Morphometry of Dorsal Raphe Nucleus Serotonergic Neurons in Alcoholism

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Background: Reduced serotonergic function is hypothesized in alcohol abuse and dependence. Serotonergic innervation of the cortex arises predominantly from the dorsal raphe nucleus (DRN). We sought to determine the number and morphometric characteristics of DRN serotonergic neurons postmortem in alcoholic individuals ($n = 9$; age: 16–66years; 8M:1F) compared with psychiatrically normal, nonalcoholic controls ($n = 6$; age: 17–74 years; 4M:2F).

Methods: Brainstems were collected at autopsy, fixed and cryoprotected. Alcohol dependence or abuse was determined by psychological autopsy (DSM-IV), the presence of liver fatty changes or cirrhosis and/or high blood alcohol level. Tissue was sectioned at 50 μm ($-25\,^{\circ}$C). A series of 1:10 sections was immunoreacted with antiserum to tryptophan hydroxylase (TPH), the rate-limiting enzyme in the biosynthesis of serotonin. The total number of TPH-immunoreactive (IR) DRN neurons was determined by stereology. Neuron morphometry indices were determined using a video-based imaging system attached to a microscope. We identified TPH-IR neurons every 1,000 μm in each brainstem and measured neuron area, total cross sectional neuron area, and the total area and density of immunolabeled processes.

Results: Dorsal raphe nucleus neuron number (controls: 80,386 $\pm$ 10,238; alcoholic individuals: 85,884 $\pm$ 12,478) was not different between groups but TPH-IR was greater in alcoholic individuals throughout the rostrocaudal extent of the DRN. The volume of the DRN was 66 $\pm$ 9 mm^3 in controls and 55 $\pm$ 5 mm^3 in alcoholic individuals ($p > 0.05$). The average size of DRN neurons did not differ between groups (353 $\pm$ 12 μm^2 for controls vs 360 $\pm$ 15 μm^2 for alcoholic subjects). However, the area occupied by neuron processes (area of processes/DRN area) was 2.2-fold greater in alcoholic individuals compared with controls ($p < 0.05$).

Conclusions: The increased area occupied by neuron processes in alcoholic individuals may represent sprouting and, together with greater TPH-IR, be a compensatory response to impaired serotonergic transmission or cumulative effects of alcohol on the serotonin system.

Key Words: Alcohol, Serotonin, Stereology, Morphometry, Tryptophan Hydroxylase.

SEROTONIN (5-HT) IS IMPLICATED in the regulation of alcohol preference and intake in animals (for review see Higley and Bennett, 1999; McBride and Li, 1998; Naranjo et al., 1986; Sellers et al., 1992) and humans (Gorelick and Paredes, 1992; Underwood et al., 2004; Wong et al., 2003). In animals, 5-HT, its precursor 5-hydroxytryptophan, 5-HT agonists, and 5-HT reuptake blockers inhibit ethanol drinking (Krasner et al., 1975; Lu et al., 1994; Murphy et al., 1985; Myers and Tytell, 1972; Myers and Martin, 1973; Rezvani and Grady, 1994). Specific 5-HT$_{1A}$ receptor agonists (Collins and Myers, 1987; Kostowski and Dyr, 1992; Long et al., 1996; Singh et al., 1993; Svensson et al., 1993), 5-HT$_{2A}$ receptor antagonists (Lankford and Myers, 1996; Meert and Janssen, 1991; Panocka and Massi, 1992; Svensson et al., 1993), and 5-HT$_3$ receptor antagonists (Svensson et al., 1993) reduce alcohol consumption.

Clinical studies report lower levels of cerebrospinal fluid (CSF) 5-hydroxyindoleacetic acid (5-HIAA) in alcoholic individuals (Banki, 1981; Fils-Aime et al., 1996) but not homovanillic acid (HVA) and tryptophan, indicating that the low 5-HIAA is not due to a nutritional effect or altered acid metabolite transport in and out of the CSF. Ballenger et al. (1979) found low CSF 5-HIAA, but not HVA, in abstinent alcoholic individuals compared with patients with personality disorders or to alcoholic individuals in the immediate postintoxication phase and the neuroendocrine response to a 5-HT challenge is blunted in abstinent alcoholic individuals (Krystal et al., 1996) suggesting this may be a biochemical trait. Furthermore, early onset alcoholic individuals have lower CSF 5-HIAA than late onset alcoholic individuals (Fils-Aime et al., 1996) consistent with low 5-HIAA influencing the course or onset of alco-

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holism and not a consequence of alcoholism. Fluoxetine, a serotonin reuptake inhibitor, decreases depressive symptoms in alcoholic individuals with comorbid major depression, but effects on alcohol consumption are discrepant (Cornelius et al., 1997, 2001; Kranzler et al., 1995). Several clinical trials, however, have found that 5-HT reuptake inhibitors decrease the desire to drink in a dose-dependent manner (Cornelius et al., 1995, 1997; Naranjo and Bremner, 1993).

One autoradiographic postmortem study reported that ^{3}H-imipramine binding was increased in some areas of the hippocampal formation in patients positive for alcohol at the time of death, compared with those without alcohol (Gross-Isseroff and Biegon, 1988). Less serotonin transporter binding in the brainstem of alcoholic individuals has been reported using SPECT imaging (Heinz et al., 1998). Thus, despite some inconsistencies, animal studies suggest that altering serotonin activity affects alcohol consumption, and so altered serotonin function in clinical studies may contribute causally to alcoholism and not just be a consequence of alcoholism.

In nonhuman primates, serotonergic innervation of the cerebral cortex and much of the forebrain is derived from serotonin-synthesizing neurons in the dorsal raphe nucleus (DRN) and the median raphe nucleus (MRN). In the human, the DRN is a large group of neurons embedded in the ventral part of the central gray matter of caudal mesencephalon and rostral pons.

Fewer serotonergic neurons were observed in alcoholic individuals with Wernicke Encephalopathy (WE) and Wernicke–Korsakoff syndrome (WKS) compared with normal controls (Halliday et al., 1993). A study in non-WKS alcoholic individuals failed to find a difference in the total number of neurons in the alcoholic individuals though less immunoreactivity was reported (Baker et al., 1996). In support of the potential causal role for these observations, alcohol preferring rats have fewer serotonin neurons than nonpreferring strains (Zhou et al., 1994).

In the present study, we sought to determine the number and morphometric characteristics of serotonergic neurons in the DRN postmortem in alcoholic individuals compared with psychiatrically normal nonalcoholic controls.

METHODS

The respective Institutional Review Board(s) for Human Use Considerations of the New York State Psychiatric Institute and the University of Pittsburgh approved the procedures for collection and use of brain tissue.

Subject Characteristics

Control subjects ($n = 6$) died of accidental death ($n = 2$) or natural causes ($n = 4$). There was no evidence of drug abuse, neuropathology, or psychopathology based on psychological autopsies, toxicological screens and autopsy findings or data obtained by the Coroner's Office staff from relatives, friends, and other sources (Table 1). Control cases ranged in age from 17 to 74 years (39 ± 9 years) and had a male:female (M:F) ratio of 4:2. One of the controls had epilepsy. The determination of DSM-IV alcohol dependence or alcohol abuse was made using the psychological autopsy method we have previously reported and validated (Kelly and Mann, 1996). We also included 2 cases with the presence at autopsy of liver changes and a positive toxicology for ethanol ($> 0.2\%$) even if they did not make DSM-IV criteria. The alcoholic group ($n = 9$) did not differ significantly in age (16–66; 43 ± 6 years; $t = 0.78$; $p = 0.45$) from controls (17–74; 39 ± 9 years), and the M:F ratio was 8:1 (Table 1). The post-mortem interval (PMI) for all cases was 15 ± 1 hours (range 9–24 hours) and was not different between groups ($t = 1.49$; $p = 0.16$).

Tissue Collection

Brainstems were collected at autopsy. All subjects were free of gross neuropathology and had negative toxicology screens in blood, urine, and bile for psychoactive and neurotoxic drugs with the exception of alcohol in the alcoholic group.

Upon removal of the brain from the cranium, the cerebellum was removed and the brainstem separated with a transverse cut at the anterior border of the superior colliculi. The brainstem was then fixed in formalin (10%) for 2 weeks. Following initial fixation in formalin, the brainstem was manually sectioned into approximately 3 cm thick blocks. The most anterior block, from the superior colliculi to the middle cerebellar peduncles, contained the DRN in its entirety. Tissue blocks were then infiltrated for 5 to 7 days each in increasing concentrations (10–30%) of cryoprotectant sucrose in 0.1% formalin. A final 5-day infiltration in 30% sucrose was done before freezing and storing tissue at $-80\,^{\circ}$C. The anterior block, containing the DRN, was then sectioned with a sliding microtome (Microm, Model HM400, Heidelberg, Germany) and two 50 μm sections from every 1,000 μm were used for this study (one for Nissl and one for PH8 immunostaining).

Immunocytochemistry

Serotonergic neurons were identified using antibodies (provided by R. C. Cotton) to phenylalanine hydroxylase (PH8), which has substantial sequence homology to tryptophan hydroxylase (TPH) (Törk et al., 1992). The relative specificity of the PH8 antibody and cross-reactivity for tyrosine hydroxylase and TPH labeling in the raphe, ventrotegmental area, and substantia nigra has been previously addressed (Haan et al., 1987; Törk et al., 1992).

Serotonin neurons were labeled according to the method and modifications of Underwood et al. (1999). All cases were stained independently; with approximately 20 sections/case it was not possible to assay all cases simultaneously. Incubations and labeling were performed on free-floating sections. Sections were treated with Triton X-100 and incubation with the primary antibody (1:50,000 dilution) lasted 7 days to enhance penetration of the antibody and preserve morphometry. The primary antibody was labeled using the avidin–biotin peroxidase method (Hsu et al., 1981) with a Vectastain ABC kit (Vector Laboratories, Burlingame, CA). The secondary biotinylated antimouse antibody was used at a 1:200 dilution. The DAB was used at a concentration of 0.05% and stopped the reaction after 5 minutes for all cases.

Counting

The total number of TPH-IR DRN neurons was determined stereologically using a personal computer-based imaging workstation equipped for stereology (MicroBrightfield Inc., Williston, VT). The computer is attached to a microscope (Leica, model Diaplan; Wetzlar, Germany) fitted with a motorized stage (Ludl Electronic Products, Hawthorne, NY) and stage position encoder. The suggested protocol for the determination of neuronal population by

3 dimensional stereology using the fractionator method (e.g., Gundersen et al., 1988a, 1988b) was followed. The DRN boundary on each slide to be sampled was defined at low magnification (×16–×40). The contour was drawn by the image analyst with the use of the computer pointing device and the microscope motorized stage. The contour corresponded to the recognized DRN boundary (e.g., Baker et al., 1991, 1996; Törk and Hornung, 1990) capturing the vast majority of labeled neurons belonging to the DRN. Diffusely distributed neurons beyond the conventional contours of the DRN were not included.

The volume of the DRN (VDRN) was determined using the Cavalieri method. The first slide to be sampled was selected randomly from one of the first 3 slides in which the DRN first appeared and the others sampled every 1 mm thereafter in systematic random fashion. The disector began 3 μm below the cut edge of the section. Immunopositive neuron "tops" were defined at a total magnification of ×400.

Morphometry

Neuron morphometry was examined every 1 mm throughout the rostrocaudal extent of the DRN. The measurement of the size and shape of neurons was performed using a video-based, computer-assisted image analysis system (MCID, Imaging Research Inc., St. Catherine's, Ontario, Canada). The pixel dimension was calibrated using a graticule on a microscope slide to provide the linear distance in the *x* and *y* axes. All neurons with a clearly defined perimeter (e.g., not overlapping another neuron in a different focal plane) were analyzed. Each neuron was reviewed under the microscope, the video image of the microscope field was captured, and the neuron measured at an optical magnification of ×200 with an additional electronic zoom of the digitized computer image of ×4 for optimum clarity of the cell perimeter.

The morphologic measurements were computer assisted. The measurement of neuron size was (for each neuron): neuron area (μm^2). Neuron arborization density was determined using an image analysis system and CCD camera (Dage MTI, Model 72) attached to a microscope (Zeiss, Model Universal; Carl Zeiss Inc., Goettingen, Germany) to differentiate the immunolabeled neurons and neuronal processes from the unlabeled background. Sampling was performed under bright field illumination with a green filter to enhance contrast. Every other microscope field (at ×125, approximately 400 μm×400 μm) was digitized and sampled. Each image was sampled twice. The first sample measured the entire area occupied by immunolabeled product. Cell soma had a markedly greater optical density than neuronal processes, enabling a second sample to define the area of the neuron cell bodies. The process density was determined by subtracting the area of labeled neuron soma from the total area of immunolabeling as follows:

$$\text{Neuron processes} = (\text{neuron processes} + \text{cell bodies}) - (\text{cell bodies})$$

Personnel blind to the group assignment of the case did all sampling.

Data Analysis

Data are presented as mean ± SEM. Comparisons of means between the alcohol and control groups were done using 2 sample *t*-tests. This method works reliably even when the standard deviations between the groups are substantially different. The effect of age on neuron number and density was assessed using an analysis of covariance, adjusting for group (alcohol or control) and its interaction with age.

The number of cases needed and the hypothesized difference between groups (a priori) and resulting anticipated statistical power was based on our previous study (Underwood et al., 1999) examining DRN neurons in depressed suicide (nonalcoholic). In the present study of alcoholic individuals, a 2-tailed analysis was performed using a normal distribution 2-sample with unequal variance power (0.8, $p = 0.05$) calculation. The power analysis indicated a minimum group size of 5 was required.

RESULTS

Neuron Number

The number of DRN serotonergic neurons in alcoholic individuals was 85,884 ± 12,478 and not different from controls (80,386 ± 10,238; $t = -0.87$, $df = 13$, $p = 0.932$). Neither DRN volume (66 ± 9 mm^3 in controls; 55 ± 5 mm^3 in alcoholics individuals $t = 0.955$, $df = 13$, $p = 0.357$), nor length (15 ± 2 mm in controls; 18 ± 1 mm in alcoholic individuals; $t = -1.21$, $df = 13$, $p = 0.249$) differed between the 2 groups (Table 1). The number of neurons counted along the rostrocaudal extent of the DRN was examined to determine the distribution of the neurons (Fig. 1). In controls, the DRN had more neurons in the rostral portion anterior to the trochlear decussation compared with the caudal portion due to the enlargement of the DRN and the coexistence of several of the DRN subnuclei.

Neuron Morphometry

The average size of DRN neurons does not differ between groups (352 ± 12 μm^2 for controls vs 360 ± 15 μm^2 for alcoholic individuals; $t = -0.419$, $df = 13$, $p = 0.682$). However, a marked difference in the staining intensity between controls and alcoholic individuals is apparent (Fig. 2). We therefore sought to characterize the staining difference and determine whether it is due to morphologic features related to the area occupied by the processes of the neurons stained. As an index of the amount of TPH, we examined the optical density (TPH-IR) of DRN neurons. Alcoholic individuals had a 42 ± 11% greater TPH-IR than in controls ($t = -2.505$, $df = 12$, $p = 0.028$, Fig. 3). Tryptophan hydroxylase-immunoreactive did not correlate with age, severity of alcoholism/abuse, or duration of alcoholism. There was also no effect of age in either group, and there was no age:group interaction ($F = 2.945$, $p = 0.114$).

To determine the morphology of stained DRN neurons, we measured the area occupied by neuron processes. The area of the DRN occupied by neuron cell bodies is not different between controls and alcoholic individuals ($p > 0.05$). In contrast, the density of neuron processes (area of processes/DRN crosssection area) is 2.2-fold greater in the entire DRN in alcoholic individuals compared with controls ($t = -2.499$; $df = 13$, $p = 0.03$) and the difference is present throughout the rostrocaudal extent of the DRN (Fig. 4). Neuron process density does not correlate with alcohol severity rating (not shown), but increases with duration of alcoholism (Fig. 5, $r = 0.73$, $p = 0.06$).

Table 1. Comparison of Alcoholics and Controls: Demographics and Serotonin Neuron Indices

Sex[a] (M or F)	Age (y)[b]	PMI[c]	Race[d] (W:B)	Cause of death[e]	Diagnosis[f]	DRN length (mm)	DRN volume (mm^3)	Neuron number	Alcoholism duration (mo)
Controls									
M	22	24	W	Natural	None[g]	12	21	38,138	—
M	56	12.5	W	CV	None	11	77	88,756	—
M	43	17.5	B	CV	None	22	92	122,895	—
M	19	14	W	MVA	None	19	79	84,248	—
F	74	12	B	CV	None	18	63	77,168	—
F	17	19	W	MVA	None	7	64	71,113	—
Mean	39	17	4:2			15	66	80,386	—
SEM	9	2				2	9	10,238	—
Alcoholic									
M	66	16.5	W	CV	Paranoid	23	58	152,661	510
M	55	12	B	CV	None	15	57	86,602	NA[h]
M	25	12	W	MVA	None	12	24	44,145	NA
M	41	12	W	CV	None	16	52	18,418	276
F	58	9	W	Hemorrhage	None	15	65	81,011	84
M	24	9	W	Drown	Antisocial	20	55	90,748	18
M	56	21.5	W	CV	None	24	81	119,735	456
M	47	12	W	CV	Bipolar	16	40	72,024	336
M	16	9	B	MVA	ADHD	17	66	107,611	12
Mean	43	13	7:2			18	56	85,884	242
SEM	6	1				1	5	12,478	78

[a]Sex: M, male; F, female.
[b]Age: age in years, values are mean ± SEM.
[c]PMI: postmortem interval (time from death to fixation in hours).
[d]Race: B, black; W, white.
[e]Cause of death: CV, cardiovascular; MVA, motor vehicle accident; GSW, gun shot wound.
[f]Diagnosis: ADHD, attention deficit hyperactivity disorder.
[g]Epilepsy.
[h]NA: reliable data not available for 2 cases.

PMI and Comorbid Psychiatric Diagnosis

The PMI did not differ between groups ($p = 0.106$), and there was no relationship between PMI and the number of DRN neurons, DRN volume, DRN length, process density, or optical density ($p = 0.132$–0.935). The 5 alcoholic cases without additional comorbid psychiatric diagnosis were compared with the 4 cases with an additional comorbid psychiatric diagnosis for the number of DRN neurons and all other DRN morphometrics and no differences were found ($p > 0.05$).

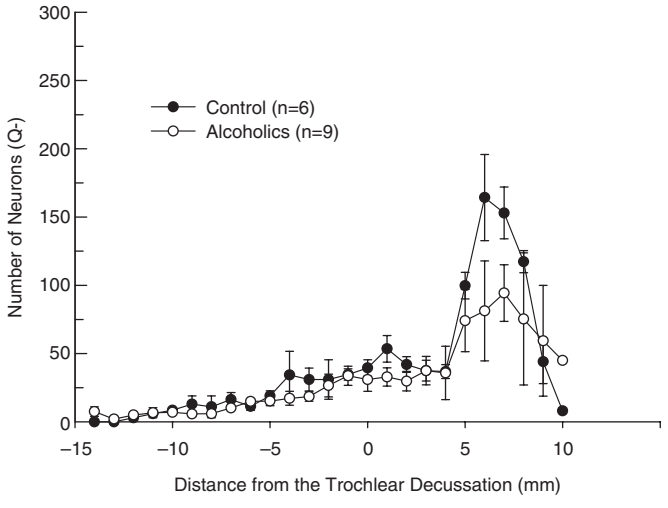

Fig. 1. Distribution of the number serotonin neurons in the dorsal raphe nucleus (DRN) in alcoholic individuals (○) and controls (●). Tryptophan hydroxylase-immunoreactive neurons were counted along the anteroposterior extent of the DRN and plotted as a function from their distance from the trochlear decussation. Rostral is to the right and caudal to the left.

DISCUSSION

In the present study, we tested the hypothesis that fewer serotonin synthesizing neurons in the DRN contribute to the decreased serotonergic function hypothesized in alcoholism. No difference was found in the number of DRN neurons, however greater TPH-IR was observed in alcoholic individuals along with a positive relationship between neuron process density and duration of alcoholism.

Our finding of no difference in the total number of DRN TPH-IR neurons is consistent with the observations of Baker et al. (1996) in non-Wernicke's alcoholic individuals, but not with the observation of fewer DRN neurons in a noncomplicated alcoholic made by Halliday et al. (1993). In our study, the alcoholic individuals as a group were observed to have more variability than the controls, particularly in the estimation of the total number of DRN neurons. The methods used could contribute to the observed differences in group variability and explain the discrepancy with Halliday et al. (1993). One possibility is that there is a bias in quantitation of the total number of neurons by counting immunostained neurons, because the

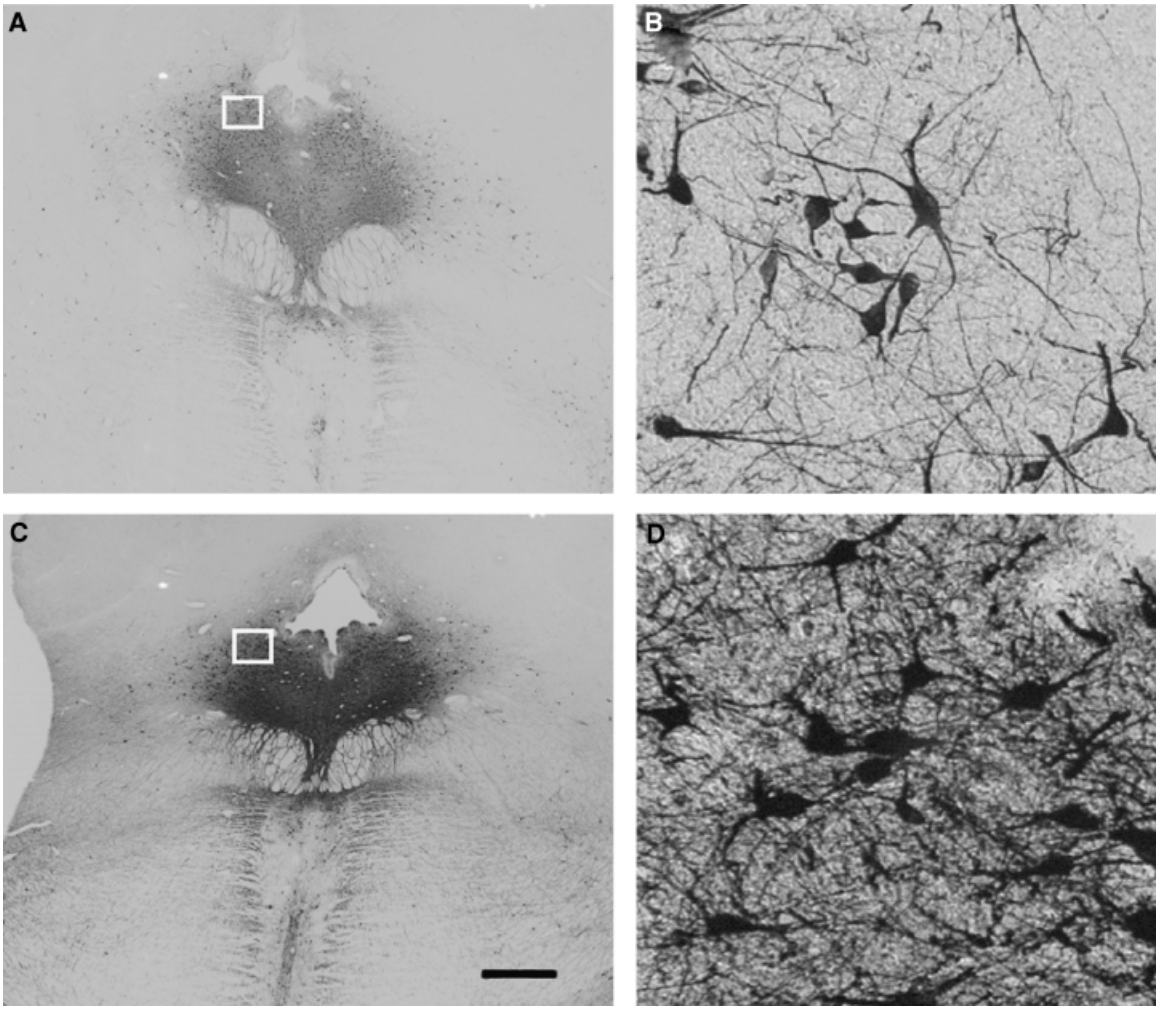

Fig. 2. Tryptohan hydroxylase immunoreactivity in the dorsal raphe nucleus (DRN) in a representative control (**A**, **B**) and alcoholic (**C**, **D**) at low (×16, A, C) and high (×400, B, D) magnification. Tryptophan hydroxylase was labeled using an antibody to phenylalanine hydroxylase, as described in the text. The high power photomicrograph was taken from the dorsal subnucleus, as indicated in (A) and (C). Note that the intensity of staining is greater in the DRN of the alcoholic subject. The increase in staining extends to neuronal processes. Scale bar represents 1 mm in the low magnification panes and 80 μm in the high-magnification panels.

staining intensity is markedly different between groups, with the alcoholic individuals having more staining. The increased staining intensity in alcoholic individuals could result in more neurons being counted than in controls, where there could be more lightly stained neurons and fewer counted as a result. Therefore, there is the possibility that the alcoholic individuals do have fewer, but more darkly stained, neurons than controls, thereby masking a difference in number. A difference in staining, or more important physiologically, an increase in the amount of TPH, will be important to distinguish from the total number of neurons in understanding the pathology and potential compensatory mechanisms taking place in the serotonergic system of alcoholic individuals. Another possible source of bias is in the anatomy of the DRN and in the stereologic method of sampling used in this study. The variable topography of the DRN in its distribution in the brainstem could also contribute to the variability. The DRN varies in size, being larger at its rostral end and narrow at its caudal end. A lack in the uniformity of the size and shape of the DRN and a heterogeneous distribution of neurons within the discrete subnuclei could therefore contribute to variability in the estimation of total number as well as to the distribution of neurons determined using stereology because of differences in sampled neurons.

In addition to the decrease in the number of DRN neurons in alcoholic individuals observed by Halliday et al. (1993), the largest decreases in neuron number were observed in the medullary and pontine serotonergic cell groups, raising the possibility that serotonergic alterations are widespread and involve other functions mediated by other brain regions. We did not examine serotonergic cell groups other than the DRN. However, the possibility that serotonin alterations exist in serotonergic nuclei other than the DRN would be of considerable importance to understanding the etiology of the alcohol-related changes.

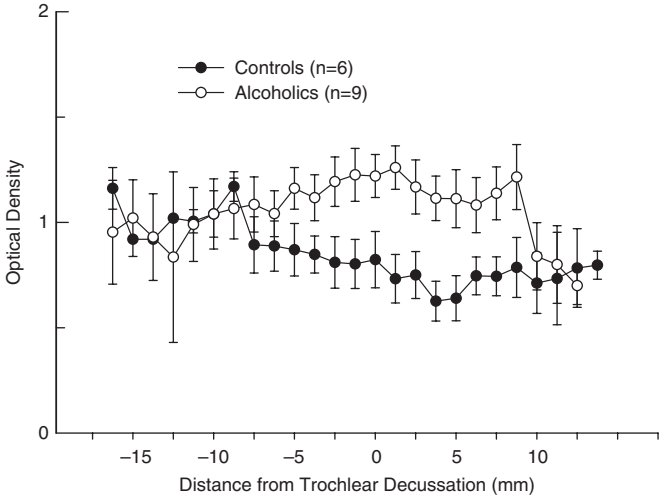

Fig. 3. Distribution of the staining intensity of serotonergic dorsal raphe nucleus (DRN) neurons in alcoholic individuals and controls. The optical density of neurons was measured and plotted along the rostrocaudal extent of the DRN. Note that alcoholic individuals (○) have a higher staining intensity of serotonergic neurons throughout most of the rostrocaudal extent of the nucleus. Values for each point are means ± SEM.

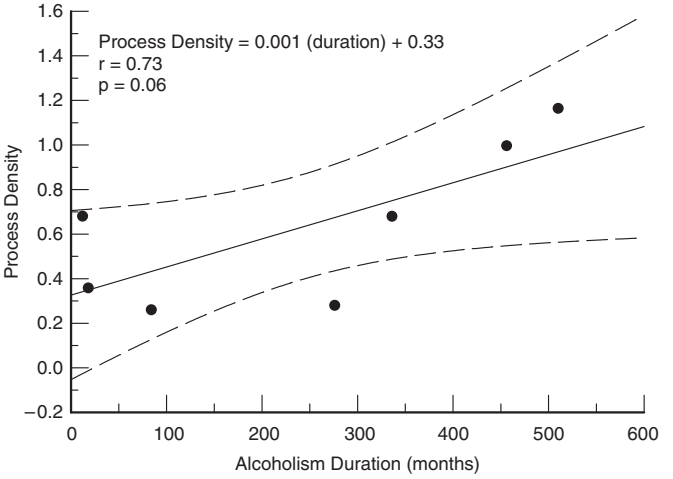

Fig. 5. Relationship between duration of alcoholism and serotonergic process density in the dorsal raphe nucleus. The duration of alcoholism was determined by psychological autopsy, data were not available for 2 cases. Process density was the mean across the rostrocaudal extent of the DRN for each subject. Note the increase in process density with increase in the duration of alcoholism.

Our finding of increased TPH-IR is consistent with the findings of Bonkale et al. (2006) in alcohol-dependent depressed suicides in the midbrain DRN dorsal subnucleus using immunoautoradiography. In contrast, Baker et al. (1996) observed less TPH-IR in the stained DRN neurons. The reason for the discrepancy is not clear. We used the same antibody used by Baker and colleagues reducing the possibility of a methodological effect. Interestingly, we, but not others (Bonkale et al., 2004) observed greater TPH-IR in DRN neurons in depressed nonalcoholic suicides (Underwood et al., 1999). We subsequently replicated this finding using an immunoautoradiography technique (Boldrini et al., 2005), raising the possibility of a state-dependent similarity between depressed suicides and alcoholic individuals, such as stress in the immediate antemortem period. Stress (Azmitia et al., 1993; Chamas et al., 2004) and dexamethasone (Azmitia et al., 1993) have been demonstrated to increase TPH in the DRN of rodents. The most frequent cause of death in the subjects of our study was an acute cardiovascular event in both the alcoholic individuals and the controls (see Table 1), reducing the likelihood of differences between groups due to death-related stressors. Such stress-related effects could be manifest differently in the phenotypically different classes of type I compared with type II alcoholic individuals. We do not have sufficient data on which to make such determinations of classification. Similarly, the different expression could be related to as yet undefined different TPH2 genotypes with different resulting TPH enzyme isoforms. However, there are no reports indicating the existence of functional TPH2 polymorphisms.

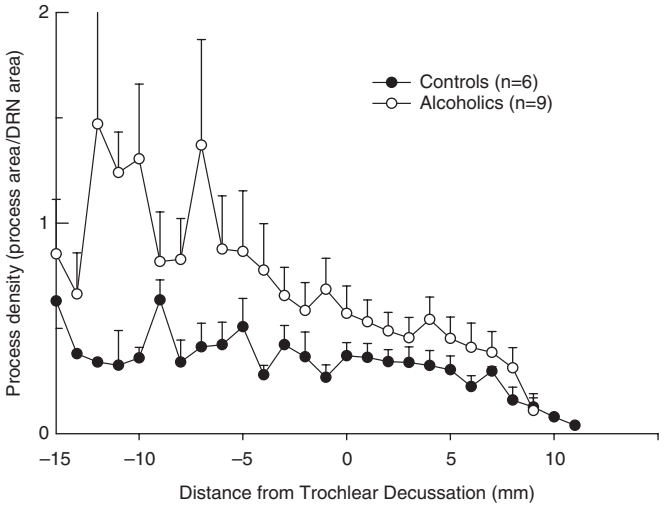

Fig. 4. Distribution of serotonergic neuron process density in the dorsal raphe nucleus (DRN) in alcoholic individuals. The density of tryptophan hydroxylase immunoreactive processes was determined by measuring the area of the DRN occupied by labeled processes. Note that the process density was greater in alcoholic individuals throughout the length of the DRN.

An increase in 5-HT immunoreactivity in the DRN and a decrease in the number of 5-HT immunoreactive DRN cell bodies has been reported (Matsushita et al., 1999) in pyrithiamine-induced thiamine deficient mice, raising the possibility that altered diet or metabolism could contribute to our findings. No other evidence of thiamine deficiency (or WKS) was found in our sample of alcoholic individuals (see ''Methods''), nor was there thiamine deficiency or WKS in the alcoholic individuals examined by Baker et al. (1996). It remains possible that the cases here could have a subclinical thiamine deficiency that could contribute the staining difference.

Suicide as a cause of death cannot explain the findings because suicide was not the cause of death in any of the alcoholic individuals. Another important difference between our observations here in alcoholic individuals and those in suicides lies in the increase in number and density of DRN neurons in suicides, but not in alcoholic individuals. Such differences indicate the likelihood of different etiologies and compensatory responses to the hypothesized 5-HT deficits in alcoholism and suicide. Likewise, the TPH-IR increase in alcoholic individuals is not likely due to changes associated with major depressive disorder (MDD) because MDD was not diagnosed in any of the alcoholic individuals; a single case was bipolar, but could not explain the differences. In addition, the alcoholic cases without additional psychiatric diagnosis were compared with the cases with an additional psychiatric diagnosis and no differences were found further suggesting that differences in the psychiatric comorbidities do not contribute to, or explain the findings. The causes and contributors to the differences will be important for understanding the different ways in which serotonin neurons may compensate for neurotransmission abnormalities.

The observation of increased TPH-IR has several implications for understanding the pathobiology of serotonin function in alcoholism. The observation suggests more TPH enzyme. Tryptophan hydroxylase is the rate-limiting enzyme in 5-HT synthesis and more TPH suggests an increase in the overall synthesis of 5-HT. However, lower CSF 5-HIAA in alcoholic individuals suggests the contrary, namely that the TPH enzyme may not be fully functional, or that the synthesized 5-HT is not released. Lower serotonin function in alcoholic individuals is not due to fewer 5-HT–synthesizing neurons or lack of TPH protein.

Neuron size was measured to determine whether differences in neuron function, as measured by TPH-IR might be reflected in the size of the neuron. We observed no difference in neuron size, but neuron process area, as a percentage of total area was increased. This may be a measure of neuron process sprouting or a loss of neuropil. The latter is more probable, because we observed greater neuron process density, but not area. Moreover, there is no evidence for alcohol promoting neuronal sprouting. In contrast, alcohol exposure in early life has been shown to inhibit and limit normal neuron arborization (Sari et al., 2001).

This study has several limitations. The first is the small sample size. The limited availability of well characterized postmortem cases is a limitation in postmortem research in general and can result in sample in-homogeneity and higher variability. For example, our study sample had a large age range. This range of age allowed for examination of the relationship between duration of alcoholism and the effect on 5-HT neurons. In the present study, effort was taken to have comparable age range, PMI, and sex ratio; as such, these variables are not likely to account for the observed differences. The number of cases needed is empirical and dependent on the anticipated effect size and standard deviation of the measurement. The findings in the present study are largely consistent with the literature and the group sizes are comparable with those used elsewhere.

Lower levels of the 5-HT metabolite 5-HIAA in the CSF are found in abstinent alcoholic individuals (Banki, 1981; Fils-Aime et al., 1996), and interestingly, early onset alcoholic individuals have lower CSF 5-HIAA than late onset alcoholic individuals (Fils-Aime et al., 1996), raising the possibility of either progressive disruption of 5-HT neurotransmission with continued alcoholism or a serotonergic dysfunction predisposing to alcohol abuse and early onset as a result.

Our study did not have sufficient numbers of cases with early onset or abstinence or Type I or Type II alcoholism to make meaningful comparisons. In nonhuman primate models, monkeys raised under adverse rearing conditions have developed lower CSF 5-HIAA and self administer alcohol more readily (Higley and Bennett, 1999; Higley et al., 1991, 1996). Alcohol preferring rats have fewer serotonin neurons than nonpreferring strains (Zhou et al., 1994). These studies, taken together, indicated reduced serotonin neurotransmission as a predisposing factor to the effects alcohol and/or a consequence of the direct actions of alcohol.

Less serotonin transporter binding in the brainstem of alcoholic individuals has been reported using SPECT imaging (Heinz et al., 1998). Given our finding of no difference in the number of neurons, it would suggest there is less transporter binding per serotonin neuron. Reduced local reuptake of 5-HT might magnify the effect of released serotonin and perhaps contribute to further reduce 5-HT neuron firing through increased 5-HT at DRN 5-HT_{1A} inhibitory autoreceptors.

While future studies are needed to elucidate these possibilities, overall they indicate a marked degree of plasticity in DRN 5-HT neurons. Taken together, greater TPH-IR in a normal number of DRN 5-HT neurons in alcoholic individuals is more consistent with a homeostatic response to serotonin depletion or deficiency and not an underlying cause of decreased serotonergic neurotransmission.

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