Research Report

Dystrophic serotonergic axons in neurodegenerative diseases

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\begin{abstract}
Neurodegenerative diseases such as Parkinson’s disease (PD), frontal lobe dementia (FLD) and diffuse Lewy-body dementia (DLBD) have diverse neuropathologic features. Here we report that serotonin fibers are dystrophic in the brains of individuals with these three diseases. In neuropathologically normal (control) brains (\textit{n}=3), serotonin axons immunoreactive (IR) with antibodies against the serotonin transporter (5-HTT) protein were widely distributed in cortex (entorhinal and dorsolateral prefrontal), hippocampus and rostral brainstem. 5-HTT-IR fibers-of-passage appeared thick, smooth, and unbranched in medial forebrain bundle, medial lemniscus and cortex white matter. The terminal branches were fine, highly branched and varicose in substantia nigra, hippocampus and cortical gray matter. In the diseased brains, however, 5-HTT-IR fibers in the forebrain were reduced in number and were frequently bulbous, splayed, tightly clustered and enlarged. Morphometric analysis revealed significant differences in the size distribution of the 5-HTT-IR profiles in dorsolateral prefrontal area between neurodegenerative diseases and controls. Our observations provide direct morphologic evidence for degeneration of human serotonergic axons in the brains of patients with neurodegenerative diseases despite the limited size (\textit{n}=3 slices for each region (3) from each brain (4), total slices was \textit{n}=36) and the lack of extensive clinical characterization of the analyzed cohort. This is the first report of dystrophic 5-HTT-IR axons in postmortem human tissue.

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\end{abstract}

1. Introduction

The anatomy and plasticity of 5-HT projecting axons in experimental animals has been extensively studied. The fine 5-HT axons were first visualized in the rat brain with histochemical fluorescence (Fuxe, 1965). Immunocytochemical analyses with antibodies raised against 5-HT later revealed the full global projections of the raphe neurons, showing also that 5-HT fibers-of-passage are thick and unbranched while the terminal fibers are fine, highly branched and varicose (Steinbusch, 1981; Azmitia and Gannon, 1983). Dense innervation is apparent throughout the neuroaxis and it has been suggested that every cell in the rat cortex is near a 5-HT-containing axon (Molliver, 1987).

It has been difficult to study the morphology of the 5-HT axonal system in the adult human brain. Postmortem autolysis and the usual methods of postmortem brain fixation result in the loss of 5-HT from axonal storage sites, which makes histochemical fluorescence and 5-HT immunocytochemistry ineffective. Antibodies raised against monoamine...
hydroxylase enzymes label 5-HT cell bodies and processes in the human brainstem, but fail to reveal the distal terminal fibers (Craven et al., 2005; Underwood et al., 1999; Baker et al., 1991; Haan et al., 1987).

A better approach may be to focus on the 5-HT transporter protein. The 5-HT transporter (5-HTT) protein is specific to 5-HT axons in the adult brain, although occasional astrocytic staining can be observed. The axonal site was visualized in experimental animals by autoradiography after uptake of 3H-5-HT by the transporter protein in situ (Descarries et al., 1975) or in vitro in fresh brain slices (Azmitia, 1981). Antibodies to 5-HTT allowed the morphology of 5-HT terminal axons to be studied immunocytochemically in brains of non-human animals (Qian et al., 1995; Zhou et al., 1996). In humans, radioactive ligands specific for the 5-HT transporter permit the gross regional distribution of 5-HT axons to be revealed in vivo by PET (Abi-Dargham et al., 1996; Szabo et al., 1996) or in postmortem tissue by film autoradiography (Varnas et al., 2004; Chinaglia et al., 1993). 5-HTT immunoreactive (IR) axons of normal morphology were seen in the postmortem brainstem and prefrontal cortex of individuals without known neurological disorders (Qian et al., 1995; Austin et al., 2002). The 5-HTT-IR axons were described as fine, highly branched and varicose, similar in appearance to those described in non-human animals.

In animal studies, serotonin axons can be damaged under a variety of conditions. Immunocytochemical analysis or immunofluorescence labeling with antibodies raised against 5-HT detects dystrophic immunoreactive fibers in rats after neurotoxic injections (Wiklund and Bjorklund, 1983); ingestion of designer-drugs of abuse (Wilson and Molliver, 1994); and in aged animals (van Luijtenaar et al., 1989). Immunocytochemistry with antibodies to 5-HTT also shows dystrophic 5-HTT immunoreactive axons after neurotoxic injections (Zhou et al., 1996). These dystrophic axons are enlarged, bulbous or splayed after acute damage to 5-HT fibers, suggesting an active degeneration process. In aging animals, 5-HT fibers may appear abnormally fine, varicose and tightly clustered suggesting atrophy and withdrawal from their forebrain targets (van Luijtenaar et al., 1980).

Despite a preponderance of pharmacological, neurochemical, and molecular evidence that the 5-HT system is disrupted in many clinical disorders, dystrophic serotonergic axons in the human brain have never been described. There is indirect evidence that 5-HT axons are damaged in neurological disorders such as FLD (Sparks and Markesbery, 1991; Menza et al., 1999); PD (Halliday et al., 1990; Marksteiner et al., 2003); Alzheimer’s disease and ischemic heart disease (Stout et al., 2003); and DLBD (Ballard et al., 2002). For example in DLBD, Lewy bodies occur in the dorsal raphe nucleus, and serotonin levels are markedly reduced in the striatum, neocortex and frontal cortex (Langlais et al., 1993; Ohara et al., 1998; Perry et al., 1993).

In this study, we obtained well-characterized postmortem neurodegenerative brains from patients with PD, FLD and DLBD, as well as from neuropathologically normal (control) individuals. In neuropathologically normal brains, 5-HTT immunocytochemistry showed typical 5-HT axonal morphology in abundant axons, including both projecting fibers and terminating regions, and only rare dystrophic fibers. By contrast, dystrophic 5-HTT-IR fibers were frequent and wide-
spread in all brains from patients with degenerative disease. Using a morphometric software system, we identified significant alterations in the number and sizes of 5-HTT-IR axonal structures in the prefrontal terminal regions of all three neurodegenerative diseases. To our knowledge, this study provides the first direct evidence that 5-HT axons in the human brain are vulnerable to degeneration in neurodegenerative states.

2. Results

2.1. 5-HTT patterns in neuropathologically normal brains

5-HTT-IR fibers were seen in all examined regions of neuropathologically normal brains. The 5-HTT-IR axons formed large, dense bundles in the rostral midbrain especially within the medial lemniscus (arrows, Fig. 1A). The 5-HTT-IR fibers-of-passage were thick, unbranched and either long and straight (possibly myelinated fibers) or wavy in appearance (Fig. 1A).

2.1.1. Ascending projections

We observed fine and varicose, or coarse and non-varicose 5-HTT-IR axons in all regions examined. The non-varicose fibers were particularly abundant in brainstem areas associated with major ascending pathways as well as in the forebrain in corpus callosum, fornix, and perforant path fibers. The 5-HTT-IR fibers in these forebrain pathways ascended into the gray terminal areas of the cortex where the fibers became thinner and more branched. In addition, a dense fiber plexus of tangential projecting fibers was found in layer I of entorhinal (Fig. 1B) and prefrontal cortical regions (Fig. 1C). These fibers were in close proximity to the pia layer and could be seen extending into layers II and III.

2.1.2. Terminal distribution

In the terminal areas within the midbrain (region of the Substantia Nigra), the 5-HTT antibody labeling resolved thin, varicose, and highly branched 5-HTT-IR axonal fibers. Distinct 5-HTT-IR boutons were visualized at the terminals of these fibers. In entorhinal and prefrontal cortices, the distribution of 5-HTT-IR fibers extended throughout all cortical layers, with extensive branching seen in deeper layers near large pyramidal neurons. The 5-HTT-IR axons were fine, highly branched and formed irregularly spaced varicosities (Figs. 1B–C). The terminals were frequently seen closely surrounding large pyramidal neurons in what can be described as pericellular plexuses.

Occasional dystrophic 5-HTT-IR axons (Fig. 2) were seen in the parahippocampal terminal regions, especially obvious in the oldest brain examined (B3573; male, 79 years of age, postmortem interval of 15.3 h). The abnormal 5-HTT axons – tight grouping of small varicosities (clustering) (Fig. 2A, arrows) and enlarged varicosities (Figs. 2B–C, arrows) – were scattered among typical fibers, especially in the hilus of the dentate gyrus (Fig. 2B) and the deep entorhinal layers (layers V–VI) (Fig. 2C) and in the CA layers of the hippocampus (not shown). However, there was no evidence in control material of advanced neurodegeneration (splayed endings or dark aggregates of stained material) or a marked reduction in fiber density.

2.2. 5-HTT patterns in the brain in neurodegenerative diseases

5-HTT-IR axons were found in all regions of the brains from individuals with any of the three neurodegenerative diseases examined. In the brainstem from these cases, 5-HTTIR axons appeared normal, although occasional abnormal fibers were

![Image](image-url)
seen. Similarly, in the corpus callosum of the forebrain, long 5-HTT-IR axons could be followed with no apparent evidence of pathology, although in the gray matter from this brain region and other cortical gray areas the number of 5-HTT-IR axons appeared markedly reduced. Significant numbers of 5-HTT-IR axons were dystrophic in shape in prefrontal and temporal cortical areas. The hippocampus and entorhinal and prefrontal cortices showed four main types of abnormal profiles: (1) enlarged, twisted and swollen varicosities, sometimes appearing as ballooned profiles; (2) fine fibers forming tight clusters; (3) isolated splayed fibers with an irregular shape; and (4) densely labeled aggregates and degenerating profiles.

2.3. **Diffuse Lewy-body dementia**

In brainstem sections from cases of DLBD, the 5-HTT-IR fibers appeared less dense than normal and occasionally the processes were abnormal in appearance and darkened (arrows, Figs. 3A–B). In both the entorhinal cortex (Figs. 3B–C) and prefrontal (Figs. 3E–F), 5-HTT-IR axons in layer I, close to the pia,
2.4. Parkinson’s disease

The axonal pathology in Parkinson’s patients was severe and seen in all brain regions examined, including the rostral midbrain

(Fig. 4). The 5-HTT-IR axonal density appeared to be reduced in all areas, especially the temporal (Figs. 4C–D) and prefrontal (Figs. 4E–F) regions. In brainstem sections from cases of PD (Fig. 4A), the 5-HTT-IR fibers appeared shorter and more fragmented than those seen in neuropathologically normal brains and frequently exhibited enlarged varicosity swellings (Fig. 4B).

In terminal brain areas, 5-HTT fibers exhibit dystrophic morphologies similar to those in DLBD. Layer I showed an apparent reduction in 5-HTT-IR fibers. Dystrophic 5-HTT-IR axons (swollen varicosities) were seen in all regions of the cortical gray matter and in the hippocampus. Many of the 5-HTT-IR axons in the cortex also terminated in the form of clusters of very fine fibers. Enlarged vesicles (Fig. 4C), splayed ending (Fig. 4D), degenerating profile (Fig. 4E), and clustering (Fig. 4F) abnormalities of 5-HTT-IR axons were found in temporal and prefrontal areas.

2.5. Frontal lobe dementia

In the brains of patients with FLD, the 5-HTT-IR axonal innervation, especially around the SN, appeared relatively normal although fiber staining was possibly stronger than in neurologically normal cases (Figs. 5A–B). Dystrophic axons were infrequent in layer 1 of entorhinal and FLD cortices but were more numerous in the deep layers of prefrontal cortex where they appeared with abnormal swollen varicosities (Figs. 5C, F) clustered (Fig. 5E), splayed and degenerating axons (Fig. 5D). These dystrophic fibers were often seen among normal looking 5-HTT-IR fibers (Figs. 5C, F).

2.6. Morphometric analysis of pathologic changes

Morphometric analyses of the prefrontal cortex region from all cases showed that 5-HTT-IR labeled profiles per unit area were fewer in each group of diseased brains than seen in the control brains (1109±141 profiles/0.5 mm², n=3). These decreases were statistically significant for the DLBD and Parkinson’s diseased brains (FLD—80% of control, n.s.; DLBD—61% of control, p<.05; PD—56% of control, p<.01) (Fig. 6A). Typical axons were distinguished from pathological axons by measurements of axon area (Fig. 6B). A histogram of the cross-sectional areas of all 5-HTT-IR profiles for each section (12,233 profiles examined) was measured and expressed as histograms. In control brains, a greater percentage of the total number of profiles (46%) was smaller (<10 μ²) than in the disease brain groups (FLD—27%, p>.05; DLBD—36%, p<.01; PD—37%, n.s.) (Fig. 6B). Conversely, the proportions of large (30–75 μ²) irregular axonal profiles in control brains were lower (24%) compared to the diseased brains (FLD—37%, p<.01; DLBD—31%, n.s.; PD—31%, n.s.). The differences might be more dramatic and statistically significant if expressed as a ratio of large to small. Thus computer-assisted morphometric analysis confirmed that the 5-HTT-IR axons in prefrontal cortex from each group of diseased brains were statistically distinct from those labeled in the neuropathologically normal brains.

3. Discussion

Our results support previous reports on the immunocytochemical detection of fine 5-HTT axons in human postmortem tissue (Austin et al., 2002; Qian et al., 1995). The 5-HTT-IR
axons in postmortem brain sections from individuals without a diagnosed neurological or psychiatric disease were similar to those described previously in humans and animal studies. We detected extensive neuropathology of 5-HTT-IR axons, however, in brains of patients with Parkinson’s disease, frontal lobe dementia or diffuse Lewy-body dementia. This is the first report of 5-HT axonal pathology in humans and shows that the 5-HT system in the human brain is vulnerable to degeneration in various neurodegenerative disorders, as shown previously in animal models of disease.

3.1. Serotonin system and neurodegeneration

Although numerous pathologies in late-age onset neurodegenerative diseases have been identified with markers of specific pathologies (e.g. β-amyloid, ubiquitin, hyperphosphorylated-tau or α-synuclein reactive), there are relatively few examples of a transmitter-specific neuronal degeneration in neurodegenerative diseases (see reports on acetylcholine: Bossy-Wetzel et al. (2004), Perry et al. (1993) and norepinephrine: Haglund et al. (2006)). Even in Parkinson’s diseases, we could not find a description of dystrophic dopaminergic fibers (see Dickson et al. (1994)) although the normal distribution of projections from dopaminergic neurons to the human caudate has been described (Kung et al., 1998).

We now report that serotonin fibers, immunocytochemically labeled with antibody against the 5-HTT, show extensive and wide-spread pathology in the brains of patients with PD, FLD or DLBD. In animal studies, serotonin fibers degenerate when exposed to a variety of environmental and neurotoxic factors (e.g. van Luijtelaar et al. (1989)—aging; O’Hearn et al. (1988)—MDMA and related drugs; Zhou et al. (1994)—alcohol; Liu and Nakamura (2006), Aucoin et al. (2005)—amyloid; Baumgarten and Bjorklund (1976), Frankfurt and Azmitia (1984)—5,7-DHT). Our findings demonstrate that human serotonin axons are also vulnerable to degeneration in pathological states.

3.2. Comparison of axonal pathology

Depletion of normal fibers and appearance of degenerating profiles were evident in all three groups of brains from patients with degenerative diseases. There was no evidence that gender or postmortem interval contributed to these observations. Recent unpublished work using 5-HTT immunostaining (n = 22 brains from neurologically typical normal subjects, range 32–85 years, average age 57.2 years and n = 3 female) showed no evidence of the frequent severe 5-HTT-specific axonal pathology reported here in the brains from patients with neurodegenerative diseases. The 5-HTT-IR dystrophic axonal profiles in this report are similar in appearance to those described after systemic administration of neurotoxin (Baumgarten and Bjorklund, 1976) and designer-drug (Molliver and Molliver, 1990) induced degeneration of 5-HT fibers in rat brain. The dystrophic axons seen in the deep layers of FLD prefrontal cortex exhibited increased caliber, reduced branching, and swollen varicosities and resembled those seen after 5,7-DHT neurotoxin intracerebral injections into the 5-HT fibers-of-passage in MFB (Frankfurt and Azmitia, 1984) or cingulum bundle (Zhou and Azmitia, 1986). The 5-HTT fibers in DLBD and PD brains show degenerating fibers characterized by varicose swelling and clustering of fine terminals. This pattern of degeneration is seen in aged rats (van Luijtelaar et al., 1989) and S100B knockout animals (unpublished observation). The dystrophic axonal pattern suggests that these terminal fibers may be retracting from a region in which levels of trophic factor are reduced.

3.3. Possible relevance of 5-HTT axon degeneration to symptoms of neurodegenerative diseases

Dystrophic degeneration of serotonergic axons may contribute to the development of many of the symptoms of neurodegenerative diseases such as mood, motor, sensory, autonomic, cognitive, and sleep disorders (see Sandyk and Fisher (1988)). In PD patients, selective 5-HT reuptake inhibitors (SSRIs) improve bradykinesia treated with L-dopa (Rampello et al., 2002). In our study, six of nine patients with neurodegenerative disease had histories of late-life depressive symptoms. Depression is believed to be a risk factor for the onset of Alzheimer’s disease (Green et al., 2003; Chen et al., 1999; Kokmen et al., 1991; Kral and Emery, 1989). Affective disorders are often comorbid with neurodegenerative disorders that are associated with dementia (Allen and Burns, 1995; Schreizner et al., 2005; Lauterbach et al., 2004). Affective symptoms are frequently part of the initial presentation in neurodegenerative diseases (Ishihara and Brayne, 2006; Kessing and Andersen, 2004), and, in some cases, may be the dominant presenting symptoms (Ballard et al., 2002). A history of major depression, without specification

<table>
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<tr>
<th>Brain #</th>
<th>Diagnosis</th>
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<th>Depression</th>
<th>Antidepressant treatment</th>
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<tr>
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<tr>
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<td>75</td>
<td>23.5</td>
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<tr>
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<td>67</td>
<td>14.85</td>
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Table 1 – The diagnosis, demographic, postmortem interval (PMI) history of depression and antidepressant treatment
of episode-related cognitive impairment, appears to be a risk factor for subsequent onset of dementia (Kessing and Andersen, 2004). This is supported by early work with twins, which found that depression and psychiatric illness were risk factors for developing dementia (Wetherell et al., 1999). Mood may be improved in depressed cognitively impaired older patients by treatment with antidepressants including selective serotonin reuptake inhibitors (SSRI) even in the absence of improvement in cognitive or motor domains (Schrag, 2006; Ishihara and Brayne, 2006; Nebes et al., 2003). The actions of serotonin in the development and treatment of impairments seen in neurodegenerative diseases require further investigation (Gruden et al., 2007; Schmitt et al., 2006; Truchot et al., 2007).

3.4. Study limitations

Although our findings demonstrated consistent differences in the extent and character of 5-HT axonal pathology in the nine neurodegenerative disease cases compared to the three control cases analyzed, the small size of the sample precluded a precise matching of the cases for age and postmortem interval. Recent unpublished work using 5-HTT immunostaining now includes 22 brains from neurologically typical normals, three of which are female, age range is 32–85 and average age is 57.2. No evidence of the frequent 5-HTT-specific axonal pathology reported here in the brains from neurodegenerative diseases is seen. The occasional 5-HTT-specific axonal pathology in the temporal cortical regions of neurologically normal subjects is confirmed (unpublished observation). Moreover, in contrast to the extensive neuropathological evaluation, the limited clinical characterization of the subjects in the study precluded a complete assessment of psychiatric history and medications in all cases. Nevertheless, these initial findings on 5HT fiber pathology in neurodegenerative diseases provide a strong rationale for more extensive analyses of the relationship between cognitive or affective parameters and 5-HT pathology in a larger cohort of clinically well-characterized subjects.

4. Experimental procedures

4.1. Brains

Brains were obtained from the Harvard Brain Tissue Resource Center at McLean Hospital in Belmont, Massachusetts. We obtained 36 tissue blocks for our study from 12 brains, which met diagnostic criteria for the neurodegenerative diseases of Parkinson’s disease (PD, n=3), Frontal Lobe Dementia (FLD, n=3), and Lewy-body dementia (DLBD, n=3). The patients all met pre-mortem and postmortem criteria for the diagnosis made at Harvard Brain Tissue Resource Center. The duration of the illness or specific treatments were not available from the records for all cases in this study. A majority of the patients with a neurodegenerative disorder also had a psychiatric history of depression (6/9), and in three cases, the information was unavailable and cannot be considered negative for depressive disorder. Neuropathologically normal brains (n=3) from donors with no medical or psychiatric history of illness were studied for comparison (Table 1).

4.2. Selection of regions for study

We used anterior brainstem blocks, which contained the substantia nigra and red nucleus, and the fiber tracts of the medial forebrain bundle, dorsal raphe cortical tract and the medial lemniscus. The temporal block we used contained the hippocampus (subiculum, CA fields and dentate gyrus) and the entorhinal cortex and associated white matter. The frontal block we used contained the dorsolateral prefrontal cortex (Brodmann layer 9) with all six cortical layers and a significant amount of the corpus callosum. We examined all layers of entorhinal and prefrontal cortices. In the hippocampus, we looked at the hilus of the dentate gyrus and the stratum radiatum of CA3 (paying particular note to the mossy fibers) and stratum radiatum of CA1. We also examined the fibers-of-the perforant path connecting the entorhinal cortex to CA1 and molecular layer of the dentate gyrus.

4.3. Orientation

We developed a method to produce a full section dark-field montage (Fig. 7) using a prototype condenser (Leitz Microscopic Company, Germany) for viewing with a 1.6× objective. Pictures were taken with a Kodak-6.0 million pixel camera (DCL 760). The montage of the various brain regions required between 6–25 images to assemble and was used to establish precise locations within the brain section.

4.4. Immunocytochemistry

Immunocytochemical studies were performed as previously described (Shiurba et al., 1998) using rabbit polyclonal antibodies raised against serotonin transporter (synthetic peptide
from the carboxy-terminus (aa 602–622: G9LKERIKSTPETP-TEIPC) of the cloned rat serotonin transporter (AB177L, Calbiochem.). This site-specific antibody has been demonstrated to be highly selective for the 5-HTT proteins in immunocytochemistry and western analysis (Zhou et al., 1996; Qian et al., 1995). Dilutions of antibody were calculated to be in the linear range of staining intensity (1/1000–1/10,000 for 5-HTT). Brain tissue used for immunocytochemical analyses was immersion-fixed in cold 10% phosphate-buffered formalin (0.15 mol/l), pH 7.4. Immunocytochemistry was performed by the avidin-biotin complex (ABC) method using Vectastain kits (Vector Labs, Inc., Burlingame, CA). Free-floating vibratome 50 μm sections were treated for 30 min with 3% methanolic hydrogen peroxide to block non-specific endogenous peroxidase activity and rinsed in 0.05 M Tris-buffered (pH 7.4) saline (TBS) containing 0.4% Triton X-100. The sections were treated for 30 min with 20% normal rabbit serum (NRS) to reduce non-specific background staining. Sections were then incubated in TBS with 1% NRS and 0.4% Triton X-100 with appropriate dilutions of primary antisera (AB-1, 1/5000) overnight at room temperature. The tissue was incubated first in biotinylated-secondary antibody (1:200 dilution) and subsequently in preformed ABC (90/d/10 ml avidin and 90/d/ml biotin). The final reaction was achieved by treating the sections with 0.02% hydrogen peroxide and DAB (0.5 mg/ml) in 0.1 M TBS, pH 7.4, for 5 min. Vibratome sections were mounted on gel-coated slides and air-dried following any immunocytochemical or histological procedures. All sections were dehydrated in a series of ethanol to xylene and coverslipped with Permount. Immunocytochemical controls consisted of either incubating tissue in non-immune sera or omitting incubation in primary antisera.

4.5. Morphometric methods

Non-stereologic, morphometric measures of particle number and area were performed on each brain (n=3) for statistical analysis. Stereologic methods were not used because of the limited number of brain sections available, and the high density of 5-HTT-IR axons. The sections were viewed with a Leitz orthoplan microscope with Kohler illumination and photographed with a Nikon DCL760 digital camera for the analysis of 5-HTT-IR axonal number and area. Morphometric analysis was performed with the UTHSCSA Image Tool for windows (version 3.00) using pictures taken with a 25× PLFluotar Objective with 8× column magnification. At least three pictures were randomly taken in the prefrontal cortical layers (II–VI) for each section from all groups. The photographs were untouched except for conversion into Grayscale before performing a threshold selection for illumination density measures of 5-HTT-IR labeling (setting of 150 on a 1–255 scale). All 5-HTT-IR labeled structures were automatically selected by the computer (10 pixel minimum cut-off) for counting, and measures of area. In the morphometric analysis of particles, normal varicosities that were assumed from the images obtained to have an area of <10 m2 profiles were estimated to have an area of between 30–75 m2. The application of a stereologic method of analysis is precluded by the unavailability of representative sections throughout the entire dorsolateral prefrontal area.

4.6. Statistical analysis

All values were transferred to Excel worksheet and averaged for each region from the four groups. To prepare histograms, the object area from each section was assigned to a bin of ascending area from 10–1500 μm2, and the percentage of objects in each bin was calculated for each section, and then averaged or each group. ANOVA followed by student’s t-test was performed and p<0.05 used to establish significance. Histograms and statistical tests were carried out using the Data Analysis System on Microsoft Office XP Excel.

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