 1). Smaller neurons and glia tended to have fewer or no grains and approximated background levels. Sections assayed with the sense probe resulted in background levels of silver grains throughout the entire tissue section, demonstrating the specificity of the antisense probe. The mean number of neurons counted per anatomical level was not significantly different between the groups (Figure 2). The coefficient of error was the same for both the

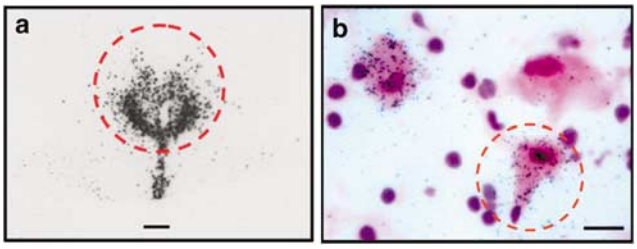

Figure 1 (**a**) *In situ* hybridization film autoradiogram of tryptophan hydroxylase-2 (TPH2) in the human dorsal (DRN) and median raphe nuclei (MRN), Bar = 1 mm. (**b**) High magnification ($\times 400$) photomicrograph of an emulsion-dipped section of a TPH2 *in situ* hybridization slide demonstrating silver grains specific to the neurons of the dorsal (DRN) and median raphe nuclei (MRN). An unlabeled neuron, where very few silver grains are found, is also visible above the encircled neuron, Bar = 25 μm.

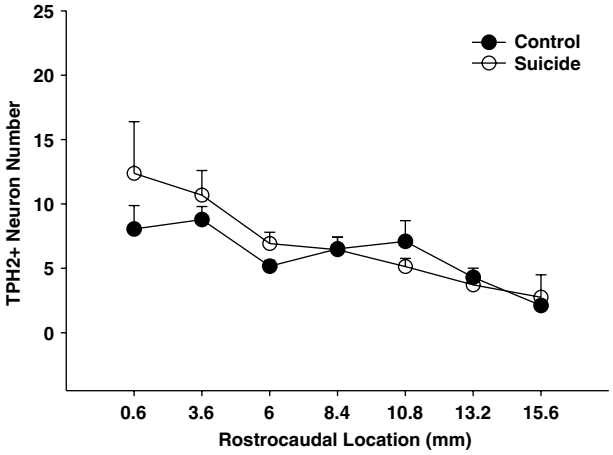

Figure 2 Mean number of neurons counted at each anatomical interval for suicides and controls. No significant difference in the number of neurons counted between suicides and controls was found ($P > 0.05$).

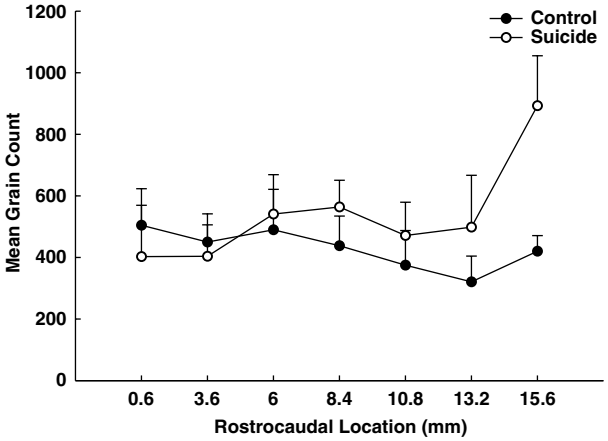

Figure 3 Mean grain count at 2.4 mm intervals along the rostrocaudal axis of the dorsal raphe nucleus (DRN). Suicides have more tryptophan hydroxylase-2 (TPH2) grains per neuron than matched controls ($P = 0.03$). The largest difference is seen at more caudal levels of the DRN.

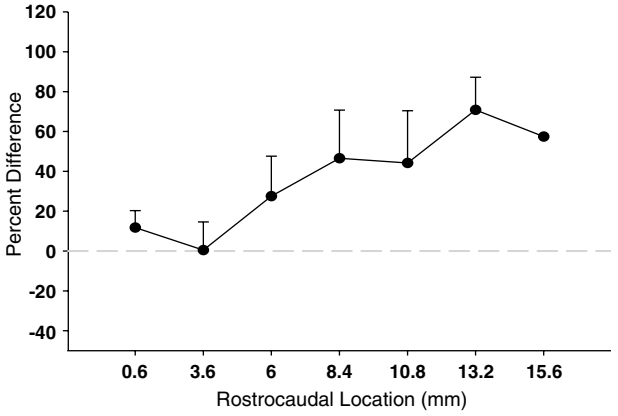

Figure 4 Mean difference in tryptophan hydroxylase-2 (TPH2) grain density between suicides and controls across the rostrocaudal axis in the dorsal raphe nucleus (DRN). Difference in expression is determined as the mean percent of control at 2.4 mm intervals across the rostrocaudal axis. The mean change in grain density per neuron is 48 % higher in suicides as compared to controls, and is present throughout the extent of the DRN and median raphe nucleus (MRN), but peaks at caudal levels ($t = 3.90$, $P = 0.008$, d.f. = 6).

control and depressed suicide group (0.22). Our previous analysis of TPH2 films from these subjects demonstrated no difference in total area or volume of the DRN/MRN between depressed suicides and controls.[13]

Since the grains were quantified using a fixed area target sampling circle placed over the soma of individual neurons (see Figure 1b), density measures per unit area are equivalent to grain density per neuron. Mean TPH2 grain density per neuron was higher in the DRN and MRN of depressed suicides as compared to matched controls (d.f. = 5.19, $P = 0.03$). The average grain density of DRN/MRN neurons throughout the DRN/MRN in depressed suicides was 26.5% greater than controls (see Table 1).

Mean TPH2 grain density did not correlate with age, or PMI as determined by regression analysis. Neither mixed model analysis nor ANCOVA, with age, sex or PMI as covariates, showed an effect of demographic variables on TPH2 expression ($P > 0.05$ for all variables).

Background density in the fixed area circle sampled inside the DRN, but not including a neuron, was higher in depressed suicides than controls (controls, 0.005 ± 0.001 vs suicides, 0.012 ± 0.003 grains per μm^2, $t = -2.34$, d.f. = 7, $P = 0.05$) This represents the elevation in TPH2 expression in neuronal processes within the DRN. The other four background counts taken outside the raphe nuclei did not differ between suicides and controls.

When the grain density per neuron was examined at 2.4 mm intervals across the rostrocaudal axis of the DRN/MRN (Figures 3 and 4), the elevation in grain density per neuron in suicides appeared to have the largest difference in caudal levels of the DRN/MRN. Therefore, we divided the DRN into rostral (0–8.4 mm) and caudal (8.4–18.0 mm) portions recognizing and respecting the anatomical organization of the nucleus. Mixed model analysis of grain density at these two anatomical levels indicated that the difference between depressed suicides and controls was present in the caudal (d.f. = 6.06, $P = 0.02$), but not in the rostral DRN ($P = 0.46$). The greatest difference was located at 13.2 mm caudal from the most rostral section of the DRN ($168 \pm 13\%$ of control).

We analyzed the individual DRN subnuclei in autoradiograms and further defined the anatomy of the difference in expression of TPH2 mRNA density in the DRN subnuclei. TPH2 expression in suicides was greater in magnitude than in controls in all five subnuclei ranging from 107 to 145% of control, but failed to reach significance ($P > 0.05$) in the caudal subnucleus.

Discussion

We have expanded on our original report of elevated TPH2 gene expression in the DRN and MRN of depressed suicides,[13] greater density of serotonergic neurons in depressed suicides[14] and greater amount of TPH protein in depressed suicides.[15] We now report that elevated TPH2 gene product in suicides is the result of transcriptional upregulation at the level of individual serotonergic neurons. It appears that depressed suicides may have more serotonergic neurons, as previously shown by immunocytochemistry[14] with higher TPH2 transcriptional capacity. We further determined that in suicides, transcriptional upregulation of TPH2-expressing neurons is more pronounced at caudal levels of the DRN and in the MRN. Specifically, the only subnucleus with increased TPH2 expression in the DRN of suicides was the caudal subnucleus. The pattern of distribution of change in grain density per neuron expression

of TPH2 in suicides across the rostrocaudal axis correlates[13] with our previous densitometric analysis of TPH2 *in situ* hybridization autoradiograms ($r = 0.96$, $P = 0.002$).

Elevated TPH2 expression in suicides does not correlate with age, sex, pH or PMI, signifying that the statistically significant differences in TPH2 grain counts between controls and suicides are due to the biology of the disorder rather than an effect of demographic variables. Although we cannot discount the affects of drug therapies prescribed before death, or the consequence of alcohol or nicotine use on TPH2 expression, we can, at least, conclude that at the time of death, peripheral and brain toxicological screens were negative, and therefore, the elevation in TPH2 expression may be a result of MDD and/or suicide.

Upregulation of TPH2 gene expression may account for higher levels of DRN TPH immunoreactivity in depressed suicides.[14,15] The original finding of greater TPH protein immunoreactivity in suicides reflected an increase in the number TPH immunoreactive neurons and also a darker reaction product in individual neurons in suicides as compared to controls. Combined, an increase in TPH2 transcription and a larger number of immunoreactive neurons in suicides both contribute to more expression and more protein. Our previous study indicates that there are more neurons expressing TPH2 transcript in depressed suicides compared with non-psychiatric controls. However, since an estimate of number of neurons was not the goal of this study, we did not employ an unbiased counting approach, and thus estimation of the total number of neurons in the DRN was not possible in this study.

Since TPH is the rate-limiting 5-HT biosynthetic enzyme and is increased in depressed suicides, it remains to be determined why brain serotonin and cerebrospinal fluid levels of 5-hydroxyindole acetic acid levels are low in suicides and serious suicide attempters.[1,27] One possible explanation is that the TPH isoenzyme in depressed suicides is mutated and has lower catalytic activity. In fact, one functional missense variant ARG441HIS has been identified in the coding region of the TPH2 gene and found to be present in approximately 10% of a population of elder major depressives, and has not been detected in a cohort affected with bipolar disorder.[28] When expressed in PC12 cells, this genetic variant was found to decrease serotonin levels by 80% compared to wild type, demonstrating that this single nucleotide polymorphism (SNP) is a loss of function mutation. This SNP represents a rare functional polymorphism.[29–33] However, *in vitro*, it has been shown to have decreased catalytic activity, stability and solubility as compared to wild type.[34] Future studies in a large postmortem sample can assess whether elevated TPH2 expression correlates with genotype.

We found that the largest change in TPH2 expression was in caudal levels of the DRN. In mice, it has been shown that in response to swim stress, 5-HT levels decrease in the lateral septum, increase in the striatum and do not change in the cortex.[35] A retrograde labeling study determined that the DRN innervation of these serotonergic targets is regionally restricted. Interestingly, the lateral septum, in which 5-HT levels were found to decrease in response to stress, is innervated by the caudal subnucleus, at least in the rodent.[36] Elevated TPH2 expression was found throughout all DRN subnuclei in suicides, and did not appear regionally restricted. However, the larger increase at caudal levels compared to more rostral levels suggests that the caudal subnucleus, which predominates at these levels, may be critically involved. Perhaps, the caudal subnucleus is most sensitive to low 5-HT and therefore has the largest increase in TPH2 expression in response to low 5-HT. In fact, corticotrophin-releasing factor type 2 receptor agonist treatment in rats results in increased c-FOS expression in TPH immunoreactive neurons in mid- and caudal levels of the DRN.[37]

Other evidence has been found for the functional selectivity of DRN subnuclei. In ovariectomized rats, estrogen increased TPH2 expression only in mid- and caudal regions of the DRN and was correlated with decreased anxiety behaviors.[38] In stress-sensitive female monkeys, SERT mRNA was found to be decreased specifically in the caudal subnucleus.[39] Although 5-HT levels were not measured in these studies, both of these findings are consistent with a compensatory response to deficient serotonergic neurotransmission.

In rodents, acute stress increases TPH2 transcript and protein expression,[40,41] suggesting that depression and suicide-related stress may account for elevated TPH gene expression.[42] Hyperactivity of the corticotrophin-releasing hormone (CRH) and diminished functioning of the hypothalamic–pituitary–adrenal axis have been implicated in depression and suicidal behavior.[43] Future studies are necessary to explore the relationship between CRH and TPH2 elevation in depression and suicide.

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