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Brannan O'Neill 4/20/11

Autonomic dysreflexia and plasticity in mouse sympathetic preganglionic neurons after spinal cord injury

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April 20, 2011

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a thesis submitted to the Faculty of Emory College of Arts and Sciences
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Abstract

Autonomic dysreflexia and plasticity in mouse sympathetic preganglionic neurons after spinal cord injury

By Brannan O'Neill

Autonomic dysreflexia (AD), a neurological condition associated with spinal cord injury (SCI), is associated with a loss of descending monoaminergic modulation on sympathetic output of the autonomic nervous system. Plasticity of the central nervous system and specifically the spinal cord is known to occur after injury, yet the anatomy of the mouse spinal cord post-SCI is relatively unexplored. Using a mouse model of AD, distribution of serotonergic 5HT2A receptors and dopaminergic D2 and D3 receptors were explored in control cords and following chronic injury at high thoracic levels T1 or T2. Immunochemistry showed labeling of processes adjacent to the intermediolateral nucleus (IML) for 5HT2A receptors and somatic/perisomatic labeling of D2 and D3 receptors in the IML in control tissue. Post-SCI, all receptors showed decreased expression close to the injury site (thoracic levels T2-T7) and dopaminergic receptors D2 and D3 continued to show little to no expression further from the injury site (thoracic level T9-T12). Serotonergic receptor 5HT2A showed increased expression in spinalized tissue in a rostrocaudual gradient moving away from the injury site, with greatest expression in lowest thoracic levels. Spinal cord plasticity was further demonstrated in the dorsal horn on injured spinal cords with calcitonin gene-related peptide and Substance P staining, both heavily implicated in pain perception. Presynaptic (D2, D3) and postsynaptic (5HT2A) modulation of autonomic output could mediate AD symptoms, possibly with the use of a 5HT2A agonist for treating effects due to colon distention and baclofen for spinal spasticity.

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EMORY COLLEGE DEPARTMENT OF BIOLOGY

Autonomic dysreflexia and plasticity in mouse sympathetic preganglionic neurons after spinal cord injury

Undergraduate Honors Thesis

Brannan O'Neill
Adviser: Dr. Shawn Hochman
Spring 2011

Autonomic dysreflexia (AD), a neurological condition associated with spinal cord injury (SCI), is associated with a loss of descending monoaminergic modulation on sympathetic output of the autonomic nervous system. It is characterized as a hypertensive crisis initiated by activation of peripheral pain fibers. Plasticity of the central nervous system and specifically the spinal cord is known to occur after injury, yet anatomical plasticity in the mouse spinal cord post-SCI is relatively unexplored. In rat, it is known that loss of the descending monoamine transmitters serotonin, dopamine, and norepinephrine can lead to drastic changes in their receptor function. Using a mouse model of AD, the distribution of serotonergic 5HT2A receptors and dopaminergic D2 and D3 receptors were explored in control cords, and following chronic injury at high thoracic levels (T1 or T2). I focused analysis on the intermediolateral nucleus (IML), the location of sympathetic preganglionic neurons. Immunochemistry showed labeling of processes adjacent to the IML for 5HT2A receptors and somatic/perisomatic labeling of SPNs for D2 and D3 receptors in control tissue. Post-SCI, all receptors showed decreased expression close to the injury site (thoracic levels T2-T7). D2 and D3 receptor expression decreases extended further from the injury site (thoracic level T9-T12). In contrast, serotonergic receptor 5HT2A showed progressively increased expression in spinalized tissue in a rostrocaudal gradient moving away from the injury site, with greatest expression in lowest thoracic levels. The loss of inhibitory influences via D2 and D3 receptors and concomitant increase in excitatory influence of 5HT2A receptors is consistent with changes that would increase autonomic activity, as observed in AD.

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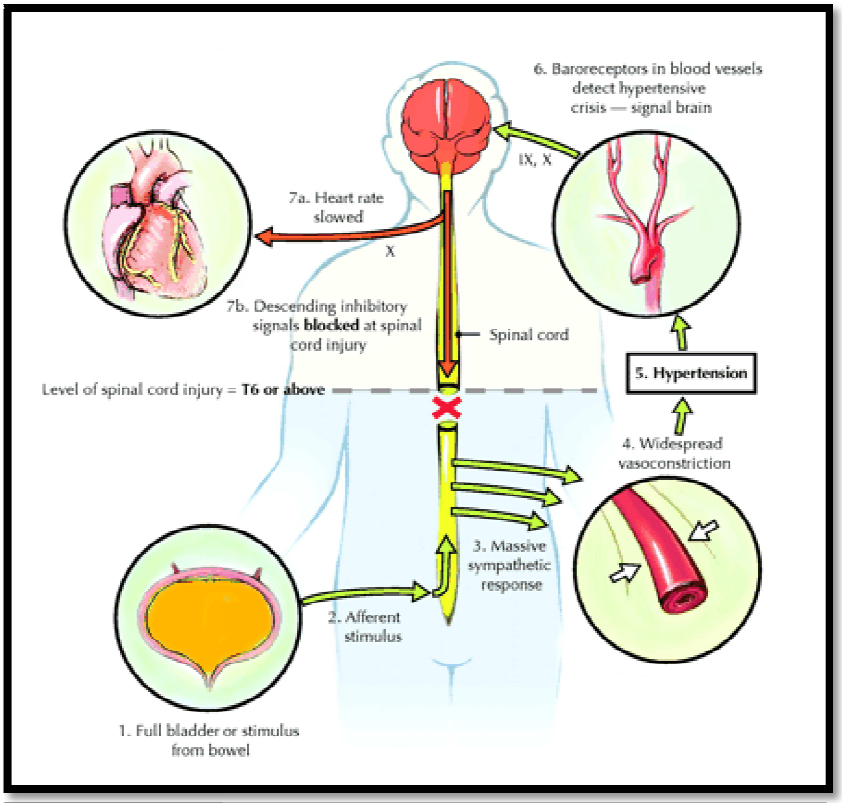
INTRODUCTION

Autonomic Dysreflexia

Autonomic dysreflexia (AD) is characterized by an extensive sympathetic discharge, often associated with spinal cord injury (SCI) or disease. The mechanism thought to trigger AD is the activation of pain-encoding, or nociceptive, afferent stimuli originating below the level of the lesion of the spinal cord[4]. These nociceptive stimuli activate the sympathetic nervous system (SNS) to elicit and perpetuate an increase in blood pressure by means of a vasoconstriction in muscle, skin, and splanchnic vascular beds. The blood pressure increase is so severe that it is potentially life-threatening[15]. AD typically occurs in patients with SCIs above the T6 thoracic spinal cord level, but can be observed in association with transections as low as T10 level (Figure 1).

Figure 1: Autonomic Dysreflexia



Noxious stimulus sends signals via afferent pathways
past lesion of spinal cord, resulting in a massive
nse and widespread vasoconstriction. Baroreceptors
on and signal brain; inhibitory signals sent by brain
at site of lesion.

The risk of developing AD is greatest with cervical spinal cord lesions[12]. The initial onset of AD can occur anywhere from a few weeks to a number of years after SCI takes place, but approximately 80% of patients develop their first episode within a year post-injury. As stated above, AD is initiated by activity in pain fibers occurring below the level of transection[16]. In a normal case, the nociceptive stimulus triggers nerve impulses to also be sent to the brain where the blood pressure increase can be controlled; with a complete SCI, the transmission of these impulses is impaired and cannot travel past the injury. The result is a spinal cord reflex to the SNS, leading to an unregulated increase in blood pressure[22]. These noxious stimuli can be anything from a bedsore to a bladder infection[33].

Autonomic dysfunction is obviously a significant source of decreased quality of life, in addition to paralysis and neuropathic pain[8]. Finding relief from these problems has been cited as a higher priority than recovering motor function in some patients[1]. However, understanding of this disease is substantially limited, and the capacity to treat these neurological consequences is accordingly restricted[28]. In order to uncover new and better ways of treating these conditions, or perhaps even prevent their development after SCI, the mechanisms by which these phenomena develop post-SCI must be explored. Specifically, a more fundamental understanding of the neurophysiological substrates responsible for these conditions and the mechanisms of post-SCI plasticity that produce these states is needed.

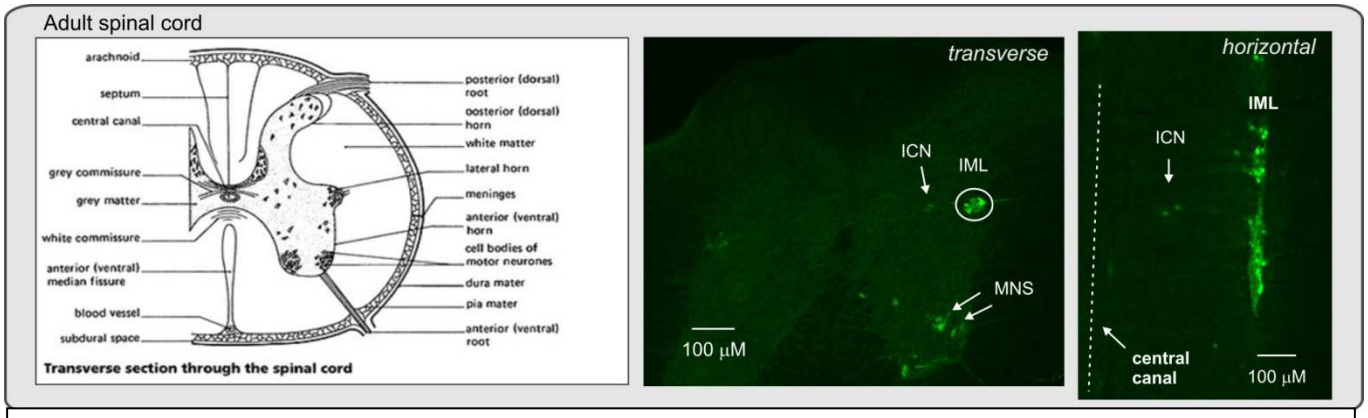
Sympathetic preganglionic neurons

While advancements have been made in regards to identifying sensory anatomical changes that would generate AD, the real focus should be concentrated on the output characteristics of the spinal sympathetic preganglionic neurons (SPNs) of the SNS that enable the expression of the disease. However, this aspect of AD remains virtually unexplored as the plasticity of the SPNs themselves post-SCI has barely been studied. Synaptology has been investigated following SCI[20], yet receptor expression and distribution has yet to be fully characterized. While many studies have explored the anatomical

changes following spinal injury in rats[7,14], knowledge of the topic as it pertains to mouse is considerably restricted. Mice show advantages over rats in their capacity for transgenic modifications as well as exhibiting an approximately 85% genetic similarity to humans[3]. I therefore sought to initiate the first studies on autonomic plasticity after SCI in mouse.

There are several anatomically distinct populations of SPNs in thoracic segments, such as the intermediolateral nucleus (IML) which contains the highest number and density of SPNs[27], and the intercalated nucleus (ICN) and central autonomic regions (Figure 2)[6].

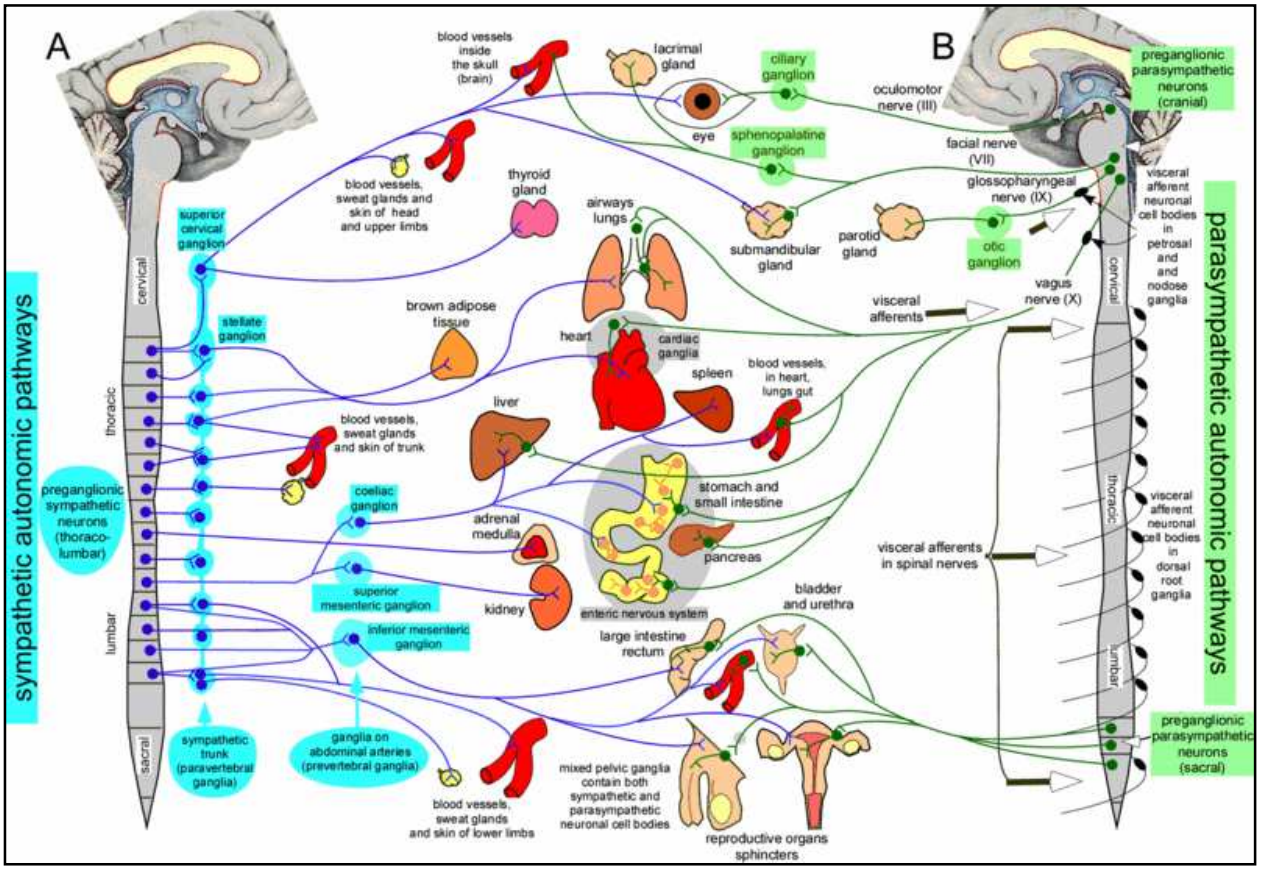
Figure 2: HBP/GFP labeling



Labeling in HB9/GFP transgenic mice; green signifies GFP+ neurons, shown here to be specific to IML, ICN, and MNs due to HB9 transcription factor which is expressed by all autonomic and somatic neurons. Left panel: cross-sectional schematic of spinal cord anatomy. Middle panel: 10x magnification of adult mouse spinal cord cross-section through thoracic segment. Right panel: 10x magnification of adult mouse spinal cord, horizontal slice through thoracic segments. (ICN: intercalated nucleus, IML: intermediolateral nucleus, MNs: motoneurons)

The rostrocaudal gradient of the spinal sympathetic nervous system features a ladder-like system of organ innervation, with thoracic SPNs projecting to visceral organs (heart, liver, lung, intestine, spleen, stomach). Because the actions of the sympathetic nervous system (SNS) and parasympathetic nervous system (PNS) are largely antagonistic, organ innervation is achieved through both (Figure 3). The SNS, when activated, elicits bronchodilation, increased heart rate/blood pressure, and decreased GI and reproductive activity. The SPNs are cholinergic, and project onto noradrenaline-releasing post-ganglionic neurons that innervate tissues.

Figure 3: Autonomic Nervous System

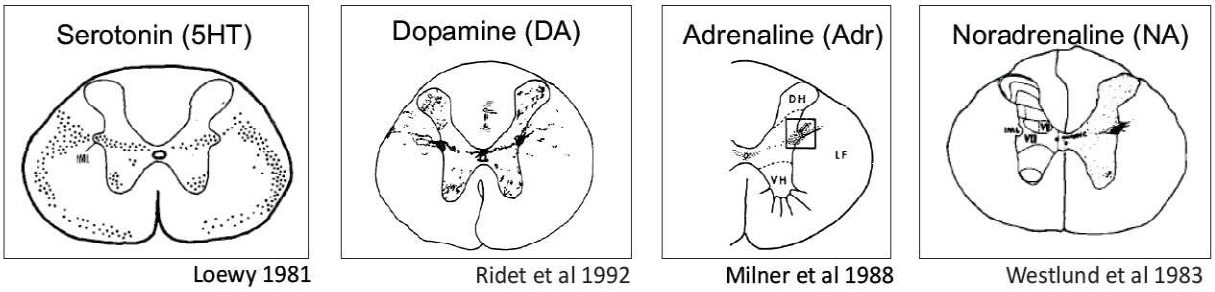


Schematic of autonomic nervous system. A. Sympathetic autonomic pathways. B. Parasympathetic autonomic pathways. Blessing, Gibbins (2008) Centre for Neuroscience, Flinders University, Australia.

Monoamines

The monoamine transmitters, namely dopamine, serotonin (5HT), and noradrenaline (norepinephrine), feature descending pathways that have been implicated in the modulation of both sympathetic and motor systems, and noradrenergic, serotonergic, and dopaminergic fibers densely innervate the IML and lamina IX, where SPNs and motoneurons (MNs) reside, respectively (Figure 4). Autonomic and somatic MNs express the common transcription factor HB9, enabling these neurons to be identified using HB9-GFP transgenic mice (Figure 2).

Figure 4: Descending Monoaminergic Pathways



Monoaminergic pathways descend through the spinal cord from the brain and project through IML region.

Spinal cord injury leads to a loss of all descending projections onto the SPNs[7], which raises interest as to the plasticity in monoaminergic receptor expression in this region. In preliminary screenings in the autonomic area, a strong distribution of serotonergic $5HT_{2A}$ and dopaminergic D_2 and D_3 receptors was observed to be notably specific for the IML. The serotonin $5HT_2$ receptors, specifically $5HT_{2B}$ and $5HT_{2C}$, have been shown to become constitutively active in spinal motoneurons after long-term spinalization[24,25], implying the receptors remain turned on even in the absence of neurotransmitters. If the same can be shown for $5HT_{2A}$ in the SPNs, then pharmacological approaches that selectively control this receptor activity could be used to alleviate symptoms. For example, as $5HT_2$ receptor activity is known to alter SPNs, the serotonergic receptor $5HT_2$ agonist dimethoxy-4-iodamphetamine (DOI) increases resting mean arterial pressure (MAP) and blocks colon-distension induced hypertension in rats exhibiting AD, while the $5HT_2$ antagonist ketanserin decreases MAP allowing for a colon-distension induced pressor response[7]. Anatomical findings of receptor expression relating to these receptors would further the use of these drugs with known responses in assuaging AD symptoms. As with changes in $5HT_2$ receptor activity mentioned, increased expression of $5HT_2$ receptors could also warrant the use of antagonists to treat the hyperexcitability observed with the AD phenotype. In contrast, decreased $5HT_2$ expression post-SCI might necessitate agonists for this receptor in an effort to block adverse effects due to colon distension.

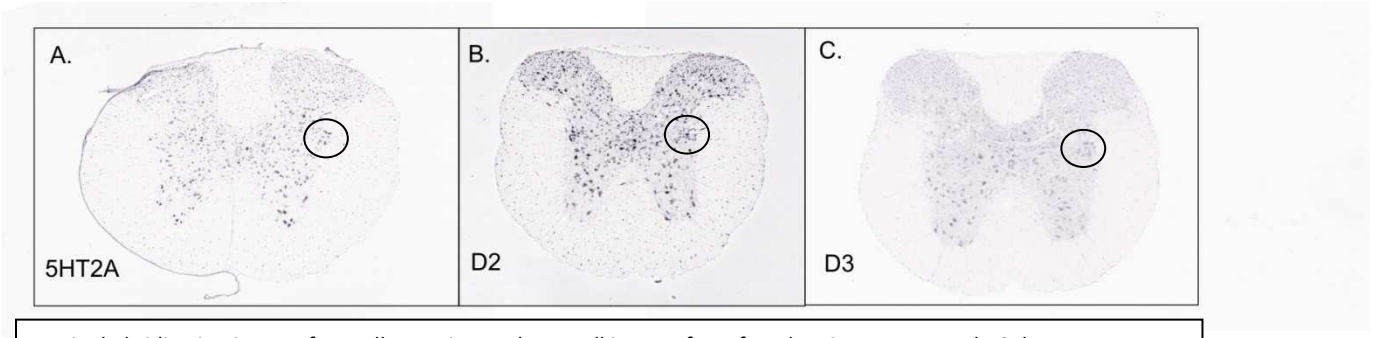
Dopaminergic receptors have long been implicated in Restless Leg Syndrome (RLS)[23]. The disorder is characterized by an overwhelming urge to move one's body to stop odd sensations analogous to burning, itching, or tickling in the muscles. An increase in sympathetic autonomic activity has been hypothesized to contribute to the genesis of this disorder[5]. Dopamine receptor agonists acting preferentially on D_2/D_3 receptors (and the dopamine precursor levadopa) are used to treat RLS. I hypothesize that D_2/D_3 receptor activation may similarly be used to treat AD neuropathology. However, this may only work if D_2/D_3 receptors are present on SPNs after injury. It is conceivable that SCI leads to a reduced expression of D_2 and D_3 receptors in the IML region in an attempt to compensate for the loss of descending dopaminergic drive, or to limit inhibitory actions as SCI individuals are normally hypotensive.

On this basis I propose that dopaminergic receptors D_2 and D_3 and serotonergic receptor $5HT_{2A}$ are of high interest in terms of elucidating possible therapies for AD. The D_2-like family of dopamine receptors, of which D_2 and D_3 both belong to, are G_i-coupled metabotropic receptors known to have inhibitory functions[30] while the $5HT_2$ receptors are G_q-coupled and known to have excitatory functions[9]. By obtaining a grasp for the anatomy and distribution of these receptors post-SCI, rational strategies for the testing of potential agonists and antagonists for AD symptoms can be considered.

To provide a preliminary knowledge on the distribution of the many monoamine receptors in the IML, the Allen database was consulted. The Allen Brain Database catalogs mRNA expression patterns using *in situ* hybridization for a variety of receptors in the mouse spinal cord, including monoaminergic receptors, at postnatal 56 days (Figure 5). Based on these findings, the dopaminergic receptors seem to have specific labeling of the neurons of interest in the IML region. The D_3 dopaminergic receptor shows a more specific labeling of autonomic and motor regions while the D_2 receptors have a more widespread distribution. This suggests that a lower side effect profile could be achieved through drugs targeting D_3 receptors. It is important to note that while the database examines

mRNA expression, the immunochemical approaches used in this project target protein expression. Therefore, an absence of expression reported by the database does not necessarily predict an absence of protein expression in the IML, as the receptors could originate from descending dopaminergic neurons that express the receptors and traffic them to axonal projections in the spinal cord. Conversely, the presence of receptor mRNA does not in and of itself indicate expected protein localization in the IML, as the receptors may selectively transport to distal dendrites or axonal terminals elsewhere. Nonetheless, observations of both protein and mRNA expression on SPN cell bodies would constitute convincing evidence of receptor localization.

Figure 5: Allen Database *In Situ* Hybridization



In situ hybridization images from Allen Brain Database. All images from female mice at postnatal 56 days, cross section of upper thoracic segments. Circles highlight expression in IML region. A. Serotonergic receptor 5HT2A expression. B. Dopaminergic receptor D2 expression. C. Dopaminergic receptor D3 expression.

Immunochemical Approach

As observed for motoneurons[2], it is anticipated that complete SCI will lead to plasticity of monoaminergic receptor function in sympathetic preganglionic neurons. This project uses immunocytochemical approaches to specifically examine the distribution of $5HT_{2A}$, D_2, and D_3 receptors and changes in their expression after complete high thoracic spinal cord transection in adult mice. Because of their greater loss of inputs and injury proximity, SPNs located closer to the injury site are expected to undergo a greater injury response and the changes in receptor density are also expected to be prominent at rostral thoracic levels nearest the transection site, specifically levels T_2-T_4[19,21]. This expectation is predicated by prior observations of a preferential loss of synaptic input to SPNs at more

rostral thoracic segments after high thoracic spinal transection[18]. Accordingly, I hypothesize that the greatest changes in receptor expression will be found closer to the injury response.

Immunohistochemistry is the process of detecting antigens in cells of a tissue section, and will be the primary method employed in this study. The technique utilizes primary antibodies that bind to specific antigens in the tissue of interest and its principal purpose is to identify the distribution and cellular localization of expressed proteins. The interaction of antigen with antibody can then be visualized using various approaches, with fluorophore-tagged secondary antibodies raised against the species generating the primary antibody. These fluorophores fluoresce to be visualized when excited with the appropriate wavelength of light.

A powerful method of immunochemistry visualization is to use confocal microscopy. This optical imaging technique increases the optical resolution and contrast of a microscoped-image. Spatial filtering techniques remove out-of-focus light, specifically through point illumination and a spatial pinhole. Only light produced by fluorescence very close to the focal plane can be detected which creates a resolution much better than that observed with conventional microscopes.

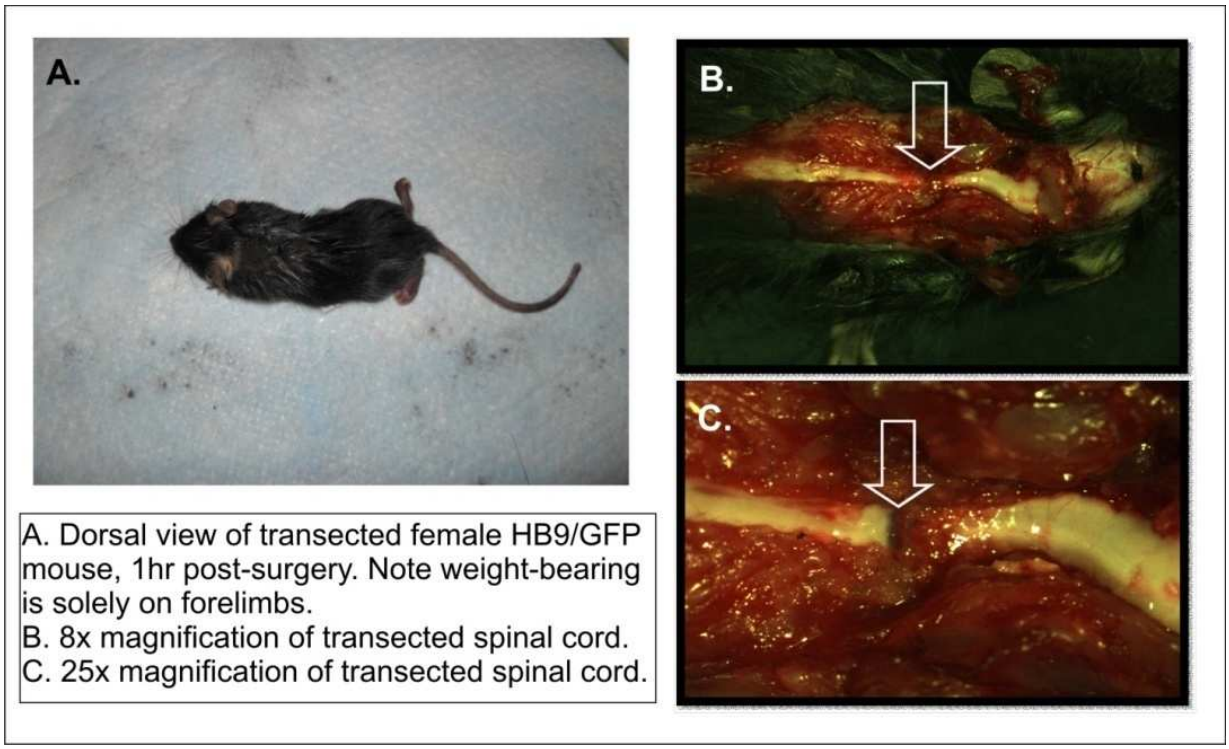
METHODOLOGY

Full compliance with the National Institutes of Health guidelines for animal care and the Emory Institutional Animal Care and Use Committee was observed. Female heterozygotic HB9/GFP mice (n=9 for this study) were obtained from the Jackson Laboratory in Bar Harbor, Maine. This mouse strain is a derivation of the C57BL/6 strain, which always undergoes AD 2 weeks post-SCI[11].

Surgical Transections

AD is most often cited in clinical reports approximately 2-3 months post-injury in humans[13,15]. In mice with T2 transections, the disease was present in 100% of the subjects 2 weeks post-injury[11]. Female adult HB9/GFP mice (postnatal approximately 56 days) were anesthetized with isoflurane

Figure 6: Surgical Transections



(NovaPlus) by inhalation. Dorsal laminectomies of the third thoracic vertebrae exposed the upper thoracic spinal cord, which were transected at T1 or T2 with surgical scissors. The lesions were packed with Gelfoam (Pfizer) and skin was sutured shut using 6-0 nylon sutures. The completeness of the transection was verified by visual inspection (Figure 6).

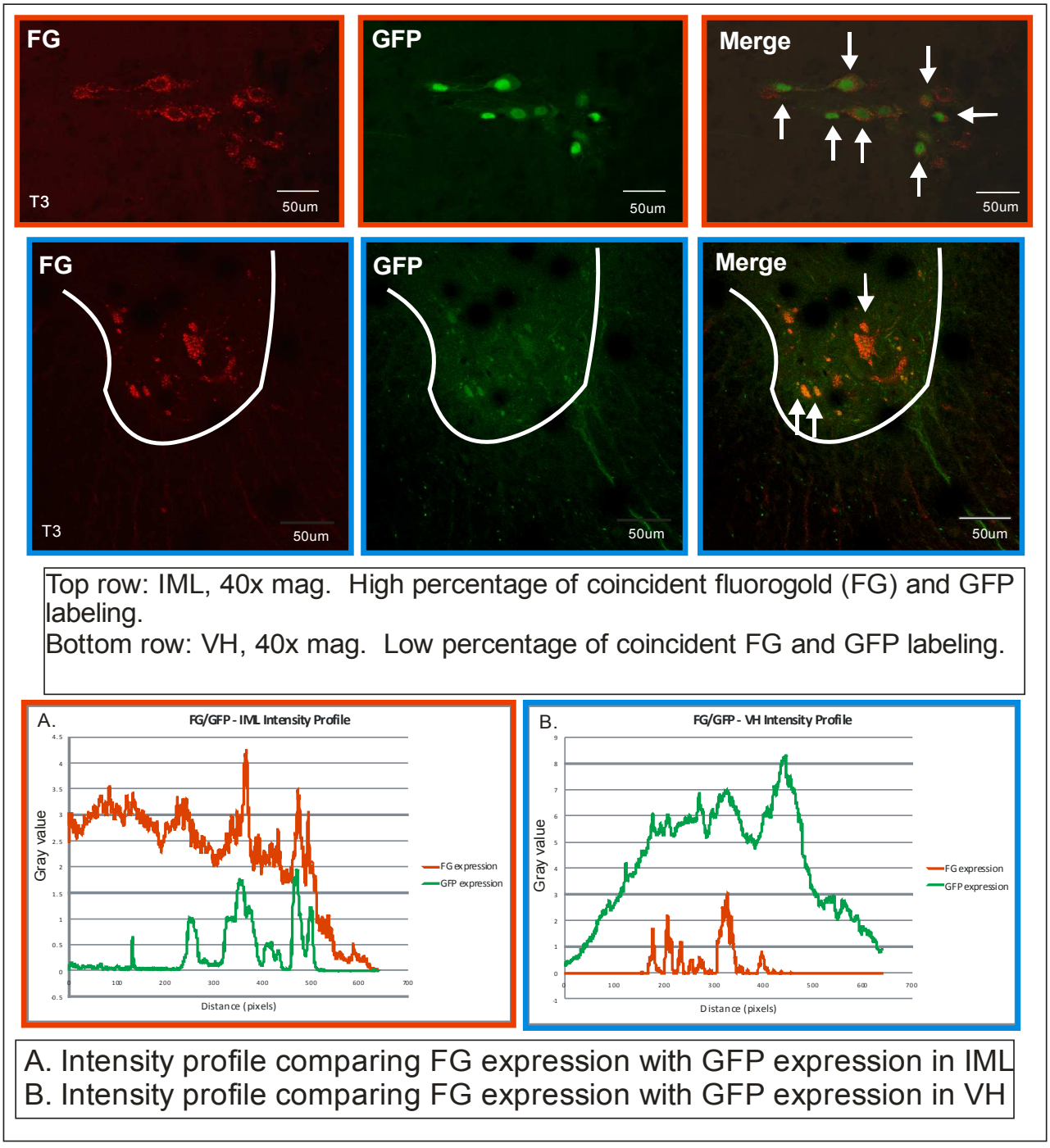
Mice were housed in separate cages following surgery and placed on heating pads to maintain body temperature for three weeks. They were given two daily injections of lactated ringer's (.5 mL, Baxter Labs) subcutaneously for two days post-surgery. Injections of buprenorphine (.05-.1mg/kg) were given subcutaneously for two days following surgery. Weight was monitored to ensure sufficient percentages of body weight were maintained and bladders were emptied twice daily by manual compression. Animals were anesthetized with urethane by a 2mg/kg intraperitoneal injection and perfusion fixed with 1:3 volume/body weight of pre-fixative (0.9% NaCl, 0.1% $NaNO_2$, 1 unit/ml heparin)

followed by equal volume/body weight of Lana's fixative (4% paraformaldehyde, 0.2% picric acid, 0.16 M PO_3; pH 6.9) 3 weeks post surgery.

Immunochemistry

Female adult HB9/GFP mice (all postnatal approximately 56 days, 3 weeks post surgery for spinalized animals) were anesthetized with urethane by a 2mg/kg intraperitoneal injection, perfusion fixed with 1:3 volume/body weight of pre-fixative (0.9% NaCl, 0.1% $NaNO_2$, 1 unit/ml heparin) followed by equal volume/body weight of Lana's fixative (4% paraformaldehyde, 0.2% picric acid, 0.16 M PO_3; pH 6.9). All spinal cords (n=8, 4 control and 4 spinalized) were isolated and post-fixed in Lana's fixative for 2 hours, then stored in 10% sucrose, 0.1M PO_3 (pH 7.4). Sectioning of the cords on a cryostat gave 10 µm thick slices. Slices were mounted on Superfrost Plus microscope slides (Fisher Scientific, Pittsburgh, PA) and stored at 4°C until immunoprocessed. All incubations and washes for immunoprocessing occurred in 0.1M PO_3-buffered saline containing 0.3% triton X-100 (PBS-T). Tissue was hydrated in PO_3-buffered saline (PBS) for approximately 4 hours. Tissue was then permeabilized overnight in PBS-T at 4°C followed by incubation in primary antibody for 72 hours (chicken anti-GFP 1:500 [Abcam, Cambridge, MA] and either rabbit anti-D2L 1:250 [Chemicon, Temecula, CA], rabbit anti-D3 1:250 [Chemicon, Temecula, CA], or rabbit anti-$5HT_{2A}$ 1:250 [ImmunoStar, Hudson, WI]). Slices were then washed three times for 30 minutes and incubated in biotin-SP-conjugated anti-rabbit secondary antibody at 1:250 (Jackson ImmunoResearch, West Grove, PA) with dylight488 anti-chicken secondary antibody (Jackson ImmunoResearch, West Grove, PA) for 1.5 hours. Slices were again washed three times for 30 minutes and incubated in Cy3 anti-rabbit conjugated secondary antibody at 1:1000 (Jackson ImmunoResearch, West Grove, PA). Slices were washed for 30 minutes in PBS-T followed by two 30 minute washes in 50mM Trizmabase hydrochloric acid (Tris-HCl). Slides were coverslipped and imaged with an Olympus FluoView 1000 confocal microscope.

Figure 7: Fluorogold/GFP Labeling



To determine the extent the HB9-GFP transgenic line labels adult SPNs, one female mouse (postnatal 56 days) was injected intraperitoneally with fluorogold (FG), 1% in saline from FluoroChrome LLC. Intraperitoneal FG is known to label all neurons with axonal projections outside the spinal cord, and the

number of HB9-GFP+ SPNs can be calculated as a percentage of FG+ neurons observed in autonomic regions. To do this, the spinal cord was isolated, sliced on a cryostat, and incubated in both anti-FG (Jackson ImmunoResearch [West Grove, PA], rabbit, 1:1000) and anti-GFP (Abcam [Cambridge, MA], chicken, 1:1000) (Figure 7). The IML region showed approximately 75% coincident labeling of FG and GFP, whereas the ventral horn (VH), the location of FG+ motoneurons, showed approximately 50% coincident labeling.

As controls, primary antibodies were pre-absorbed in the antigens they were raised against (Chemicon) to verify the selectivity of the antibody labeling. Primary antibodies were also omitted with each round of staining as a form of control to verify that the secondary antibodies produced no labeling in and of themselves.

Microscopy and Analysis

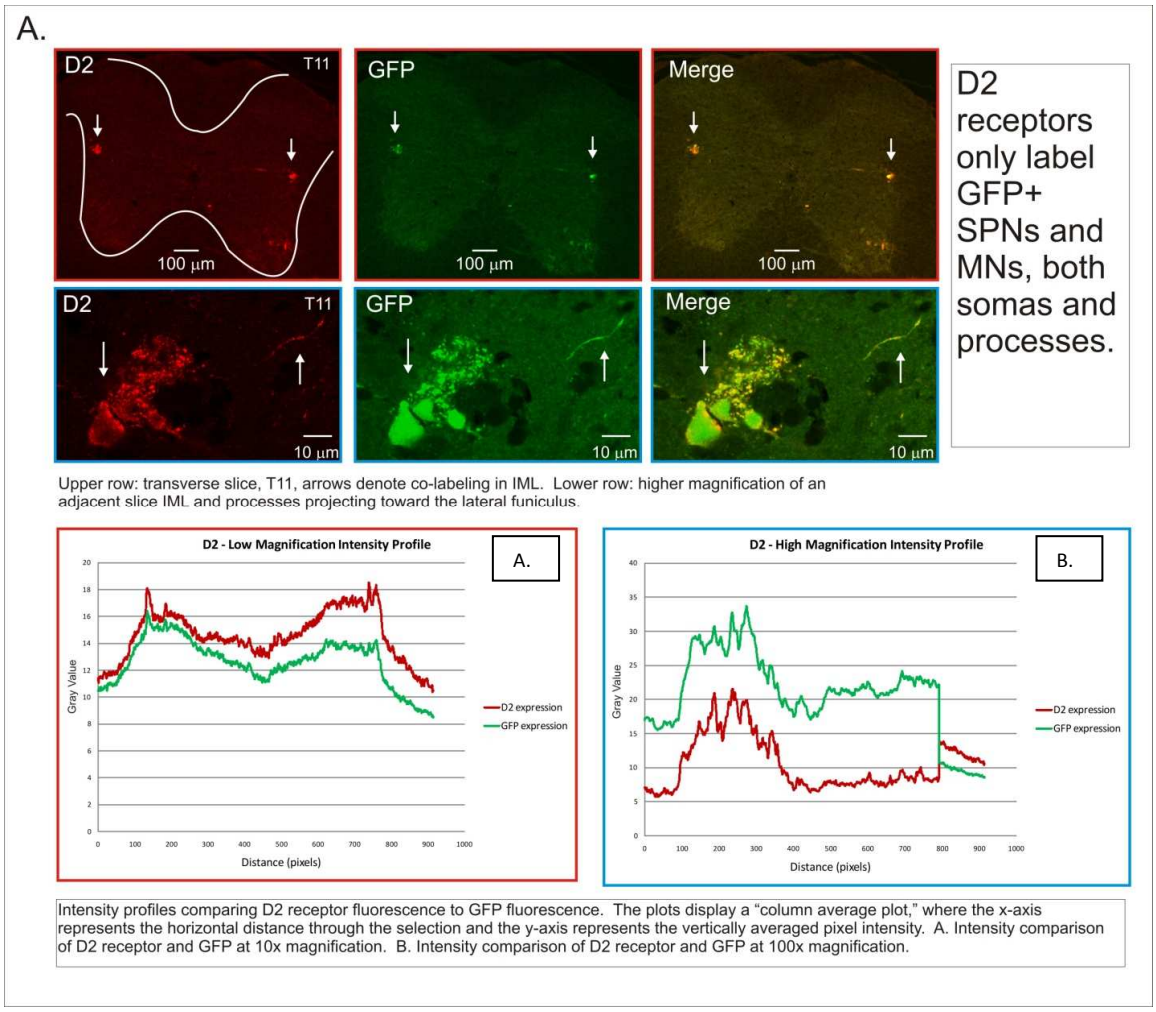
All slices were imaged on an Olympus Fluoview confocal microscope with objective lenses of either 10x, 20x, 40x, 60x, or 100x magnification. Objective lenses of 10x and 20x magnification were air-based, while 40x, 60x, and 100x objective lenses were oil-based. All analyses of pixel intensity were performed with ImageJ software (National Institutes of Health). Horizontal axes represent pixel distances along image while vertical axes represent the vertically averaged pixel intensities. Omission control slides served as sources of reference for background staining and were used with ImageJ software to subtract the background staining from all images. This enabled detection of real staining and eliminated all artifact of secondary and tertiary staining.

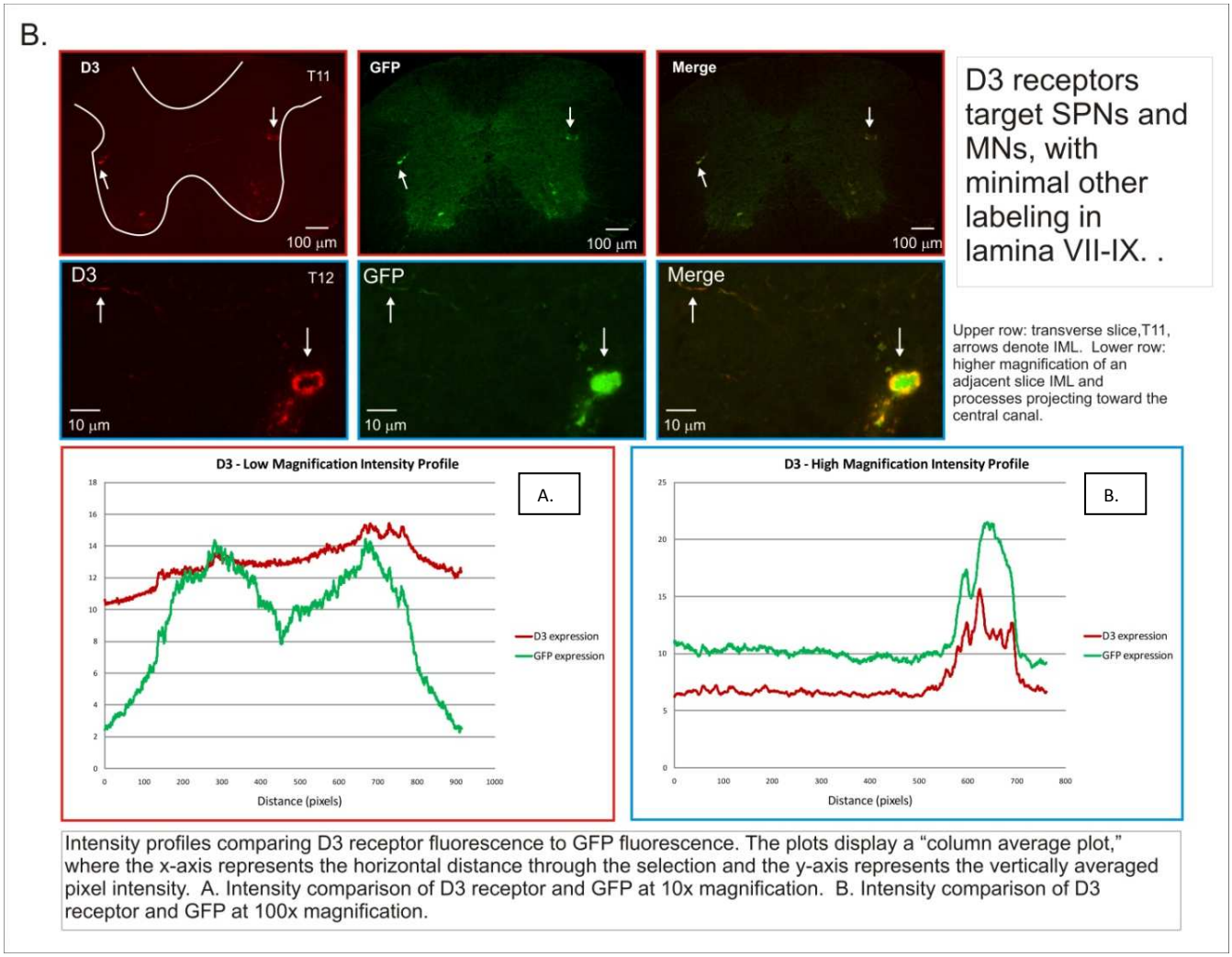
RESULTS

1. Dopamine receptors label both SPNs and MNs.

G_i-coupled dopamine receptors (D_2 and D_3) were found to rather selectively label SPNs and MNs (Figure 8). Specifically in the IML region, the pixel intensity of the D_2 receptor fluorescence matched the GFP fluorescence with greater than 50% coincidence as demonstrated by the column average plots in Figure 8.

Figure 8: Dopamine receptor labeling; sy mpathetic preganglionic neurons express D2 & D3 receptors





Intensity profiles comparing D3 receptor fluorescence to GFP fluorescence. The plots display a "column average plot," where the x-axis represents the horizontal distance through the selection and the y-axis represents the vertically averaged pixel intensity. A. Intensity comparison of D3 receptor and GFP at 10x magnification. B. Intensity comparison of D3 receptor and GFP at 100x magnification.

The perisomatic labeling of the neurons limited the coincident labeling percentage from exceeding 50%, but the evidence of D_2 expression in SPNs is confirmed by the horizontal tracing (as GFP expression inflates, D_2 expression also increases).

These results describe more specific targeting than reported with in situ hybridization and the Allen database. A total of four control spinal cords were immunoprocessed and imaged, and the dopaminergic specificity for the SPNs was highly reproducible and confirmed in approximately 10 slices per cord.

Figure87 displays representative labeling patterns for D_2 and D_3 receptor labeling seen throughout the thoracic spinal segments in control animals. D_2 receptor expression is clearly strongest

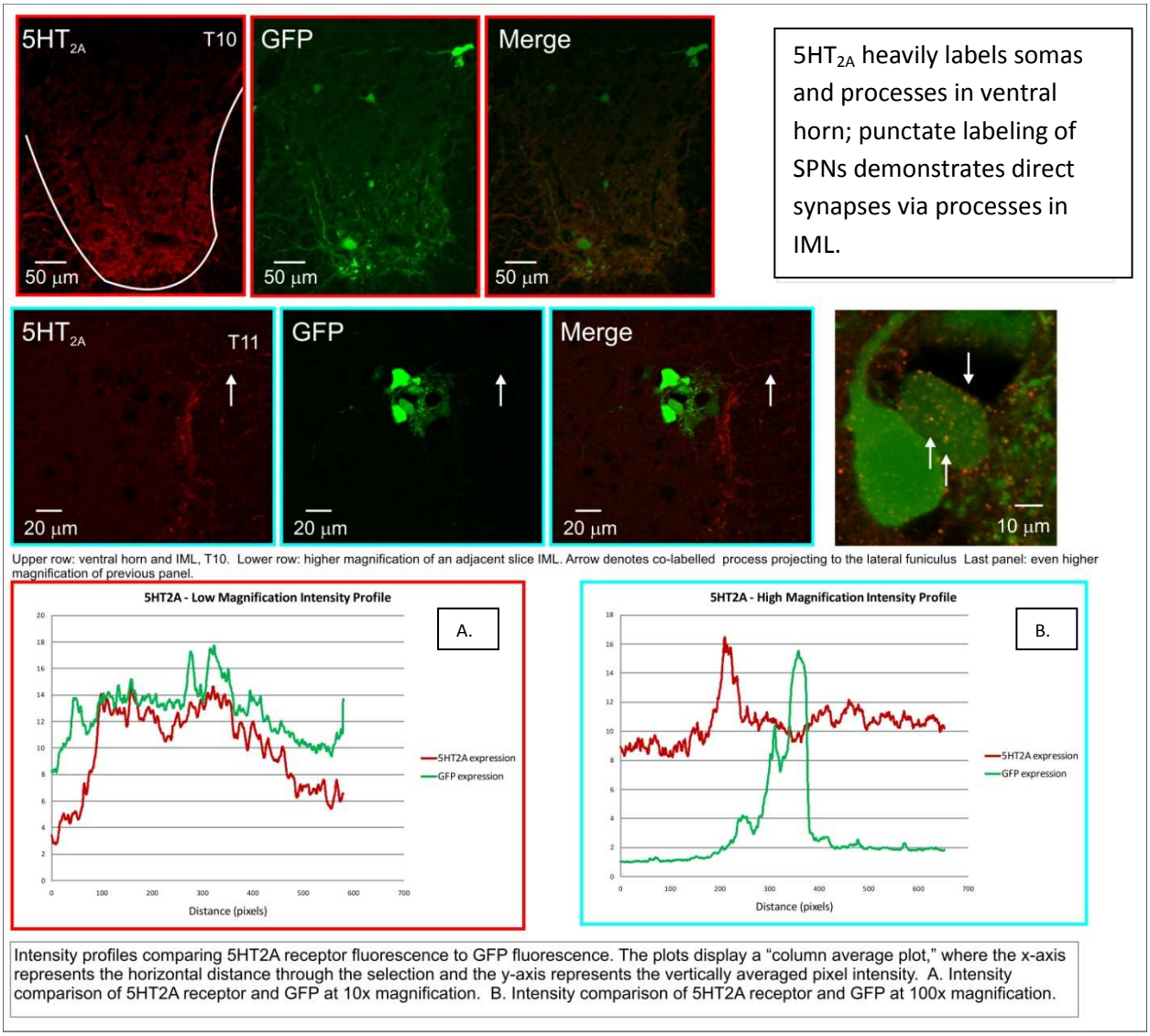
in SPNs and motoneurons (Fig8A top row). Higher magnification images clearly demonstrate that D_2 receptors are preferentially found in SPN cell bodies with clear evidence of additional receptor expression in medially projecting dendrites. Intensity profiles comparing GFP to D_2 fluorescence labeling confirms the remarkable correspondence in labeling of D_2 receptors in SPNs and motoneurons. A very similar distribution was found with D_3 receptors though receptor expression was not as strong, approximately 75% that of D_2 expression (Fig 8B). Overall, SPNs rather selectively express the dopamine D_2 and D_3 receptors perisomatically as well as displaying distinct dopaminergic receptor positive labeling of processes.

2. Serotonergic 5HT2A receptors label processes adjacent to IML.

As alluded to earlier, the presence and distribution of $5HT_{2A}$ receptors in the IML and ventral horn is suggested by Allen Database findings. Here, serotonergic $5HT_{2A}$ receptor immunolabeling was observed to be strongly expressed in the ventral horn and was always adjacent to the IML in ventrally projecting processes (Figure 9), possibly SPN axons (n=4, labeling pattern confirmed in approximately 10 slices per cord).

Direct $5HT_{2A}$ receptor-containing processes and puncta were found associated with SPNs (see high magnification picture at far right). Most $5HT_{2A}$ labeling appears distinct from GFP+ SPNs suggesting that the $5HT_{2A}$ receptors are associated with presynaptic axonal sites. However, some somatic GFP/$5HT_{2A}$ double labeling (~20%) is apparent at higher power, consistent with the Allen database predictions that SPNs express $5HT_{2A}$ receptors. Coupling the limited somatic $5HT_{2A}$ labeling in SPNs with *in situ* suggestive evidence that these neurons synthesize $5HT_{2A}$ may indicate that most SPN $5HT_{2A}$ receptors are found in SPN axon terminals. SPN axons are known to project through the ventral horn and out ventral roots to innervate postganglionic neurons.

Figure 9: Serotonergic labeling; in vicinity of SPNs, with direct synapses via processes in IML



3. Effects of spinal cord transection on Dopamine D2 and D3 receptor expression.

In this series of experiments, control and SCI tissue sections were co-processed to facilitate reliable comparisons of immunolabeling differences. At three weeks post spinal cord lesion, adult mouse spinal cords showed a drastic decrease of 86% in expression of dopaminergic D_2 and D_3 receptors (n=4). Control spinal cords showed strong coincident labeling of dopamine receptors with cells of the IML somatically and perisomatically. In contrast, spinalized tissue showed virtually no expression (gray

values within the range of 0 to 1.5 as opposed to values from 3 to 11 for control tissue) of dopamine receptors at either high or low thoracic levels (Figure 10).

Table 1 compares relative receptor expression between control and SCI cord by using a thresholding technique for pixel intensity. In both upper and lower thoracic cord there is clearly a profound significant reduction in D_2 immunolabeling as compared to control cord (p=.00052 for upper thoracic, p=.005 for lower thoracic). The table also clearly shows a lack of rostro-caudal differences in expression after injury with sites close to (upper thoracic) and further from (lower thoracic) the lesion being equally altered (p=.518). Similar patterns were observed with D_3 immunolabeling with a 66% reduction in expression at sites close to the injury and an 89% reduction in expression further from the lesion site. Table 3 compares relative D_3 receptor expression between control and SCI cord, with significant differences seen in comparing control to injured cord (p=.0058 for upper thoracic, p=.0069 for lower thoracic). No rostro-caudal differences in expression after injury were observed with sites close to (upper thoracic) and further from (lower thoracic) the lesion being similarly altered (p=.621).

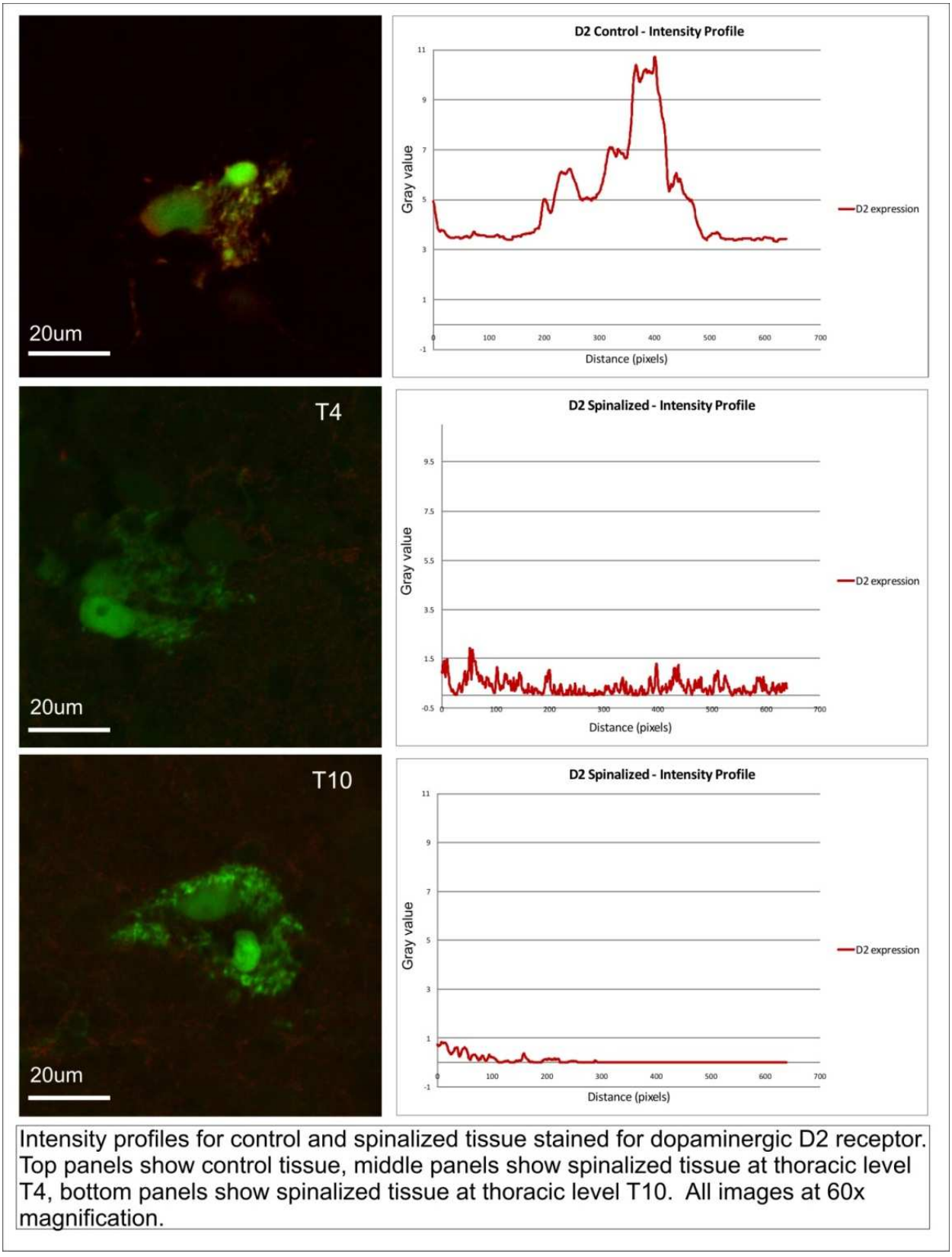
Table 1: D2 Receptor Fluorescence

D2: Pixels above threshold	Area 1	Area 2	Area 3	Area 4	Number of pixels fluorescing above threshold for four identical areas of IML in control cord, spinalized upper thoracic cord, and spinalized lower thoracic cord. Threshold defined using omission controls in which no primary antibodies were used. * denotes statistically significant (<.05) from control using Student's T-Test; no significant difference in upper thoracic and lower thoracic
Control	355	369	336	296	
Spinalized: Upper Thoracic	*32	*65	*30	*56	
Spinalized: Lower Thoracic	*56	*19	*28	*37	

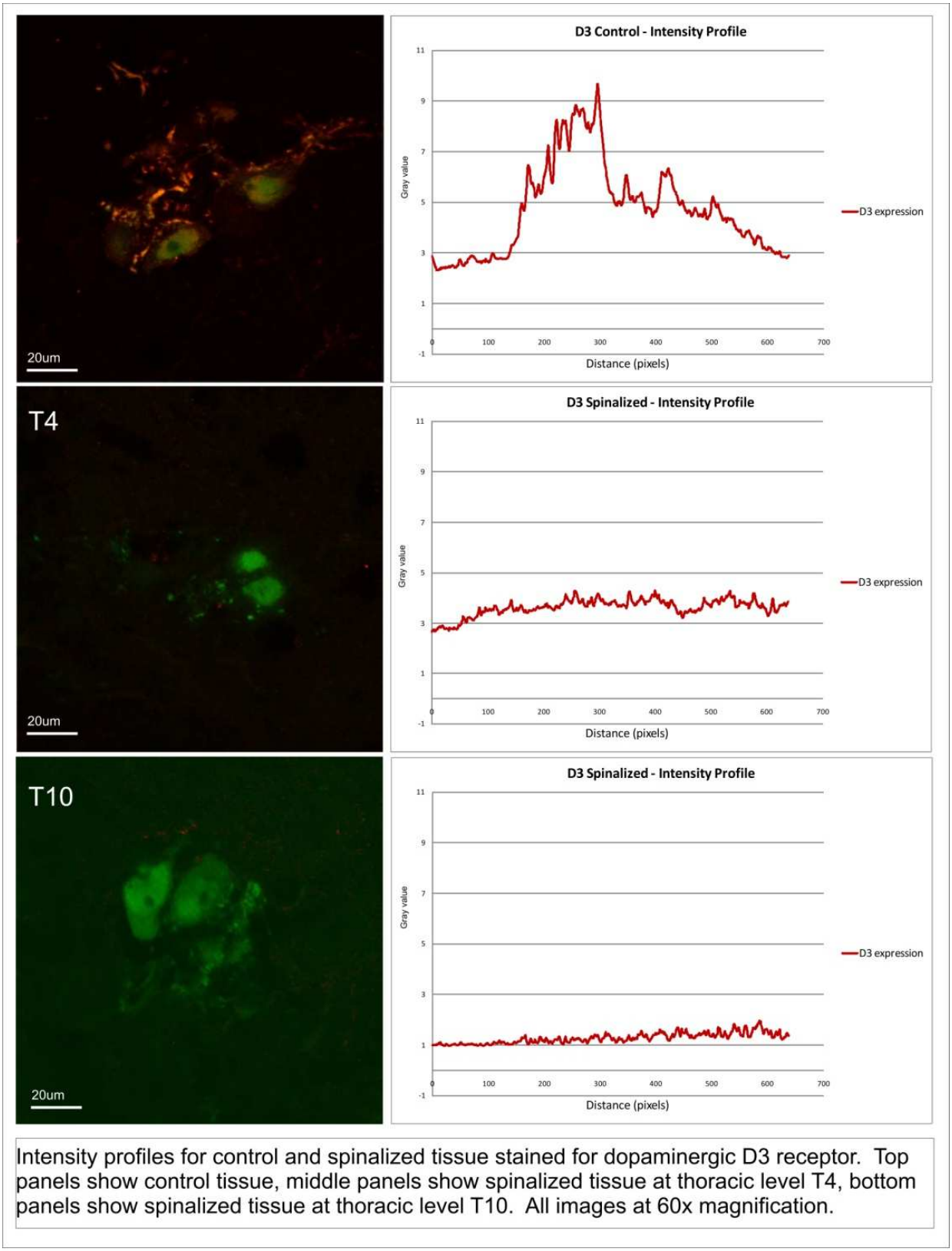
Table 2: D3 Receptor Fluorescence

D3: Pixels above threshold	Area 1	Area 2	Area 3	Area 4	Number of pixels fluorescing above threshold for four identical areas of IML in control cord, spinalized upper thoracic cord, and spinalized lower thoracic cord. Threshold defined using omission controls in which no primary antibodies were used. * denotes statistically significant (<.05) from control using Student's T-Test; no significant difference in upper thoracic and lower thoracic
Control	299	427	572	439	
Spinalized: Upper Thoracic	*29	*46	*24	*10	
Spinalized: Lower Thoracic	*31	*34	*14	*64	

Figure 10: Dopaminergic labeling post-SCI; Expression lost throughout cord



Intensity profiles for control and spinalized tissue stained for dopaminergic D2 receptor. Top panels show control tissue, middle panels show spinalized tissue at thoracic level T4, bottom panels show spinalized tissue at thoracic level T10. All images at 60x magnification.

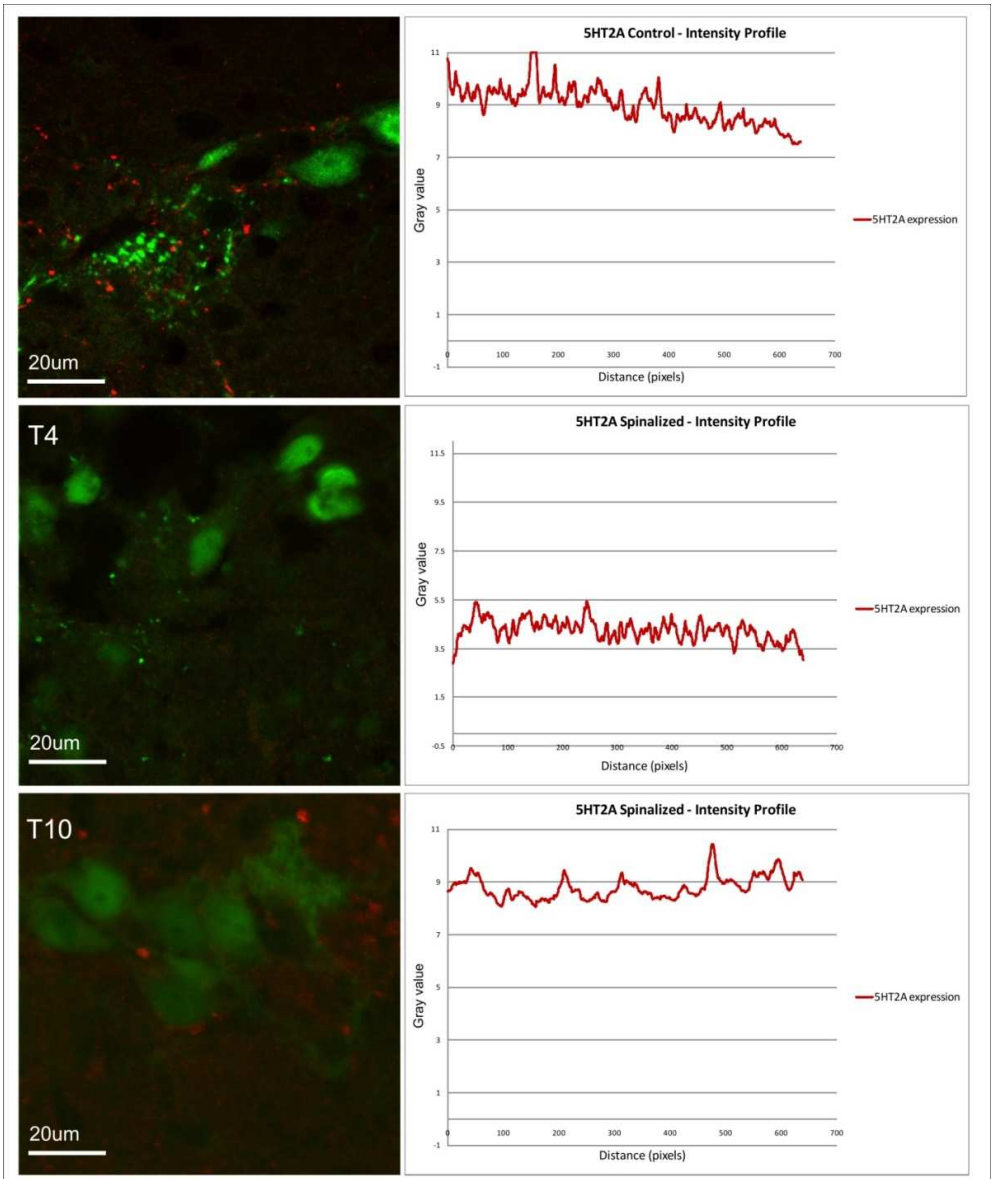


Intensity profiles for control and spinalized tissue stained for dopaminergic D3 receptor. Top panels show control tissue, middle panels show spinalized tissue at thoracic level T4, bottom panels show spinalized tissue at thoracic level T10. All images at 60x magnification.

4. Serotonergic 5HT2A receptor expression decreases post-SCI in a caudorostral gradient.

Post-SCI, adult mouse spinal cords (n=4) show dramatically decreased expression of approximately 60% of serotonergic $5HT_{2A}$ receptors in upper thoracic tissue.

Figure 11: Serotonergic labeling post-SCI; Rostrocaudal gradient extends from injury site



Intensity profiles for control and spinalized tissue stained for serotonergic 5HT2A receptor. Top panels show control tissue, middle panels show spinalized tissue at thoracic level T4, bottom panels show spinalized tissue at thoracic level T10. All images at 60x magnification.

Table 3: 5HT2A Receptor Fluorescence

5HT2A: Pixels above threshold	Area 1	Area 2	Area 3	Area 4	Number of pixels fluorescing above threshold for four identical areas of IML in control cord, spinalized upper thoracic cord, and spinalized lower thoracic cord. Threshold defined using omission controls in which no primary antibodies were used. * denotes statistically significant (<.05) from control using Student's T-Test; ^ denotes statistically significant (<.05) from lower thoracic using Student's T-Test
Control	451	783	824	301	
Spinalized: Upper Thoracic	*^43	*^30	*^47	*^63	
Spinalized: Lower Thoracic	208	237	279	290	

Staining for serotonergic $5HT_{2A}$ receptors in the adult uninjured spinal cord showed a strong presence (80% of IML region) of processes adjacent to the IML projecting towards the ventral horn (Figure 11). At high thoracic levels (T2-T7), serotonergic $5HT_{2A}$ receptor expression diminished greatly post-SCI. However, at low thoracic levels, (T9-T12), considerable receptor expression remained (81% as compared to control tissue). Table 3 compares relative $5HT_{2A}$ receptor expression between control and SCI cord. A significant difference, p=.026, exists between control and upper thoracic spinalized tissue, yet no significant difference is seen between control and lower thoracic spinalized tissue (p=.081). It is important to note the significant difference observed between upper thoracic spinalized tissue and lower thoracic spinalized tissue (p=.00085), confirming the rostro-caudal gradient of $5HT_{2A}$ expression recovery.

DISCUSSION

Monoaminergic receptors $5HT_{2A}$, D_2, and D_3 give both pre-synaptic[30,31] and post-synaptic[9,30] modulation of sympathetic output in the mammalian spinal cord, providing possible sources of therapeutic targeting in alleviating exaggerated sympathetic discharge induced by an AD phenotype.

Evidence of decreased $5HT_{2A}$, D_2, and D_3 receptor expression post-SCI may be a compensatory response associated with the loss of descending serotonergic and dopaminergic projections from the brainstem onto spinal neurons after complete SCI. In terms of the dopaminergic system, whether this occurs as a result of compensating for the loss of descending dopaminergic projections that occurs with SCI or to limit inhibitory signaling to reduce consequential hypotension post-SCI is unclear. As these

dopaminergic receptors are also autoreceptors, both presynaptic and postsynaptic inhibitory modulation can occur following their activation[30]. Because there is a lack of D_2 and D_3 expression in the IML post-SCI, a loss of D_2-like dopaminergic modulation onto autonomic output is signified. The coincident staining in control tissue for D_2 and D_3 dopaminergic receptors showed clear perisomatic labeling for each, with traces of nuclear HB9-GFP labeling, in SPNs. The postsynaptic nature implied by this staining confirms what is known about these receptors. This, in conjunction with the absence of D_2/D_3 expression post-SCI, proposes that dopamine-targeting therapeutics are not of high priority.

Loss of D_2 and D_3 dopaminergic modulation of sympathetic output and preferentially stronger reduction in serotonergic $5HT_{2A}$ modulation in the upper thoracic spinal cord raises important considerations regarding the use of monoaminergic drug therapies that target these receptors, and questions the necessity of antagonists for $5HT_{2A}$, D_2, or D_3 receptors. However, as expression of $5HT_{2A}$ receptors recovers in a rostrocaudal gradient extending from the site of injury, serotonin $5HT_{2A}$ receptor antagonists could elicit some relief from hypertension, or agonists for hypotension seen in SCI. The rostro-caudal gradient observed here supports the use of drugs acting on $5HT_{2A}$ receptors as AD symptoms have been rescued in rats using DOI in a dose-dependent manner[7]. Baclofen, an agonist for $GABA_B$ receptors, has been used intrathecally to treat severe spinal spasticity[26] in patients with multiple sclerosis (MS) or high SCI with little adverse effects and high efficacy.

This treatment for spasticity in conjunction with small doses of DOI for blocking colon-distension induced hypertension[7] could prevent AD symptoms by pre-emptively limiting a noxious stimulus causing a dramatic blood pressure increase. However, more knowledge remains to be gained in terms of anatomical plasticity of spinal cord receptors post-SCI and electrophysiological characteristics of injured versus uninjured spinal cords.

This novel immunochemical examination of mouse spinal cord anatomy post-SCI showed a loss of monoaminergic expression of $5HT_{2A}$, D_2, and D_3 receptors in the upper thoracic cord close to the site

of injury. Decreased dopaminergic D_2 and D_3 expression extended throughout the cord, but serotonergic $5HT_{2A}$ expression exhibited some recovery in a rostrocaudal gradient extending from injury site. I only looked at receptor distribution at three weeks post-SCI, and it is conceivable that receptor expression recovers or ultimately increases from baseline with more time. Further studies to understand the monoaminergic spinal cord system include immunochemical characterizations of other receptors including adrenergic receptors α_{1A} and α_{2A}, serotonergic receptor $5HT_7$, and dopaminergic receptor D_5. This study examined plasticity with complete spinal cord lesions, but incomplete transections raise interest as to what plasticity the spinal cord would undergo with retention of some descending pathways, as this is the more common condition seen clinically[32]. Studies on time-dependent changes could provide further insight into the plasticity phenomenon and different time lapses post-SCI should be explored. Anatomical examinations of spinal cord tissue above the site of lesion should also be undertaken to determine if receptor labeling is consistent with what is seen in control cords, as would be expected because there is no loss of descending projections rostral to the injury site. Due to hormonal differences in male and female species, specifically the effects of progesterone and estrogen in inflammatory responses, similar studies should be undertaken in male mice to elucidate observable significant differences, if any.

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