Decreased density of NOS interneurons in the striatum of individuals with Tourette Syndrome

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This research was conducted at Yale University's Child Study Center from June 29th, 2010 to August 19th, 2010. A laboratory setting was required to do all research. Instruments used included materials used for immunohistochemistry and a microscope with a camera.

Direct supervision was present at all times, although the work was done primarily by the student. Dr. Yuko Kataoka supervised the research under the leadership and guidance of Dr. Flora Vaccarino, the Principal Investigator of the laboratory. Allyson Vermaak, a research assistant in the laboratory, helped to clarify procedures and elucidate the functions of certain chemicals when Dr. Kataoka was not present.

Introduction

Overview of Tourette Syndrome

Tourette Syndrome (TS) is a neurological disorder occurring in approximately 31-157 out of 1000 and characterized by symptoms such as involuntary movements and vocalizations called tics[1]. Usually, these tics tend to manifest themselves in childhood, around age 7.[2] Tourette Syndrome has also often been associated with premonitory urges; that is, urges to perform the tic that occur prior to it[3]. Some patients report feelings of relief after performing the tic.[4]

There are several available treatments for TS, although there is no definite cure.[4] Medications include benzodiazepines,[1] and neuroleptics, such as haloperidol and sulpiride, which function by strengthening inhibition of overactive areas.[4] The main side effect of these drugs is sedation and sometimes irritability. Treatments that do not involve drugs have been tested. Habit Reversal Training (HRT) utilizes the technique of competing motor responses.[5] Other therapies such as hypnotherapy, relaxation, and biofeedback techniques have been somewhat successful.[4] Finally, one of the last resorts for very severe TS cases is an invasive but reversible procedure known as deep brain stimulation (DBS). DBS involves stimulation of an area known as the cingulate gyrus and attempts to regulate the hyperactive areas involved in the generation of tics.[6] There is a need to better understand TS in order to provide improved treatments.

The basal ganglia

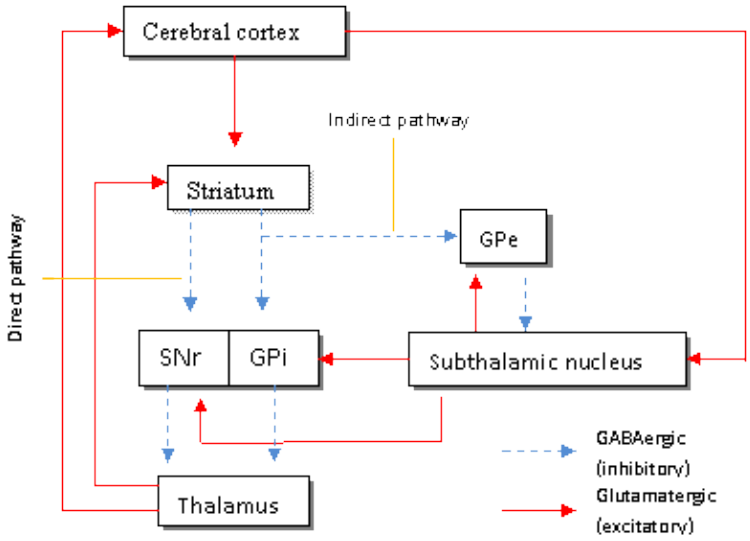

Figure 1. Schematic diagram of the basal ganglia pathways. The striatum receives input signals from areas of the cerebral cortex, integrates the information, then sends it via the direct and indirect pathways to the rest of the basal ganglia nuclei.

The human basal ganglia are crucially involved in the execution of voluntary motor tasks. This collection of nuclei (groups of neurons) located beneath the cortex has several proposed roles. One of its most important roles is its contribution to the synchrony of oscillatory activity of its neurons.[5] Synchrony is achieved when groups of neurons fire simultaneously. Lack of synchrony is believed to be important in

neurological disorders because when neurons fire completely independently of one another, dysrhythmia occurs and leads to phenomena such as tics.[5]

The basal ganglia have a few major input and output areas, where projections converge from and depart to different regions of the brain. The input areas are various parts of the cerebral cortex and the striatum.[2,4,5] Conversely, the output areas are the substantia nigra pars reticulata (SNr), globus pallidus internal (GPi), globus pallidus external (GPe),[5] and the thalamus (Figure 1).[2,4]

These input and output nuclei can be classified into direct and indirect pathways. The direct pathway is from the striatum to the SNr/GPi, then to the thalamus. The indirect pathway is from the striatum to the GPe, to the subthalamic nucleus (STN), to the SNr/GPi, then to the thalamus. The direct pathway's general effect is to inhibit output neurons, while the indirect pathway's general effect is to stimulate output neurons.[2,4,5]

The striatum

The striatum, because it is involved in integrating and processing neural information, is also a major output area of the basal ganglia. As delineated by Kataoka et al.,[7] the major anatomical landmarks of the human striatum are: caudate nucleus (Cd), putamen (Pt), internal capsule, and GPe and GPi.

The striatum has several proposed roles, some of which are relevant to TS. For example, the neostriatum (caudate and putamen only) is important in the control and execution of voluntary actions.[8]

The striatum is a convergence point for incoming signals.[9] Glutamatergic signals, involving the neurotransmitter glutamate, from the cortex and thalamus converge at the striatum with dopaminergic inputs from the midbrain. Glutamate is an excitatory neurotransmitter, while dopamine is a modulating neurotransmitter.[9]

There exist two compartments of the striatum.[10] The striosome (or patch) compartment literally consists of patches embedded within a matrix. Differences between the two compartments involve the relative concentrations of certain substances such as parvalbumin, somatostatin, and enkephalin.[7,9,11] TS has an intricate link with the striatum. Under normal conditions, most neuronal activity occurs in the matrix. This balance seems to be shifted in favor of the striosomes in individuals with TS, but the precise reason for this is not clear.[2]

Albin & Mink[2] cite multiple experiments in which the volume of the caudate nucleus, measured using brain imaging techniques, was observed to be reduced in individuals with TS. In fact, caudate volumes in childhood may be an indicator of tic severity in adolescence and adulthood in individuals with TS.[12] Smaller caudate volume seems to correlate with increased tic severity.[12]

Neurons in the striatum

There are in essence two major types of neurons in the neostriatum: projection neurons and interneurons[9]. Projection neurons are responsible for "passing on" information, while interneurons, in their broadest sense, are responsible for processing and integrating information contained in incoming signals.[9,14] Medium spiny projection neurons (MSNs) comprise over 95% of the total striatal neuron population.[9] They are the sole output in the striatum to the rest of the basal ganglia nuclei, and they are the thus the source of the direct and indirect pathways – much depends on them.[9]

Striatal interneurons, meanwhile, generally play a role in maintaining synaptic plasticity; that is, they allow synapses to adapt and change over time.[14] This study focused on striatal GABAergic interneurons, which use the neurotransmitter GABA (gamma aminobutyric acid).[9] These interneurons powerfully inhibit MSN activity by making the inside of the neuron more negatively charged than the outside, hyperpolarizing it beyond the normal membrane voltage.[15] When GABAergic neurons fire, even a single burst of an inhibitory postsynaptic potential (IPSP) can significantly pause the firing pattern of the MSNs.[8]

There are two major subtypes of GABAergic neurons: parvalbumin interneurons and NOS interneurons. Kalanithi et al.[16] found that the distribution of parvalbumin-positive (PV+) interneurons was significantly altered in the striatum of TS individuals. The researchers stained for PV in 5 control brains and 3 TS brains and calculated the density of neurons in the following regions: Cd, Pt, GPe, and GPi. Forty percent of the total PV+ interneuron population was localized in the GPi of TS samples, compared to only 15% in control samples. This enlarged proportion of GPi activity in TS samples corresponds to a diminished share of PV+ interneurons within the Cd and Pt.[16] Their finding as a whole suggests an aberrant localization of activity among TS individuals. However, the sample size used by the researchers should be increased to confirm their results. Additionally, Kataoka et al.[7] observed a statistically significant decrease in PV+ interneuron density in the striatum of TS individuals compared to control. Five TS and 5

control brains were observed. In the caudate nucleus and putamen, there were about 56% and 57% decreases in TS samples relative to control, respectively. However, in both cases, the sample sizes were also relatively small and should be augmented to confirm their results.

The second subtype of GABAergic neurons, NOS interneurons, express somatostatin (SST), neuropeptide Y (NPY), nitric oxide synthase (NOS), and NADPH diaphorase.[15] They receive both cholinergic and dopaminergic inputs from cortical areas.[15] Nitric oxide activates an enzyme called guanylyl cyclase, which then, through complex pathways, increases levels of cyclic guanosine monophosphate (cGMP). cGMP in turn is responsible for the regulation of protein kinases and ion channels. In short, NOS interneurons are implicated in the modulation of synaptic transmission.[17] Further, they are stipulated to control voluntary movement.[17] Saka et al.[18] selectively destroyed NOS interneurons and cholinergic interneurons in one hemisphere of the striatum of mice and then administered drugs that increased the activity of dopamine (DA). Rats showed increased contralateral (opposite to the side of injection) turning behavior in response to the dopamine agonists than did rats with no injection, which showed expected contralateral turning behavior. This suggests that striatal NOS and cholinergic interneurons are responsible for regulating responses to DA and therefore for controlling movement.

The goal of this study was to observe any differences in NOS interneuron density in the striatum of TS and control individuals. We hypothesized that, given the prominent role of GABAergic interneurons – more specifically, NOS interneurons – in modulating synaptic transmission, there would be a decreased density of NOS interneurons in the caudate and putamen of individuals with TS.

Methods

Brain tissue

The brain tissue used was obtained from Harvard University's brain bank before storage at Yale University. Prior to experimentation, all samples used were preserved in 4^{o} Celsius in phosphate buffered saline solution (PBS)/sodium azide (NaN_3) in six-well dishes or small compartments. Five normal control (NC) and 5 Tourette Syndrome (TS) samples were used. Each sample contained from 2-9 sections. In both the normal NC and TS conditions, the preservation times ranged from 3-12 years. The tissue was sliced by a mentor into 50 µm-thick slivers before use.

Each sliver contained cross-sections of the caudate nucleus, the putamen, some cortical tissue, and the corpus callosum. The caudate nucleus (Cd) and putamen (Pt) are visibly darker in color than the cortical tissue; therefore, their boundaries are clearly visible. The Cd and the Pt were the two regions that were analyzed.

Sources of materials

Antibodies were purchased from Millipore and Vector Laboratories. Solutions such as the Avidin-biotinylated peroxidase complex (ABC) and 3-3' diaminobenzidine (DAB) substrate kits were ordered from Vector Laboratories.

Staining procedure

The staining procedure for nitric oxide synthase (NOS) was a four-day procedure. Using fine-tipped watercolor brushes and glass rods, sections were carefully transferred into jars (all sections from a brain sample in one jar). Each jar contained approximately 100 mL of phosphate buffered saline solution (PBS)/0.1% Tween-20 (PBST). These jars were placed on a shaker for washing 3 times for 10 minutes each time. The sections were then washed together, in the same way, in 3% H_2O_2/0.1% Triton-X/PBS for 30 minutes, using about 4 mL of solution per section. Sections were incubated in 5% goat serum/0.1% Triton X/PBS for 30 minutes, using about 2.5 mL of solution per section. Finally, they were incubated in the primary antibody (anti-NOS from rabbit)/5% goat serum/0.1% Triton-X/PBS at 4^oC overnight, using about 2.5 mL of solution per section.

Sections were washed in PBST 4 times for 10 minutes each time at room temperature, using approximately 100 mL of solution per jar. They were then incubated in jars in a biotinylated secondary antibody (goat anti-rabbit)/5% goat serum/0.1% Triton-X/PBS at room temperature, using approximately 2.5 mL of solution per section. They were washed again in PBST 3 times for 10 minutes each time, using about 100 mL of solution per jar. Sections were transferred into ABC solution, which was used to amplify the antibody signal, for 60 minutes, using about 2.5 mL of solution per section. The ABC solution consisted of an "A" reagent and a "B" reagent. 1 drop of each reagent was used per section, and PBS was added to make 2.5 mL of solution per section. Sections were then washed in PBST 2 times for 10 minutes each time, then once in 50 mM Tris-HCl pH 7.5 for 10 minutes. These washes were in jars, using approximately 100 mL of solution per jar. DAB solution was prepared to react with the avidin-biotinylated peroxidase complex. DAB solution consisted of buffer stock solution and H_2O_2 (1 drop of each

per section) and DAB reagent (2 drops per section) in distilled water to give 2.5 mL of solution per section. Sections were incubated in DAB for 1 minute per section and placed in jars containing tap water on a shaker for 5 minutes to stop the DAB reaction. They were transferred to 50 mM Tris-HCl pH 7.5 then put onto microscope slides. One section fit on a single microscope slide. The slides were dried for 48 hours at room temperature.

Slides were placed onto slide holders, with 10 slides per holder, and dipped in a large jar of distilled water. Then they were transferred into large jars containing 30%, 70%, 95%, and 100% ethanol for 5 minutes each. Sections were transferred into large jars of 100% ethanol, xylene, and xylene again for 10 minutes each. Every large jar contained about 250 mL of solution. Using Permount Mounting Media, cover slips were placed on each.

Pilot study to test relative antibody efficacy

Because SST, NPY, and NOS are all expressed within one type of interneuron, three antibodies were tested to see which gave the best antibody staining. Four NC sections were used. Each of them was assigned to one of the three antibodies, and one was a negative control without the primary antibody but with all subsequent treatments. The staining procedure described above was followed. The primary antibody dilutions, determined by the manufacturer, were as follows: SST – 1:200, NPY – 1:1000, NOS – 1:4000. The secondary antibody dilutions were all 1:1000.

The relative efficacy of each respective antibody was assessed using an AxioVision-equipped microscope. The relative darkness of the staining and the clarity of the neural processes were evaluated and compared.

NOS immunostaining experiment

5 NC and 5 TS brains were used. The case numbers of the NC brains were: 98209, A07-168, A08-25, H295, and HControl. Those of the TS brains were H303, H307, H334, H298, and H339 (Table 1). All samples were taken from male individuals. Generally, the TS samples were younger at the time of death than the NC samples. Out of these 10 brains, a total of 45 sections were used. Thus, there were about 4-5 sections per brain. Every 144th section of each brain sample was taken.

Table 1. Subject information.

Cond.	Case	Age	Sex
NC	98209	59	M
NC	A07-168	65	M
NC	A08-25	50	M
NC	H295	65	M
NC	Hcontrol	47	M
TS	H303	37	M
TS	H307	34	M
TS	H334	42	M
TS	H298	54	M
TS	H339	48	M

The staining procedure described above was followed. The dilution of the NOS primary antibody was 1:4000; the secondary antibody dilution was 1:1000.

Pictures of the stained sections were taken using a 10x objective lens on an Axiovision-equipped microscope with a camera. They were assembled on Adobe Photoshop CS3. No image editing was performed. One representative picture was taken per section.

Stereological analysis

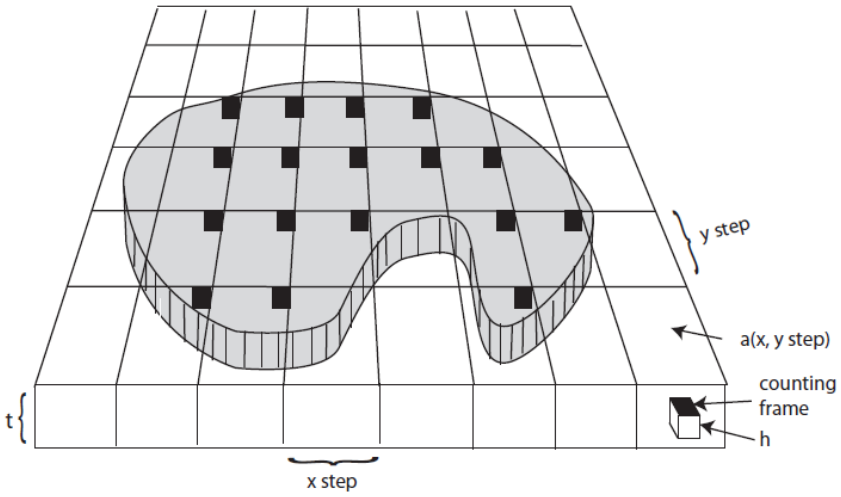

Figure 2. Diagram of the mechanisms of the Optical Fractionator probe. x and y steps represent the distances covered by one movement of the stepping motors. a(x, y step) is the area covered by one x step and one y step. The counting frames (black squares)all lie within the defined contour; each frame is of dimensions 400 µm x 300 µm x 15 µm in the x, y, and z directions. h is the depth of the counting frame. t is the measured thickness of the section.

Unbiased stereological analysis was performed using Stereo Investigator 9 software from MicroBrightfield Inc. The experimenter was unaware of the condition (NC or TS) of the tissue during counting. The caudate and the putamen were traced by the experimenter under a 2.5x objective lens using computer contour lines (Figure 2). The caudate and putamen are darker than the surrounding cortical tissue; therefore, their boundaries are clearly distinguishable. Different colored contours were used for the caudate and putamen. The software used the user-defined grid size of 2200 µm x 2200 µm to cover the area being counted. This computer-generated grid was superimposed over the section on the video monitor. The counting frame – the frame within the field of view in which cells were counted – was defined to be 400 µm x 300 µm x 15 µm in the X, Y, and Z directions. Sampling took place at each of the sites where the counting frame fell within the contour (black squares). The Optical Fractionator probe was launched to assist with the count. This probe moved the microscope stage using small motors, in the intervals x step and y step, to the locations of the sampling sites. Depending on the area enclosed by the contour, the number of total sampling sites varied from 21 to 161; larger contours contained more sampling sites than smaller contours. Under a 20x objective lens, NOS-positive cells were marked at each of the

sampling sites of all 45 sections. The thickness *t* of the tissue was also measured by the experimenter. At these sampling sites, Optical Disector was launched to measure thickness by requiring the experimenter to focus at the top, then the bottom, of the section. The top and bottom borders are clear because when either boundary is exceeded, the section appears blurry. Using the information gathered at the sampling sites and the section thickness, Stereo Investigator is able to estimate the total cell count and volume of the caudate and putamen.

The total cell count of the entire region of interest was calculated using the following formula: $N = \sum Q * \frac{t}{h} * \frac{1}{asf} * \frac{1}{ssf}$.[20] $\sum Q$ represents the total number of neurons actually counted. t is the measured section thickness of the section; h is the height of the counting frame. *asf*, the area sampling fraction, is the ratio of the counting frame's area to the area enclosed by one of the horizontal and vertical steps of the motor, a(x, y step) (shown by the area of one white rectangle). *ssf* is the section sampling fraction, which is the reciprocal of the section interval. Here, *ssf* = 1/144 because every 144th section was used.

The volume of the region of interest was computed using planimetry. The cross-sectional areas of the region enclosed by the contour was calculated, multiplied by the section interval, and then multiplied by the measured section thickness.

Statistical testing

All statistical testing was done on SPSS 17.0, on a computer running Windows XP.

Results

Testing of antibodies for visualization effectiveness

Since NOS interneurons express somatostatin and neuropeptide Y in addition to nitric oxide synthase, 3 antibodies were used to test for each of these substances. 3 TS-negative brain samples were observed for the relative efficacy of these antibodies. The evaluation involved examining the clarity of the neural processes (dendrites and axons), the intensity of the antibody staining, and the intensity of background staining. An ideal staining would comprise clear neural processes, intense antibody staining, and weak background staining. It was concluded that the NOS antibody worked best. As Figure 3 shows, the SST antibody has relatively high background staining, and the NPY antibody does not lead to clear visualization of neural processes. The NOS antibody has comparatively little background staining while maintaining clarity of the neural processes.

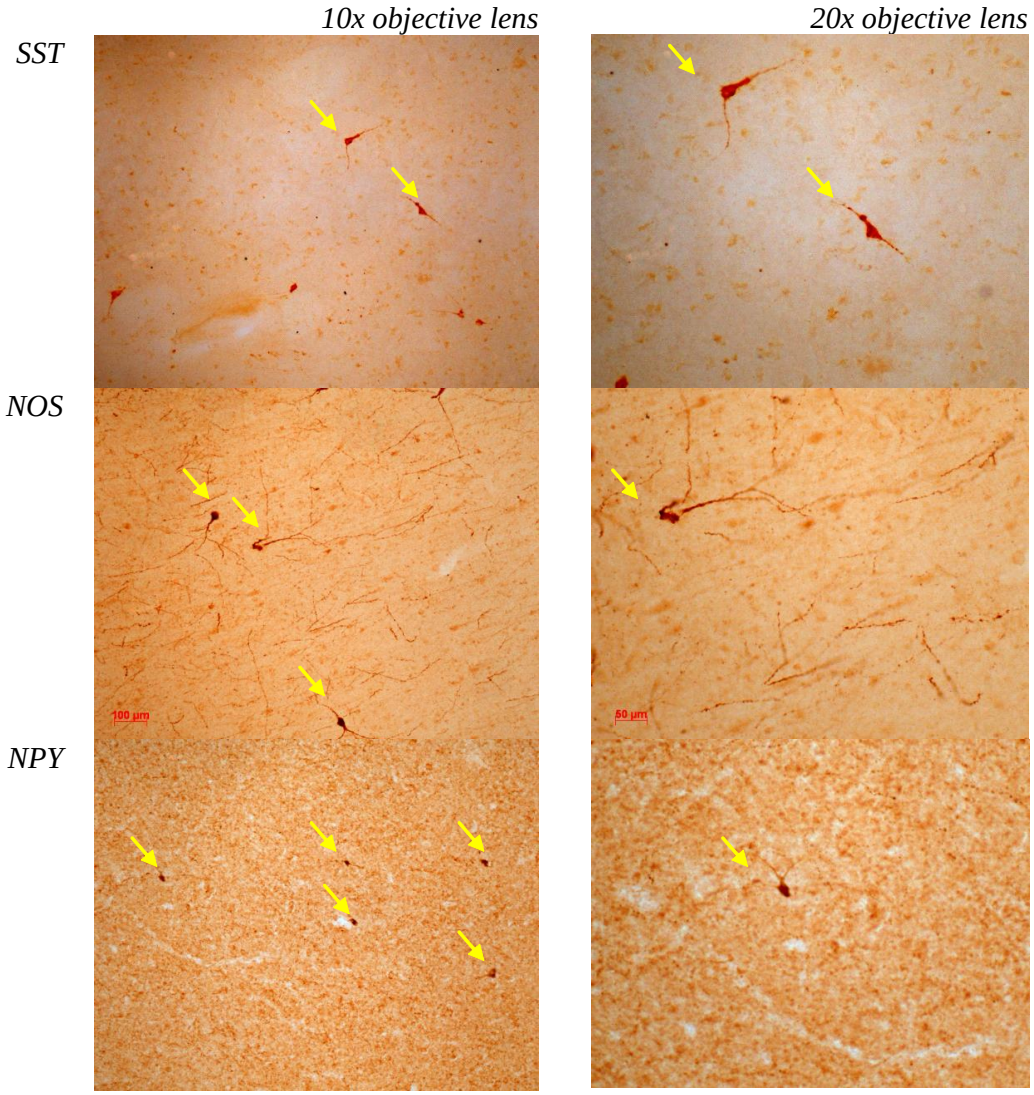

Figure 3. Qualitative evaluations of antibody efficacy under two objective lenses. Yellow arrows point to the neurons that tested positive for the antibody. SST=somatostatin. NOS=nitric oxide synthase. NPY = neuropeptide Y.

Comparison of interneuron density in control and TS cases

45 sections from 5 TS and 5 NC brain samples were stained for nitric oxide synthase using an anti-NOS antibody, a biotinylated secondary antibody, and ABC and DAB solutions. All pictures were taken with a microscope equipped with a camera and AxioVision software. These are comparable sections, in terms of their anatomical location. The sections shown are also representative – they reflect the distribution of NOS-positive cells throughout the whole striatum. In two out of three cases, there appears to be a qualitative difference between NC (normal control) and TS samples. In the sections from TS cases, NOS-positive cells appear to be more

sparsely distributed (Figure 4). As for cellular shape and morphology, there are no noticeable differences between NC and TS samples. Cells in either condition did not appear to be deformed; they exhibit distinguishable cell bodies and neural processes. Four samples are not included in the figure: HControl, H295, H298, and H339. Those sections were similar in appearance to the ones displayed.

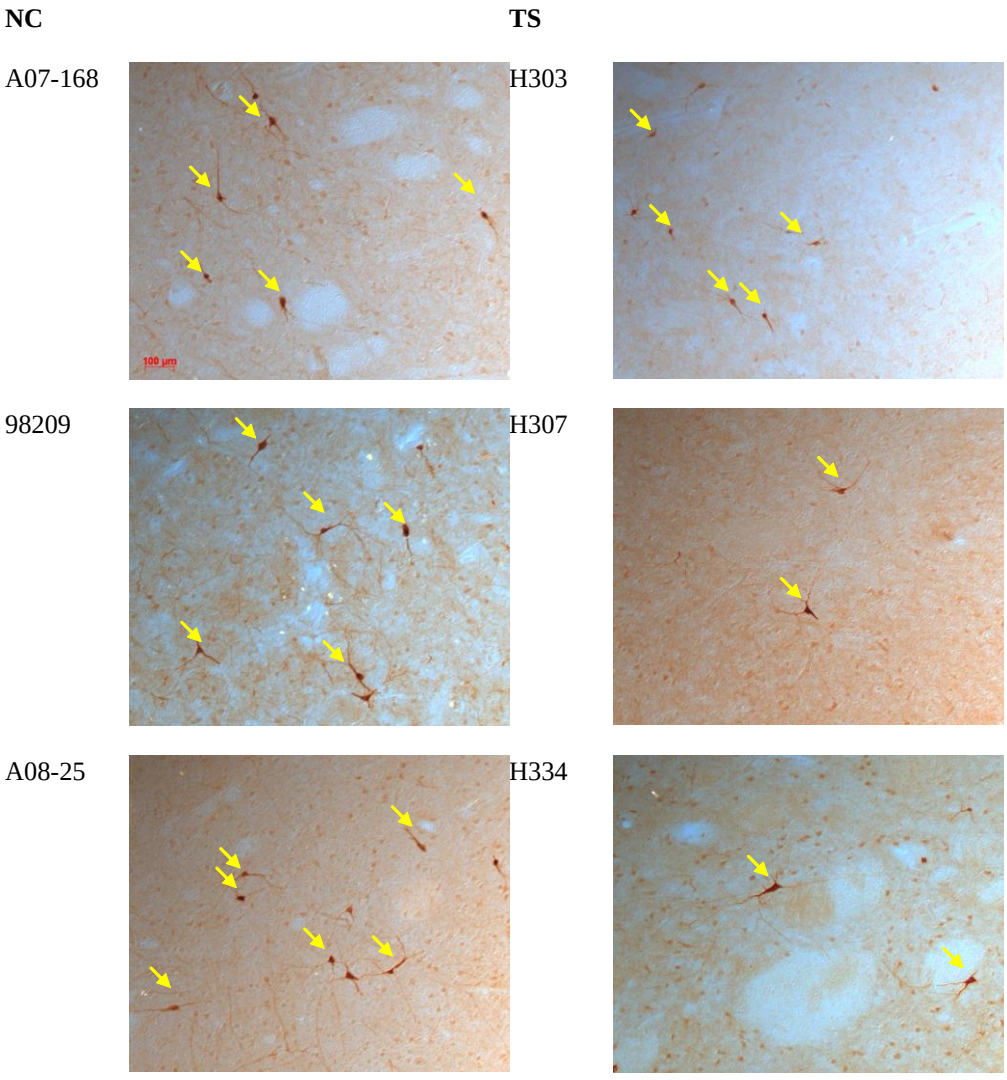

Figure 4. Pictures of comparable sections using a 10x objective lens. All pictures indicate a region within the caudate nucleus. Yellow arrows point to NOS-positive cells.

Quantitative measurements of NOS interneuron density in control and TS cases

NOS interneurons were counted in all 45 sections in both the caudate and the putamen. Stereo Investigator software was used to extrapolate an accurate estimate of cell count and section volume in the caudate and putamen based on measurements made at each sampling site.

Table 2 shows the number of sections counted and the total number of sampling sites visited for the caudate and putamen. Note that for any particular case, the number of sections does not have to be equal for the caudate and the putamen. This is because some sections were missing one of the regions.

Table 2. Total number of sections counted and total number of sampling sites visited for the caudate and putamen.

Caudate				**Putamen**			
Condition	Case	Sections	Sampling sites	Condition	Case	Sections	Sampling sites
NC	98209	4	50	NC	98209	3	69
NC	A07-168	3	58	NC	A07-168	3	60
NC	A08-25	2	50	NC	A08-25	3	80
NC	H295	3	21	NC	H295	2	42
NC	Hcontrol	3	28	NC	Hcontrol	3	85
TS	H303	4	43	TS	H303	3	117
TS	H307	4	81	TS	H307	3	76
TS	H334	4	76	TS	H334	3	65
TS	H298	9	75	TS	H298	4	58
TS	H339	7	105	TS	H339	6	161

* Some sections contained both Cd and Pt; other sections contained only one of these.

Cellular density was calculated simply by dividing cell count by volume for each of 10 cases (Table 3). There seems to be a difference in cell density between NC and TS samples both in the caudate (Cd) and putamen (Pt). There was a 45.4% and 53.2% decrease in NOS interneuron density in the Cd and Pt between the means of the five samples, respectively. The means of the densities in the caudate were 547.54 for NC and 298.81 for TS; in the putamen, they were 585.02 for NC and 273.74 for TS. The standard deviations of the data in the caudate were 146.96 for NC and 77.13 for TS; in the putamen, they were 166.30 for NC and 118.64 for TS.

In the dot graphs representing densities in the caudate and putamen, a marked decrease in NOS interneuron density in TS individuals in both regions of the brain can be seen (Figure 5). In both regions, all but one point in the TS condition were lower than the lowest point of the NC condition.

Table 3. Measured densities of NOS interneurons in NC and TS samples in the caudate and putamen.

Caudate					**Putamen**				
Cond.	Case	Cell count	Volume (μm^3)	Density (cells/mm^3)	Cond.	Case	Cell count	Volume (μm^3)	Density (cells/mm^3)
NC	98209	$2.90x10^5$	$5.89x10^{11}$	492.62	NC	98209	$2.76x10^5$	$7.30x10^{11}$	378.92
NC	A07-168	$4.12x10^5$	$7.92x10^{11}$	520.28	NC	A07-168	$4.44x10^5$	$7.28x10^{11}$	609.99
NC	A08-25	$2.12x10^5$	$5.92x10^{11}$	357.17	NC	A08-25	$5.65x10^5$	$9.68x10^{11}$	583.90
NC	H295	$1.56x10^5$	$2.54x10^{11}$	615.83	NC	H295	$2.60x10^5$	$5.03x10^{11}$	516.57
NC	Hcontrol	$2.55x10^5$	$3.39x10^{11}$	751.81	NC	Hcontrol	$8.57x10^5$	$1.03x10^{12}$	835.70
TS	H303	$1.43x10^5$	$4.79x10^{11}$	298.35	TS	H303	$4.31x10^5$	$1.38x10^{12}$	312.17
TS	H307	$1.65x10^5$	$9.33x10^{11}$	177.37	TS	H307	$9.04x10^4$	$9.11x10^{11}$	99.22
TS	H334	$2.86x10^5$	$9.26x10^{11}$	309.25	TS	H334	$2.41x10^5$	$7.83x10^{11}$	307.47
TS	H298	$3.32x10^5$	$8.49x10^{11}$	391.49	TS	H298	$2.77x10^5$	$6.61x10^{11}$	419.78
TS	H339	$3.94x10^5$	$1.24x10^{12}$	317.56	TS	H339	$4.74x10^5$	$2.06x10^{11}$	230.08

* Densities were calculated by dividing cell count by volume.

Figure 5. Dot graph of the calculated densities of NOS interneurons in the caudate (left) and putamen (right). Each small circle represents one brain sample. The horizontal bars represent the means of their respective sets.

Statistical analyses

An independent samples t-test was done for the caudate to assess whether there was a statistically significant difference between NC and TS samples ($t = 3.351$, $df = 6.048$, $p = .015$). The same statistical test was run for the putamen ($t = 3.407$, $df = 7.234$, $p = .011$). A Mann-Whitney U Test because the data cannot be assumed to be normally distributed. In both cases, $U = 1.000$, $Z = -2.402$, $p = .016$.

Discussion

The goal of the study was to provide insight into the pathology of Tourette Syndrome on a cellular level. NOS cell data was obtained through a staining process utilizing two antibodies and an avidin-biotin complex in which a primary antibody bound to the NOS antigen. A biotinylated secondary antibody then bound to the primary antibody (Figure 6). The avidin-biotin complex was added to the secondary antibody, amplifying the signal because of the affinity between avidin and biotin.[20] Finally, 3-3' diaminobenzidine was added to react with the peroxidase in the avidin-biotin complex and complete the staining process. A total of 45 brain sections from 5 control and 5 TS brain samples were analyzed with the assistance of computer software. It was observed that there were statistically significant 45.4% and 53.2% decreases in NOS interneuron density in the caudate and putamen, respectively, of individuals with TS compared to control.

One possible weakness of this study is that many TS sections were badly damaged and incomplete. The tissue used for staining was received from a brain bank and preserved in PBS/sodium azide for 3-12 years. The specific preservation durations are not known. Also, many of the TS sections were extremely fragile and ripped in the midst of the staining process. The condition of the TS tissue, therefore, started and ended up worse than that of the NC tissue in most cases. In several cases, there were missing parts of the caudate and putamen, which is why density was calculated instead of total cell count. Relying solely on cell count would tremendously misrepresent the results. Comparing cellular density, though, manages to circumvent that problem by taking into consideration the ratio of cell count to volume.

Parallels in interneuron density decreases can be seen in the findings of Kataoka et al.[7], who measured a decrease in parvalbumin and cholinergic interneuron density in the striatum of 5 TS individuals compared to controls. For parvalbumin interneurons, they noted a 60.1% decrease in the head of the caudate, and a 54.6% decrease in the body of the caudate; a 63.9% decrease in the anterior putamen, and a 43.2% decrease in the posterior putamen. A similar pattern was observed in cholinergic interneurons. The samples available were those in which TS individuals were younger in age (at time of death) than NC individuals as in this study, but using ANOVA and regression analyses, the researchers did not find age to be a factor influencing interneuron density. In addition, Kataoka et al.[7] used cresyl violet staining in order to visualize and estimate all neurons in the striatum. No significant difference in total neuron count between NC and TS individuals was found. Since the samples used for this study were similar in age and preservation conditions to those used by Kataoka et al.[7], there is reason to believe that similar results might apply. Moreover, it is important to note that the TS samples had fewer neurons and were younger in age than the NC samples. If they had been older in age than the control samples, due to possible effects of aging on neuron number, there would have been a greater concern.

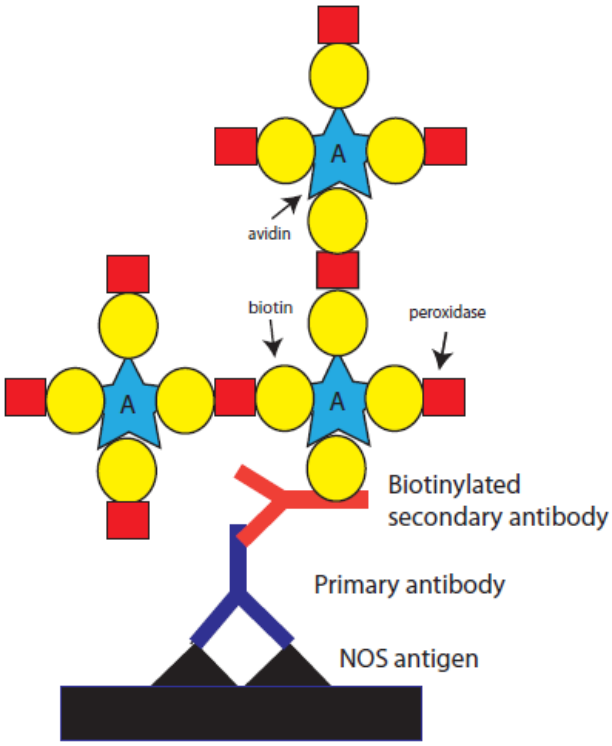

Figure 6. Schematic diagram of standard immunohistochemical procedure, modified from Bratthauer.[20]

A decrease in NOS interneuron density has several functional implications. First, all GABAergic neurons are responsible for inhibiting MSN activity.[9] GABAergic interneurons contribute to the regulation of MSNs by only allowing them to fire when a strong enough signal is received. In doing so, these interneurons provide for the modulation essential for synchrony in striatal oscillation patterns. Since the striatum is one of the major components of the basal ganglia, GABAergic interneurons are likely to contribute largely to the synchrony of the basal

ganglia as a whole. Fewer of these GABAergic interneurons can give rise to excessive firing and inappropriate behaviors such as tics, which involve unnecessary, hyperkinetic movements.[7]

Furthermore, NOS interneurons regulate the responses of MSNs to dopamine (DA).[18] An increased density of dopamine transporter (DAT) and D2 receptor was found in the frontal lobes of individuals with TS.[23] The dopamine hypothesis of TS states that in TS individuals, there is an excess of DA, an increased sensitivity to the neurotransmitter, or both.[1] The DA hypothesis is supported by the efficacy of using antagonists that block DA to treat tic symptoms[1] . Considering the role of NOS interneurons relative to dopamine, which is to regulate the release of dopamine by other cells,[1] dysfunction can result when there is an abnormal concentration of dopamine and not enough NOS interneurons present to regulate this increased concentration. In other words, there is both more dopamine (or an increased sensitivity) and fewer regulatory interneurons than normal in TS individuals, both of which compounds the effect of the dopamine and leads to disruption in neuronal circuits.

Future Research

In order to confirm these findings, more samples should be tested; these are rather preliminary results since 5 NC and 5 TS samples were used. It is critical to use more samples and samples in better condition to reproduce and corroborate these results, ensuring that they are not simply due to individual variation.

The density of NOS interneurons in the striosomes and matrix should be investigated to determine the relative balance of neuronal activity in these two regions. The study would be done using a double staining procedure, staining for NOS and enkephalin. Enkephalin can be used to distinguish between striosomes and matrix because the substance is relatively concentrated within striosomes, thus making them seem darker.[23] This study would be interesting because it is supposed that the striosomes are overly active relative to the matrix in TS individuals.[16] Therefore, it can be hypothesized that there would be fewer NOS interneurons in the striosomes in TS compared to NC. However, it is questionable as to whether there would be a significant difference in NOS interneuron density in the matrix of TS individuals compared to controls.

Next, the density of NOS interneurons in functional territories should be investigated. The striatum is essentially divided into three functional territories: sensorimotor, associative, and limbic. Although the exact boundaries of these are not clear, Kataoka et al.[7] defined them rather aptly on Stereo Investigator by using anatomical landmarks such as the globus pallidus internal

and external (GPi, GPe), and nucleus accumbens (NA). Their mapping of these functional territories would be followed. Stereo Investigator would be used to demarcate these areas and to count NOS-positive cells in each sampling site. This would be a valuable addition to the study because Kataoka et al.[7] found abnormalities in the distribution of cholinergic interneuron density in the functional territories of TS individuals. In NC individuals, cholinergic interneurons were most dense in the associative region, second in the sensorimotor, and least dense in the limbic. In TS cases, on the contrary, this gradient was completely abolished. It cannot be hypothesized yet as to which region might have the highest NOS interneuron density due to a lack of extensive research done on this type of interneuron.

Conclusion

The aim of this study was to observe any existing differences between NC and TS samples in NOS interneuron density in the neostriatum (caudate and putamen). NOS interneuron density was investigated by staining human tissue samples, using antibodies for nitric oxide synthase in 5 NC and 5 TS samples, a total of 45 brain sections. Computer software was used to estimate the NOS-positive cell count and the volume of the region being counted, which was used to calculate cell density. It was observed that there was a decreased magnitude in NOS density in TS individuals as compared to NC. In TS cases, NOS interneuron density was 45.4% lower in the caudate and 53.2% lower in the putamen compared to control. This decrease in NOS was statistically significant; it also has functional implications. The finding is promising in terms of understanding the neurobiology of Tourette Syndrome. GABAergic NOS interneurons play a major role in the striatum by coordinating the activity of MSNs, the main output neurons of the striatum. The striatum is involved in coordinating much of the activity of the basal ganglia, and the basal ganglia, finally, are largely responsible for the execution of voluntary movements. Therefore, insufficient NOS interneurons to regulate the firing of MSNS could contribute to hyperkinetic movements such as tics.

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