Synthesis, transport, and metabolism of serotonin formed from exogenously applied 5-HTP after spinal cord injury in rats

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Li Y, Li L, Stephens MJ, Zener D, Murray KC, Winship IR, Vavrek R, Baker GB, Fouad K, Bennett DJ. Synthesis, transport, and metabolism of serotonin formed from exogenously applied 5-HTP after spinal cord injury in rats. J Neurophysiol 111: 145–163, 2014. First published September 25, 2013; doi:10.1152/jn.00508.2013.—Spinal cord transection leads to elimination of brain stem-derived monoamine fibers that normally synthesize most of the monoamines in the spinal cord, including serotonin (5-hydroxytryptamine, 5-HT) synthesized from tryptophan by enzymes tryptophan hydroxylase (TPH, synthesizing 5-hydroxytryptophan, 5-HTP) and aromatic l-amino acid decarboxylase (AADC, synthesizing 5-HT from 5-HTP). Here we examine whether spinal cord caudal to transection remains able to manufacture and metabolize 5-HT. Immunolabeling for AADC reveals that, while most AADC is confined to brain stem-derived monoamine fibers in spinal cords from normal rats, caudal to transection AADC is primarily found in blood vessel endothelial cells and pericytes as well as a novel group of neurons (NeuN positive and TTX resistant). Blocking monoamine oxidase (MAO) markedly increases the sensitivity of the motoneurons (LLR) to 5-HTP, more so than the D cells described elsewhere (Vavrek et al. 2005). In normal rats, spinal monoamines are primarily produced in the terminals of descending brain stem fibers (Carlsson et al. 1964; Jacobs and Azmitia 1992), which contain the necessary biosynthesis enzymes, including tryptophan hydroxylase (TPH) that converts tryptophan to 5-hydroxytryptophan (5-HTP), tyrosine hydroxylase (TH) that converts tyrosine to l-dihydroxyphenylalanine (l-DOPA), and aromatic l-amino acid decarboxylase (AADC) that converts 5-HTP to 5-HT and l-DOPA to dopamine (DA); they also contain monoamine oxidase (MAO) that metabolizes monoamines (Best et al. 2010; Gozal 2010; Hardebo and Owman 1980; Ikemoto 2004). Transection of the spinal cord destroys all supraspinal innervation of the spinal cord, leading to the loss of most monoamine fibers caudal to the lesion site (Carlsson et al. 1964; Murray et al. 2010) together with a dramatic loss of monoamines and some of the key enzymes involved in monoamine synthesis, including TPH and TH (Clineschmidt et al. 1971; Magnusson 1973; Takeoka et al. 2010).

Despite the elimination of monoamine fiber innervation with spinal transection, repeated studies (Barbeau and Rossignol 1990; Bedard et al. 1979; Chandler et al. 1984; Guertin 2009; Hayashi et al. 2010; Tremblay et al. 1985; Viala and Buser 1971) have shown that exogenous application of the monoamine precursors 5-HTP or l-DOPA leads to synthesis of monoamines that in turn activate monoamine receptors and ultimately improve motor activity (locomotion or changes in spasms), suggesting that substantial functional AADC still remains in the spinal cord converting these precursors to monoamines. A similar situation occurs with Parkinson’s disease (PD): normal DA innervation of the striatum is lost, but somehow l-DOPA medication still leads to production of DA in the striatum (Goldstein et al. 1982; Ikemoto et al. 1997; Lidbrink et al. 1974). The origin of this AADC after loss of monoamine innervation has been extensively investigated in the striatum in relation to PD (Ikemoto 2004; Ikemoto et al. 1997; Karasawa et al. 1995; Kitahama et al. 2007; Mura et al. 1995, 2000; Ugrumov 2009) but remains uncertain after spinal cord injury (SCI) (Jaeger et al. 1983). In the striatum (and arcuate nucleus) destruction of monoamine fibers (DA fibers; with 6-hydroxydopamine) leads to an upregulation of AADC activity and in particular an emergence of a population of AADC-containing neurons, similar to the D cells described elsewhere...
further below (Ershov et al. 2005; Mura et al. 1995). This suggests that AADC activity increases when levels of monoamines are reduced, as appears to generally occur with alterations of monoamines (Berry 2004; Grandy 2007). We investigate here whether a similar compensation happens with SCI. We explore both where AADC remains in the spinal cord after injury and whether this AADC can make functional amounts of 5-HT.

In the normal spinal cord, besides the abundant monoamine fibers that contain AADC, some AADC-positive cells are found juxtaposed to the ependymal cells of the central canal, with processes extending into the lumen of the central canal. These have been termed D1 cells, together with other such cells along the neural axis (D2–D14), including in the striatum (Jaeger et al. 1983, 1984). D cells are characterized primarily by being monoenzymatic (AADC only), lacking TH and TPH, rendering them incapable of making monoamines from endogenous precursors (tyrosine and tryptophan, respectively) (Jaeger et al. 1983, 1984). They often oppose the blood vessels, central canal, or ventricles, and in this regard are thought to be part the amine precursor uptake and decarboxylase system (APUD) that has an endocrine function of discharging amines into the blood and cerebrospinal fluid (CSF) (Karusawa et al. 1995). They are anatomically reminiscent of the dopaminergic cells that contact the central canal in lower animals (Acerbo et al. 2003). D cells could be the site of monoamine synthesis with exogenous application of precursors like 5-HTP after SCI, although they do seem spatially distant from motoneurons and relatively few in number, raising a question of how they can affect motor function.

AADC is also abundant in the liver, kidney, gastrointestinal tract, and blood vessels (Berry et al. 1996). In the liver and kidney, AADC is thought to play a role in disposing of excess monoamine precursors and amino acids: here cells take up precursors, and AADC catalyzes them to form 5-HT, catecholamines, and trace amines, which are then either directly discarded into the urine (kidney) or further broken down by MAO (liver) (Berry et al. 1996). Peripheric AADC like this consumes a large fraction of systemically applied precursors 5-HTP or l-DOPA (Warsh and Stancer 1976), and thus in clinical practice it is common to block peripheric AADC with substances like carbidopa that do not easily cross the blood-brain barrier (BBB), in order to allow more effective delivery of 5-HTP or l-DOPA medications to the brain, especially in treatment of PD (Jonkers et al. 2001).

CNS blood vessels also contain some AADC as well as abundant MAO, which together are thought to perform a similar function of regulating excess monoamine precursors, as seen in the periphery (liver and kidney), and thus function as a monoamine trapping and disposal system (Hardebo and Owman 1980; Kalarria and Harik 1987; Spatz and McCarron 1998). Specifically, exogenously injected circulating monoamine precursors are normally prevented from crossing into the CNS because AADC in endothelial cells and pericytes of CNS vessels catalyzes them to form monoamines, which are then trapped in the endothelial cells and eventually broken down by MAO. Only when the concentration of blood-borne monoamine precursors is high enough to saturate activities of vessel AADC and MAO (which does not occur under normal physiological conditions) do these precursors or monoamines cross the BBB and get into CNS parenchyma (Hardebo et al. 1979b; Hardebo and Owman 1980). Under normal physiological conditions endogenous monoamines and their precursors are at much higher concentrations in the CNS than in the blood (>100-fold higher), and thus the major role of the enzymes AADC and MAO in brain vessel endothelial cells is likely to take up and metabolize excess monoamines and precursors from the CNS and thus dispose of them (Engbaek and Magnusson 1978; Hardebo and Owman 1980; Kema et al. 2000; Spatz and McCarron 1998). This role may well reverse with SCI, whereby endothelial cells may provide amines to the spinal cord, because central monoamines and their precursors are largely eliminated by injury, and this is an idea that we investigate in the present article.

While both peripheral and central AADC produce 5-HT from exogenously applied 5-HTP, this AADC is unlikely to endogenously produce 5-HT that can affect the spinal cord after spinal transection, for several reasons. First, peripherically synthesized 5-HT is mostly transported, stored in vesicles, or metabolized before it gets into the circulation; what little 5-HT does enter the circulation (from gut) is avidly transported into platelets, leaving serum 5-HT levels very low (Kema et al. 2000; Paasonen 1965), and this remaining 5-HT does not easily cross the BBB (Oldendorf 1971). Second, while 5-HTP does easily cross the BBB (by the amino acid L-transport system) (Gomes and Soares-da-Silva 1999; Hawkins et al. 2006), very little if any is normally detected in serum (Coppi and Barchielli 1989; Engbaek and Magnusson 1978; Kema et al. 2000; Tyce and Creagan 1981), and thus AADC-containing cells in the spinal cord (D cells and vessels) are unlikely to receive adequate endogenous 5-HTP to make 5-HT. Finally, there is not likely a significant source of 5-HT intrinsic to the spinal cord after transection, considering that most TPH is lost with injury, along with the associated descending 5-HT fibers (Carlsson et al. 1964; Clineschmidt et al. 1971).

In the present study we examined where AADC is expressed after spinal cord transection in rats (using immunolabeling) and whether it is upregulated in different structures, including D cells and blood vessels, to compensate for loss of brain stem monoamine innervation and associated AADC. We then explored the functional role of these sources of AADC in synthesis of 5-HT from either exogenously applied 5-HTP or endogenous 5-HTP. We found a clear upregulation of AADC expression and activity with injury, especially in blood vessels. However, D-cell AADC expression was unexpectedly decreased, and instead we found a new class of AADC-containing neurons after injury. Both these AADC-containing vessels and neurons affected spinal cord function, increasing motoneuron activity (spasms) when we added exogenous precursors, especially when we blocked MAO. However, we found no evidence that endogenous 5-HTP could produce adequate 5-HT to affect motor function. This does not, however, rule out other endogenous functions of AADC after SCI, including the production of trace amines, like tryptamine, from dietary amino acids like tryptophan (Berry 2004; Grandy 2007), as we examine in a companion paper (Li and Bennett, manuscript in preparation).

METHODS

Adult female rats with SCI were studied and compared to age-matched uninjured normal rats (3.5–5 mo old). For chronic SCI, adult
rats were transected at the S2 sacral level at ∼2 mo of age, and experiments commenced after their affected muscles became spastic (1.5–3 mo after injury), as detailed previously (Bennett et al. 1999, 2004). The affected sacrocaudal spinal cord was evaluated with histological methods and compared to normal cords and sections of cord rostral to the injury. Also, recordings were made from muscle, motoneurons, and associated ventral roots of the sacrocaudal spinal cord of spastic adult rats (Bennett et al. 2004; Murray et al. 2010). Some recordings were made from whole sacrocaudal spinal cord that was removed from chronic spinal or normal rats and maintained in vitro, while others were made with electromyogram (EMG) recordings in awake rats (Murray et al. 2010). Some rats were additionally studied only 1–2 days after sacral transection (acute spinal). All experimental procedures were approved by the University of Alberta Animal Care and Use Committee: Health Sciences.

In vitro preparation. Details of the in vitro experimental procedures have been described previously (Murray et al. 2010, 2011b). Briefly, all rats were anesthetized with urethane (0.18 g/100 g, with a maximum dose of 0.45 g), and the sacrocaudal spinal cord was removed and transferred to a dissection chamber containing modified artificial CSF (mACSF). Spinal roots were removed, except the sacral S2 and caudal C1 ventral roots and the C1 dorsal roots. After 1.5 h in the dissection chamber (at 20°C), the cord was transferred to a recording chamber containing normal ACSF (nACSF) maintained near 21°C, with a flow rate >5 ml/min. A 45-min period in nACSF was given to wash out the residual anesthetic and mACSF before recording, at which time the nACSF was recycled in a closed system.

Ventral root reflex recording and averaging. Dorsal and ventral roots were mounted on silver-silver chloride wires above the nACSF of the recording chamber and covered with a 5:1 mixture of petroleum jelly and mineral oil for monopolar stimulation and recording (Murray et al. 2011b). We evoked ventral root reflexes with a low-threshold Ca1 dorsal root root stimulation [single pulse, 0.1 ms, 0.02 mA, corresponding to 3 × afferent threshold (T)], using a constant-current stimulator (Isolux). This stimulation intensity (3 × T) is compatible with activation of low-threshold group I and II (Aβ) afferents, with a major cutaneous component (Murray et al. 2010, 2011b). The stimulation was repeated five times at 10-s intervals for each trial. The ventral root recordings were amplified (∼×2,000), high-pass filtered at 100 Hz, low-pass filtered at 3 kHz, and recorded with a data-acquisition system sampling at 6.7 kHz (Axoscope 8, Axon Instruments, Burlingame, CA). Ventral root reflexes were quantified with custom-written software (MATLAB, MathWorks, Natick, MA). That is, data were high-pass filtered at 800 Hz and rectified to allow averaging. We quantified the long-latency, long-lasting reflex (LLR) by averaging the rectified response 500–4,000 ms after stimulus, a period in which the response is mainly determined by motoneuron Ca PIC activity and not by stimulus-evoked synaptic inputs (Murray et al. 2011a, 2011b). Ventral root activity was averaged for all five stimuli in a trial. Also, background activity 300 ms prior to each stimulation was quantified similarly. This recording procedure was repeated at 12-min intervals, and agonists were added immediately after each recording, giving them time to act fully by the next recording session. Cumulative dose-response relations were computed by increasing agonist doses at these 12-min intervals (0.003, 0.01, 0.03, 0.1 μM, etc. doses used). The effects of agonists on the reflexes were reversible on washout of the agonist, but full recovery to baseline only occurred after several hours, likely because of the large size of the whole cord preparation. Thus washout of agonists was not feasible between doses of agonists used in the construction of dose-response relations.

Intracellular recording. Sharp intracellular electrodes were made from glass capillary tubes (1.5-mm OD; Warner GC 150F-10) with a Sutter P-87 micropipette puller and filled with a combination of 1 M potassium acetate and 1 M KCl. Electrodes were beveled down from an initial resistance of 40–80 MΩ to 26–32 MΩ with a rotary beveler (Sutter BV-10). A stepper-motor micromanipulator (Kopf 660) was used to advance the electrodes through the ventral cord surface into motoneurons. Penetrations were made with capacitance-over-compensation ringing. After penetration, motoneuron identification was made with antidiromic ventral root stimulation and noting ventral horn location, input resistance, and time constant (>6 ms for motoneurons) quantified snips (LLR; using Axoscope [Axon Instruments] running in discontinuous-current-clamp (DCC; switching rate 4–6 kHz, output bandwidth 3.0 kHz, sample rate of 6.7 kHz) or discontinuous single-electrode voltage-clamp (SEVC; gain 0.8–2.5 nA/mV) mode.

Slow triangular voltage ramps (3.5 mV/s voltage clamp) were applied to the motoneurons to measure their electrical properties, as detailed previously (Murray et al. 2011b). The input resistance (Rm) was measured during the voltage ramps over a 5-mV range near rest and subthreshold to PIC onset. Resting potential (Vm) was recorded with 0-nA bias current after the cell had been given ∼15 min to stabilize after penetration. The slow triangular voltage ramps were used to directly measure the PICs. During the upward portion of this ramp, the current response initially increased linearly with voltage in response to the passive leak conductance. A linear relation was fit in the region just below the PIC onset (5 mV below) and extrapolated to the whole range of the ramp (leak current). At depolarized potentials above the PIC onset threshold there was a downward deviation from the extrapolated leak current, and the PIC was estimated as the difference between the leak current and the total current (leak-subtracted current). The PIC was quantified as the initial peak amplitude of this downward deviation below the leak line (leak-subtracted current). The onset voltage for the PIC was defined as the voltage at which the slope of the current response initially reached zero (Murray et al. 2011b).

Drugs and solutions. Two kinds of ACSF were used in these experiments: mACSF in the dissection dish before recording and nACSF in the recording chamber. mACSF was composed of (in mM) 118 NaCl, 24 NaHCO3, 1.5 CaCl2, 3 KCl, 5 MgCl2, 1.4 NaH2PO4, 1.3 MgSO4, 25 d-glucose, and 1 kynurenic acid. nACSF was composed of (in mM) 122 NaCl, 25 NaHCO3, 2.5 CaCl2, 3 KCl, 1 MgCl2, 0.5 NaH2PO4, and 12 d-glucose. Both types of ACSF were saturated with 95% O2-5% CO2 and maintained at pH 7.4. The drugs added to the ACSF were 5-HT, 5-HTP, clorgyline, pargyline, carbidopa, leucine, N3S1015, and strychnine (all from Sigma-Alrichr), SB206553, APV, bicuculline, and CNQX (all from Tocris), and TTX (TTX-citrate; Alomone). All drugs were first dissolved as a 10–50 mM stock in water before final dilution in ACSF, with the exception of bicuculline, which was dissolved in minimal amounts of DMSO (final concentration in ACSF <0.04%; by itself, DMSO had no effect on LLR in vehicle controls).

Spasms in awake chronic spinal rat. Tail muscle spasms were evoked with brief electrical stimulation of the skin of the tail and recorded with tail muscle EMG. Percutaneous EMG wires (50-μm stainless steel, Cooner Wires) were inserted in segmental tail muscles at the midpoint of the tail, and recordings were made while the rat was in a Plexiglas tube, as detailed previously (Murray et al. 2011b). Muscle spasms were evoked with low-threshold electrical stimulation of the skin at the distal tip of the tail [cutaneous stimulation; 0.2 ms, 10 mA pulse; 50 × reflex threshold (50 × T); 4 spasms evoked at 10-s intervals for a trial; trials repeated at 5-min intervals], and the tail was partly restrained from moving with a piece of masking tape connecting the midpoint of the tail to a rigid stand. EMG was sampled at 5 kHz, rectified and averaged over a 500– to 4,000-ms interval to obtain the spasms (LLR; using Axoscope [Axon Instruments] and MATLAB [MathWorks]), EMG over 300 ms prior to stimulation was also averaged (background). Drugs were applied in vivo with intra-peritoneal (ip) injection and were dissolved in sterile saline. Dose-response relations were made by applying increasing drug doses at 15-min intervals, as for the in vitro preparation.

Immunolabeling. Rats were euthanized with Euthanyl (Bimeda-MTC; 700 mg/kg) and perfused intracardially with 100 ml of saline containing sodium nitrite (1 g/l; Fisher) and heparin (300 IU/l, from...
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1.000 U/ml stock; Leo Pharma) for 3–4 min, followed by 400 ml of 4% paraformaldehyde (PFA; in phosphate buffer at room temperature), over ~15 min. Spinal cords were postfixed in PFA overnight at 4°C, cryoprotected in 30% sucrose in phosphate buffer, frozen, and cut on a cryostat in horizontal or transverse 20-μm sections. We mounted spinal cord sections on slides and rinsed in Tris-buffered saline containing 0.5% Triton X-100 (TBS-TX). All subsequent antibody applications and rinses in which they followed also used TBS-TX, as we found this improved not only antibody penetration but also removal of excess antibody after incubation. Sections were incubated overnight at 4°C with the following primary antibodies: rabbit anti-5-HT (1:5,000; Sigma S5545), mouse anti-glial fibrillary acidic protein (GFAP) (1:150; Millipore MAB360), mouse anti-NeuN (1:500; Chemicon MAB377X, Alexa Fluor 488 conjugated), and sheep anti-AADC (1:200; Millipore AB119) in TBS-TX. For staining of AADC, antigen retrieval was performed by incubating slides in 10 mM citrate buffer (pH 8.5) at 80°C for 30 min prior to primary antibody incubation. To visualize the labeling of 5-HT, GFAP, and NeuN, fluorescent secondary antibodies were used, including goat anti-rabbit Texas red (1:500; Vector T-1000) and goat anti-mouse Alexa Fluor 488 (1:200; Invitrogen A11029) in TBS-TX, applied on slides for 90 min at room temperature. To visualize the AADC with fluorescent labels, tyramide amplification was additionally performed (Invitrogen TSA Kit no. 12), which included an Alexa Fluor 488 tyramide following ABC amplification (Vector PK-6101). Alternatively, to view DAB labeling of AADC or 5-HT, biotinylated donkey anti-sheep antibody (1:2,000; Millipore AP184B) or goat anti-rabbit antibody (1:200; Vector ABC kit) was applied at room temperature for 2 h in TBS-TX, followed by DAB-ABC amplification according to manufacturer guidelines (ABC, Vector PK-6101; DAB, Vector SK-4100). Image acquisition was by both conventional microscopy (for DAB) and confocal microscopy with a Leica TCS SP2 II Spectral Confocal System (for fluorescence). The latter used 1.3-μm optical sections that were collected into a z-stack over 5–20 μm and subsequently projected into a single image with maximum-intensity sorting (with ImageJ). Controls in which the primary antibody was omitted were used to confirm that the secondary antibody produced no labeling by itself. Clear labeling of monoamine fibers in normal rats was used as a positive control for AADC and 5-HT, and loss of these fibers in chronic spinal rats was a negative control.

Immunolabeling of vessels for 5-HT and AADC was quantified by counting the numbers of labeled vessels per 20-μm section and dividing by section area, to compute number of vessels per unit area. As some vessels branched in a complex pattern, each portion of the branching structure between branch points was counted as a single vessel. Because the tissue was perfused with sodium nitrite at the time of fixation, vessels were dilated and clearly distinguishable from other structures. Pairs of normal and chronic spinal rat spinal cord sections were placed on the same slide during immunolabeling, and tissue from all rats was photographed at a fixed exposure to reduce bias from differing immunolabeling. Vessels were only considered stained when they exceeded a fixed background staining threshold obtained in chronic spinal rats, and generally this was confirmed by the presence of pericytes that always stained the most strongly and lined the vessel walls. Immunolabeling of neurons and D cells for AADC or 5-HT was quantified by counting them in the same manner. Neurons were identified by their morphology and confirmed with NeuN immunolabeling. We identified D cells by their distinctive end feet that protruded into the central canal.

HPLC analysis. For biochemical analysis of 5-HT and its metabolic product 5-hydroxyindole-3-acetic acid (5-HIAA), chronic spinal rats were perfused intracardially with 100 ml of mACSF containing sodium nitrite and heparin for 3–4 min to clear blood from the spinal cord, as described above for immunolabeling. The spinal cord was then rapidly removed and placed in mACSF, as described above for in vitro recording. The cord was then incubated in varying concentrations of 5-HT in vitro for 1 h. Roots and large surface vessels were removed, and then the cord caudal to the injury was frozen in isopentane cooled on dry ice and stored at ~80°C. Subsequently, the cord was homogenized in ice-cold 0.1 N perchloric acid and centrifuged to remove precipitated protein, and the concentrations of 5-HT and its acid metabolite 5-HIAA in the supernatant were determined by high-pressure liquid chromatography (HPLC) with electrochemical detection according to the procedures of Baker et al. (1987).

Data analysis. Data were analyzed in Clampfit 8.0 (Axon Instruments) and SigmaPlot (Jandel Scientific) and expressed as means ± SD. A Student’s t-test was used to test for statistical differences before and after drug applications, with a significance level of P < 0.05. A Kolmogorov-Smirnov test for normality was applied to each data set, with a P = 0.05 level set for significance. Most data sets were found to be normally distributed, as is required for a t-test. For those that were not normal, a Wilcoxon signed-rank test was used instead, with a significance level of P < 0.05.

Standard sigmoidal curves were fit to the relation between agonist dose and reflex responses, with doses expressed in log units and with a Hill slope of unity. The dose that produced 50% effect (EC50) was measured from the curve. Also, the maximum drug-induced response (efficacy) was computed from the curve (peak of curve).

RESULTS

AADC is upregulated in microvasculature after SCI. To examine where AADC is located after SCI, immunolabeling of AADC was performed on spinal cord segments both caudal and rostral to the sacral S2 injury site in chronic spinal rats as well as at these locations in age-matched normal rats. In all spinal cord segments from normal rats, intense AADC labeling was seen mainly localized on descending monoamine fibers, which originate in the brain stem, consistent with AADC being a key enzyme in monoamine synthesis in normal cords. AADC-labeled fibers were seen descending in the white matter (WM; Fig. 1A) and traversing into the gray matter (GM). With a few exceptions, most other spinal cord structures had little distinct AADC staining, including a lack of AADC staining on most blood vessels and neurons in normal spinal cord (Fig. 1A). Occasionally, some blood vessels were weakly AADC positive, and these vessels also had perivascular AADC-positive cells, which were likely pericytes (Fig. 1A). Also, as has been described before (Jaeger et al. 1983), in these normal rats AADC-positive D cells lined the central canal, which had end feet projecting into the CSF (see Fig. 5).

In chronic spinal rats, AADC staining in monoamine fibers was eliminated caudal to the injury, in accordance with the severance of all descending fibers. However, a marked increase in AADC labeling showed up in almost all spinal vessels in both the WM and the GM (Fig. 1B), and pericytes on these vessels were densely stained by AADC labeling (Fig. 1B). These labeled vessels included spinal capillaries and small-diameter veins that all lacked a smooth muscle layer (collectively termed microvessels) (Hardebo and Owman 1980). In contrast to these microvessels, the large surface pial vessels like the anterior artery or their arteriole branches did not show AADC labeling under any conditions. To quantify the AADC in spinal microvessels, the number of AADC-labeled blood vessel branches per unit area was counted (see METHODS for threshold criteria), and found to significantly increase fivefold with injury (Fig. 1D). All spinal regions, including GM, WM, ventral horn (VH), central canal, and dorsal horn (DH), showed this fivefold increase in AADC-positive microvessels in chronic spinal rats compared with normal rats (Fig. 1, C and
The number of labeled pericytes (AADC labeled) likewise increased fivefold (not shown), as they were seen densely covering all microvessels after injury, unlike prior to injury (Fig. 1).

After injury AADC is upregulated in dorsolateral neurons associated with blood vessels. Unexpectedly, caudal to SCI, strong AADC labeling appeared in cells scattered through the GM, especially located laterally and dorsally to the central canal at the boundary of the GM and WM (Fig. 1, B, C, and F). These AADC-positive cells were presumably neurons because of their morphology, as we verify below. These AADC neurons were rarely found in normal spinal cords (on average 0.04 neurons/mm², corresponding to only a few neurons in the whole sacral cord), while there were many AADC neurons in each 20-µm transverse section after chronic SCI (Fig. 1E, significantly more). Typically, in chronic spinal rats these AADC neurons were multipolar, with soma ~10–20 µm in diameter, too small to be motoneurons and not often in the motor nucleus [motoneurons did sometimes weakly express AADC (Fig. 1C), as previously reported (Gozal 2010), though at much lower levels, near the detection threshold]. Interestingly, the AADC neurons often had fine processes that contacted blood vessels (left process of neuron in Fig. 1F; see also Fig. 2I and Fig. 3C, described below) and thus seem to have some function related to vessels. However, these AADC neurons were not D cells of the central canal, lacking contact with the central canal. Indeed, the number of D cells of the central canal decreased, rather than increased, with injury (Fig. 1E), as detailed below.

AADC labeling rostral to the lesion in chronic spinal rats was not significantly different from that in intact animals, with a similar lack of AADC-positive neurons and vessels and

Fig. 1. L-α-monoamine decarboxylase (AADC) is upregulated in microvasculature and neurons after spinal cord injury. A: immunolabeling for antibody to AADC (black, DAB) in horizontal section of spinal cord of normal rat. AADC is seen mainly confined to monoamine fibers (f; seen on expanded scale, bottom). Microvasculature (v) and pericytes (p) only weakly express AADC. D cells that are AADC positive are seen lining the central canal (CC). B: in chronic spinal rats, all descending monoamine fibers disappear together with their AADC labeling, but nearly all microvessels (v) in both white matter (WM) and gray matter (GM) are now intensely labeled with AADC, with the darkest labeling in pericytes (p) adhered to the vessel wall. Neurons (n) are also labeled for AADC. Some D cells (d) remain labeled. C: AADC immunofluorescence (green) showing distribution of AADC in transverse section of chronic spinal rat. Vessels stained with AADC are seen in the dorsal horn (DH) and ventral horn (VH). Neurons (n) expressing AADC appear with injury. D cells (d) remain only on the ventral aspect on CC (arrow). D: quantification of microvessel AADC staining, with a 5-fold increase in vessels after injury. E: quantification of number of AADC-labeled neurons and D cells per transverse section, with AADC neurons appearing only with injury and D cells decreasing in number with injury. F: expanded view of box in C, showing AADC neuron (n) and vessels (v). *Significant increase compared with control, $P < 0.05$, $n = 5$ rats/condition.

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normal numbers of D cells lining the central canal, even close to the injury site (not shown; same rats tested below injury in Fig. 1). This suggests that it is the loss of monoamine fibers, and not the injury itself, that triggers the dramatic upregulation AADC in vessels, pericytes, and neurons caudal to a chronic injury.

**Upregulated AADC synthesizes 5-HT from exogenous 5-HTP after SCI.** To determine the functionality of the upregulated AADC after SCI, 5-HT immunolabeling was carried out in both normal and SCI rats with or without 5-HTP administration in vivo (30 mg/kg ip, 25 min before perfusion, DAB label). In normal spinal cords without 5-HTP injection, only 5-HT fibers (brain stem derived) were strongly labeled by the 5-HT antibody (Fig. 2, C and G), although some diffuse background 5-HT labeling was present, as discussed below. Blood vessels were not 5-HT positive (white spaces), and 5-HT-positive neurons were not found (Fig. 2, C and G). After 5-HTP was injected in these normal animals, weak 5-HT labeling was visualized on a few blood vessels and associated with a few perivascular pericytes in spinal cord (Fig. 2, D and H). To rule out the interference of endogenous background 5-HT in characterizing the function of AADC in uninjured spinal cords, p-chlorophenylalanine (pCPA) was administered (350 mg·kg−1·day−1 ip) to deplete endogenous 5-HT. After 2 days pCPA depleted almost all endogenous 5-HT in sacral spinal cord, with only small amounts of 5-HT fibers seen near areas where monoamine fibers are normally densest (near motoneurons and central canal; Fig. 2K), and the diffuse background 5-HT staining was likewise reduced. After this 5-HT depletion with pCPA in uninjured rats, 5-HTP still failed to produce much 5-HT synthesis in microvessels, with only weak 5-HT labeling found in a small fraction of the microvessels, pericytes, and 5-HT fibers in both the WM and the GM (Fig. 2L).

![Image](http://jn.physiology.org/)

**Fig. 2. Serotonin (5-hydroxytryptamine, 5-HT) synthesis from exogenous 5-hydroxytryptophan (5-HTP) in chronic spinal rats.** A: in untreated chronic spinal rats no 5-HT immunolabeling is seen in the spinal cord caudal to the lesion (5-HT labeled black with DAB, horizontal sections). B: injection of 5-HTP (30 mg/kg ip 25 min prior to fixation) leads to synthesis of 5-HT in locations where we also see AADC, including on the microvasculature (v), in both WM and GM. However, there is also diffuse 5-HT labeling throughout the spinal cord (darker than A). Expanded image shown in F. C: in untreated normal rats, brain stem-derived monoamine fibers have 5-HT labeling, and there is some diffuse background labeling of 5-HT. Expanded image shown in G. D: injection of 5-HTP in these normal rats causes a somewhat more intense diffuse 5-HT labeling, but very few microvessels are labeled. Expanded image shown in H. E: quantification of the number of 5-HT-labeled microvessels in rats treated with 5-HTP and examined under the following conditions: normal rat caudal to injury, normal rat with endogenous 5-HT depleted with p-chlorophenylalanine (pCPA, 350 mg·kg−1·day−1, 2 days prior), chronic spinal rat caudal to injury, and chronic spinal rat rostral to injury. *Significantly greater than normal rat, †significantly less than chronic spinal caudal cord, P < 0.05, n ≥ 5 rats/condition. F: expanded image of chronic spinal rat treated with 5-HTP (30 mg/kg), showing vessels (v), pericytes (p), and neurons (n) labeled for 5-HT. Inset: the labeled neuron is from a different rat injected with a lower dose of 5-HTP (3 mg/kg). G and H: expanded images of normal rats showing 5-HT-labeled fibers with and without 5-HTP and a relative lack of 5-HT-labeled vessels. I: immunolabeling for 5-HT in spinal cord of chronic spinal rat maintained in vitro and incubated in 5-HTP (30 μM for 1 h), showing 5-HT synthesized in situ in vessels (v) and neurons (n). J: lack of 5-HT immunolabeling in normal rat treated with pCPA, except in a few fibers near the motor nucleus. K: injection of 5-HTP in pCPA-treated normal rats leads to 5-HT-labeling in only a few fibers. n ≥ 5 for A–L.

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and no production of 5-HT elsewhere, including an absence in
neurons and motoneurons (Fig. 2L).

In untreated chronic spinal rats no 5-HT labeling was ob-
served below the injury site (Fig. 2A). The tissue was so devoid
of 5-HT (white) that it was indistinguishable from tissue
processed identically for immunolabeling, but without the
primary antibody (not shown), thus providing a clear control
for the selectivity of the 5-HT antibody.

In contrast, in chronic spinal rats with 5-HTP injection (30
mg/kg ip), blood vessels and pericytes were strongly stained by
the 5-HT antibody in both the WM and the GM (Fig. 2, B and
F), consistent with their strong AADC staining (Fig. 1). Over-
all with injury, there was a significant 10-fold increase in
vessels synthesizing 5-HT from 5-HTP (Fig. 2E). Neurons
were also labeled by 5-HT, and given their morphology and
medial-lateral location we suppose that they are the same
neurons that were AADC positive (Fig. 2F, Fig. 1), as con-
firmed by double staining of AADC and 5-HT in a later section
(Fig. 3). Rostral to the spinal cord transection AADC function
was not different from normal, with no AADC neurons and
few vessels and pericytes stained with 5-HT after 5-HTP
injection, not significantly different from normal rats (Fig. 2E).

A

B

C

Fig. 3. 5-HT is not endogenously synthesized by AADC after injury. A: double immunofluorescence labeling for 5-HT (red) and AADC (green) in the spinal
cord of a normal rat (ventral horn), showing a dense network of AADC-positive monoamine fibers (f), some of which are 5-HT fibers (double-stained orange).
A few vessels (v) are weakly AADC positive, but these do not synthesize 5-HT endogenously (not labeled red). B: double labeling of untreated chronic spinal
rats, showing that AADC neurons (n) and vessels (v) do not endogenously produce 5-HT (no red). C: in contrast, injection of exogenous 5-HTP leads to
exogenous 5-HT synthesis in vessels (v) and neurons (n) double-labeled with AADC. Pericytes (p) appear to accumulate 5-HT out of proportion to the AADC
and sometimes partly detach from vessels. All images on same scale. Same rats as in Fig. 2.
Diffusely distributed uptake of 5-HT in spinal cord, separate from AADC cells. Below the injury in chronic spinal rats, weak background staining for 5-HT appeared diffusely distributed throughout the spinal cord after 5-HTP treatment (Fig. 2B, outside of vessels and neurons), unlike in the untreated chronic spinal rat (Fig. 2A), indicating that there was a weak uptake of 5-HT into many other spinal structures that do not contain AADC. In normal rats diffuse background 5-HT was also present, and this was intensified with 5-HTP application (Fig. 2, G and H) and largely eliminated by 5-HT depletor (with pCPA, Fig. 2K), suggesting that there was likewise some weak uptake of 5-HT in many structures, presumably from 5-HT escaping from 5-HT fibers. When we directly incubated isolated cords of chronic spinal rats in 5-HT itself (1 μM for 1 h in vitro), there was likewise an increase in diffuse background 5-HT immunolabeling (not shown; n = 4/4), confirming a general 5-HT uptake mechanism, separate from the main sites of 5-HT synthesis (AADC-containing neurons and vessels).

Diffusely distributed uptake of 5-HT in spinal cord, verified with HPLC. Furthermore, when the isolated cords of chronic spinal rats were incubated in 1 μM 5-HT (in vitro for >60 min), we detected 0.36 ± 0.06 μM 5-HT when we emulsified the whole cord and performed HPLC analysis (corresponding to 63.0 ± 10.6 ng/g tissue). The remaining 5-HT was accounted for as the 5-HT metabolite 5-HIAA, with a concentration of 0.61 ± 0.05 μM (or 117.5 ± 8.9 ng/g; n = 4). When we incubated the cords in MAO inhibitors (clorgyline and paroxetine, 1 μM each) together with the 1 μM 5-HT, then the HPLC analysis showed a fourfold increase in 5-HT (1.42 ± 0.73 μM, or 250.3 ± 129.8 ng/g tissue), not different from the applied 1 μM 5-HT (P = 1.0, n = 4), and consistent with 5-HT being transported and metabolized by MAO. As expected, 5-HIAA was nearly eliminated with an MAO block (0.16 ± 0.11 μM). Together these results indicate that the 5-HT must have diffused fairly uniformly into the spinal cord structures, whereas if the 5-HT had stayed only in the extracellular space a much lower overall concentration of 5-HT should have been seen with HPLC, considering that the extracellular space only makes up 10% of the total cord volume (0.1 μM 5-HT would have been seen, instead of ~1 μM) (Vargova and Sykova 2008).

In contrast, HPLC analysis of untreated cords from chronic spinal rats (below injury) gave very low 5-HT levels (4.2 ± 3.4 ng/g; n = 6), near the minimum detection limit and over 200 times lower than the 5-HT in normal cords (906.0 ± 141.8 ng/g; n = 6, significant P < 0.05). Furthermore, most of this small amount of 5-HT in chronic spinal rats was likely due to blood products (platelets) remaining in the vessels of the cord, because the HPLC gave near-zero 5-HT levels in cords well perfused and cleared of blood (<6 ng/g) and higher values (up to 30 ng/g) in cords poorly perfused. Importantly, the residual level of 5-HT in untreated rats was not significantly changed by MAO inhibitors (2.7 ± 1.3 ng/g; P < 0.05), unlike when the cords were incubated in 5-HT (see above), consistent with the residual 5-HT being stored in a location, like platelets, inaccessible to the diffusely located spinal cord MAO, and thus not likely to affect spinal cord function.

Exogenous 5-HT derived in periphery is not accumulated in AADC-containing cells. To rule out the possibility that in our experiments 5-HT was synthesized from 5-HTP in peripheral structures containing AADC (e.g., liver) and then leaked into the spinal cord, we injected 5-HT peripherally in chronic spinal rats (30 mg/kg ip, 25 min before perfusion) and then conducted 5-HT immunolabeling. With this 5-HT injection, we found that 5-HT immunolabeling did not occur in vessels and neurons, unlike with 5-HTP injections, and there was very little diffuse background staining (Fig. 2J), consistent with the relative impermeability of 5-HT across the BBB (Oldendorf 1971). This suggests that the 5-HT immunolabeling that we observe in chronic rats after 5-HTP injection is centrally produced in spinal cord blood vessels, pericytes, and AADC-expressing neurons. To further verify this conclusion, the chronically injured spinal cord was removed from the rat and then incubated in 30 μM 5-HTP in vitro (1 h). In these isolated and 5-HTP-treated cords intense 5-HT immunolabeling was visualized in vessels, pericytes, and AADC neurons (Fig. 2I), as in vivo. In contrast, incubation of isolated cords with 5-HT itself (1 μM, 1 h, in vitro) did not produce distinct 5-HT immunolabeling in vessels, pericytes, and neurons but only produced weak diffuse background 5-HT immunolabeling, as described above (not shown; n = 4/4), confirming that these AADC-containing sites are the source of 5-HT synthesis and are not simply sites that accumulate 5-HT from elsewhere.

Detailed morphology of AADC neurons, seen with in vitro 5-HTP incubation. Interestingly, with in vitro 5-HTP incubation compared with in vivo 5-HTP injections, delivery of 5-HTP to the AADC neurons appeared more effective, with cell bodies more intensely stained and morphological structure more clear, including staining of fine processes (dendrites, Fig. 2I). Importantly, these fine processes often appeared in close apposition to vessels (Fig. 2I), suggesting that AADC neurons may somehow interact with blood vessels, as already noted.

5-HT is not endogenously produced by spinal cord AADC after spinal cord injury. We next used double fluorescence immunolabeling of 5-HT and AADC to verify that synthesis of 5-HT from exogenous 5-HTP (via AADC) is the sole source of 5-HT after SCI, unlike in normal rats. In untreated normal rats, both 5-HT and AADC antibodies mostly labeled brain stem-derived monoamine fibers (Fig. 3A), with 5-HT fibers making up a substantial fraction of the total monoamine fibers (AADC labeled) while the remaining fibers were presumably of catecholamine origin. In contrast, in untreated chronic spinal rats no 5-HT fluorescence label was seen caudal to the injury (Fig. 3B, as also seen with DAB in Fig. 2), whereas in these same sections double labeling revealed AADC-positive vessels and neurons (Fig. 3B).

Application of 5-HTP (30 mg/kg ip in vivo 25 min before perfusion) in these chronic spinal rats revealed that 5-HT synthesis always colocalized with the enzyme AADC (Fig. 3C). When we lowered the 5-HTP dose to a level near the minimum to have physiological effects (3 mg/kg 25 min prior to perfusion, as detailed below) we still saw both vessel and neuronal synthesis of 5-HT (Fig. 2F, inset). This indicates that we can detect 5-HT with our immunolabeling with near-minimal functional amounts of 5-HTP, and thus the lack of 5-HT labeling in untreated chronic spinal rats indicates that there is no functional endogenous 5-HTP or accumulation of 5-HT.

Variability of 5-HT synthesis in AADC-containing neurons, vessels, and pericytes. Interestingly, even with our standard dose of in vivo 5-HTP injection (30 mg/kg), the intensity of 5-HT labeling was sometimes weaker in neurons compared...
with vessels and pericytes, even though the AADC labeling was not (Fig. 3C, Fig. 4A). We suspect that the 25 min that we waited after 5-HTP injection was not always enough to allow adequate amounts of 5-HTP to move across the BBB from vessels to neurons, because when 5-HTP was applied for longer periods, more intense and uniform neuronal 5-HT staining occurred (not shown; n = 3/3), and when cords were incubated for long periods (60 min) in vitro with 5-HTP we saw more detailed morphology labeled with 5-HT in AADC neurons, as described above (Fig. 2f).

In contrast, pericytes stood out as a location where 5-HT fluorescence was always relatively more intense than AADC

![Fig. 4. Characterization of AADC neurons that appear after spinal cord injury. A: double labeling of 5-HT (red) and AADC (green) in chronic spinal rat treated with 5-HTP (30 mg/kg ip), showing 5-HT synthesis in vessels (v) and neurons (n) also labeled for AADC. Transverse section lateral to central canal. B: in chronic spinal rats pretreated with carbidopa (50 mg/kg 30 min prior) and then given 5-HTP, AADC-labeled vessels no longer synthesize 5-HT, whereas AADC-labeled neurons continue to synthesize 5-HT. C: quantification of the number of 5-HT-labeled vessels (per mm²) and neurons (per transverse section) in rats treated in vivo with 5-HTP under the following conditions: normal rat (N), chronic spinal rat (C), chronic spinal rat treated with carbidopa (CD), and chronic spinal rat treated with leucine (Leu, in vitro 5-HT and leucine, in this case only), n = 5–8 rats/condition. D: double labeling with 5-HT and NeuN in chronic spinal rat treated with 5-HTP (and carbidopa), showing that AADC cells that synthesize 5-HT are indeed AADC neurons. E: double labeling with 5-HT and glial fibrillary acidic protein (GFAP) in chronic spinal rats treated with 5-HTP, showing that 5-HT is not synthesized in astrocytes, even though these surround the AADC-containing vessels (v) and neurons (n).]
fluorescence in 5-HTP-injected chronic spinal rats (Fig. 3C, Fig. 4A), suggesting that 5-HT is somehow accumulated in pericytes, in addition to being synthesized there. Carbidopa blocks vessel but not neuronal AADC activity. To demonstrate that AADC neurons alone have the ability to transform exogenous 5-HTP into 5-HT, carbidopa was employed to selectively impede the function of AADC in vessels. Carbidopa (50 mg/kg), which is a peripheral and vessel AADC inhibitor that does not readily cross the BBB (Hardebo et al. 1979b; Hardebo and Owman 1980), was given together with 5-HTP (30 mg/kg, 25 min prior to perfusion and 30 min after carbidopa). After this treatment with carbidopa, only the AADC neurons were 5-HT positive (Fig. 4B; not significantly different in number from without carbidopa, Fig. 4C) and vessels no longer synthesized 5-HT (significant reduction in stained vessels, Fig. 4C), consistent with previous reports that this dose of carbidopa blocks CNS vessel AADC function (Hardebo et al. 1979b). In contrast, the AADC immunolabeling staining pattern (Fig. 4B) remained similar compared with spinal cords of chronic spinal rats treated with only 5-HTP (Fig. 4A): microvessels, pericytes, and the AADC neurons were labeled. Furthermore, in the presence of carbidopa, 5-HT labeling in the AADC neurons became more pronounced (Fig. 4B) compared with that in chronic spinal rats treated with only 5-HTP (Fig. 3A): the cell bodies were more intensely stained, and fine processes of dendrites and axons were more easily visible. In addition, most of these 5-HT processes colocalized substantially with AADC (Fig. 4B). By blocking peripheral (e.g., liver) and vessel AADC with carbidopa, much less 5-HTP is known to be metabolized in the periphery, and thus much more is available to reach the spinal cord (see Discussion); this would explain the increased synthesis of 5-HT in neurons.

Leucine blocks neuronal but not vessel AADC synthesis of 5-HT from 5-HTP. We also incubated the isolated sacral cord from chronic spinal rats in vitro with 5-HTP (30 μM) together with leucine (3 mM 30 min prior to 5-HTP), which competitively blocks the movement of 5-HTP via the L-transport system, one of the major transporters of amino acids and monoamine precursors (Gomes and Soares-da-Silva 1999; Hawkins et al. 2006). In these cords, there were hardly any AADC-negative neurons labeled with 5-HTP (significant reduction compared with without leucine) but microvessels and pericytes were still strongly labeled for 5-HT (Fig. 4C). Thus transport of 5-HTP into AADC neurons is mostly dependent on the L-transport system, whereas transport of 5-HTP into vessels must utilize multiple transport systems in addition to the L-system, as has been reported before (Hawkins et al. 2006). These data also demonstrate that 5-HT can be produced in vessels alone, not depending on AADC neurons.

New class of AADC neurons are NeuN positive and GFAP negative. To further characterize the new class of AADC cells that we find after injury, we labeled AADC cells with 5-HT (after a 5-HTP injection) and double labeled with either NeuN (neuron specific) or GFAP (astrocyte specific). As shown in Fig. 4D, the 5-HT-producing AADC cells colabeled with NeuN, confirming our previous suggestion that these are AADC neurons. These rats tested with NeuN were also treated with carbidopa, to optimize the 5-HT produced in AADC neurons, as discussed above. In 5-HTP-treated chronic spinal rats, AADC cells (5-HT positive) did not colocalize with GFAP (Fig. 4E), indicating that they were not astrocytes. Additionally, vessels that synthesized 5-HT did not stain for GFAP, indicating that the vessel AADC and 5-HT synthesis was not caused by astrocytes, even though astrocytes did surround vessels (Fig. 4E).

D cells in central canal disappear with spinal cord injury. While AADC staining increased dramatically in microvessels, pericytes, and neurons after injury, the number of AADC-positive D cells in the central canal significantly decreased (Fig. 1E), as already mentioned. Furthermore, the remaining D cells were only observed at the ventral side of the central canal (Fig. 5, B and C), whereas they normally lined the whole central canal on dorsal and ventral aspects (Fig. 5A) (Jaeger et al. 1983). The remaining D cells did not endogenously produce 5-HT (not shown), as in normal rats (Fig. 5A), but could produce 5-HT when the rat was injected with 5-HTP (Fig. 5B), allowing us to further characterize these cells with double 5-HT and NeuN or GFAP immunolabeling (for technical reasons we were never able to get the AADC antibody itself to work as a colabel with NeuN or GFAP). Interestingly, we found that in these 5-HTP-injected rats (normal or injured), D cells were not NeuN positive (Fig. 5C). However, NeuN sometimes failed to even label motoneurons (not shown), so a negative NeuN finding does not necessarily rule out the original conclusions of Jaeger that these cells are neuron-like (Jaeger et al. 1983). Furthermore, we found that these D cells did not colocalize GFAP (Fig. 5D), indicating that they were not of glial origin.

We wondered whether the loss of D cells might be caused by D cells migrating away from the central canal into the GM and producing the new AADC-positive neurons that we observe after injury. Thus we examined AADC labeling at just 1–2 days after SCI. However, in none of these acutely injured rats (n = 4/4) did we detect D cells leaving the central canal area (near central canal but not in contact with CSF), and, furthermore, AADC-positive neurons began appearing far from the central canal, even at this early time point after injury (data not shown). Thus the AADC neurons likely arose from existing neurons in the GM and not from migrating D cells of the central canal.

AADC neurons and microvessels both increase spasms in 5-HTP-treated rats. Next we tested whether 5-HTP influenced the muscle spasms in chronic spinal rats and examined how AADC-containing microvessels and neurons individually contributed to spasms. In chronic spinal rats, tail spasms were evoked by brief electrical stimulation of the tip of the tail and recorded by EMG wires implanted in the segmental tail muscles (Fig. 6A). These spasms were typically composed of a large short-latency response followed by a long-lasting reflex (LLR, many seconds long), the latter of which we quantified (Fig. 6B). Application of 5-HTP dose-dependently increased the LLR up to doses of 3 mg/kg, with ~1 mg/kg producing 50% of maximal effect (EC50; Fig. 6, B, D, and G). Doses higher than 3 mg/kg decreased the reflex, similar to what has been demonstrated for the action on 5-HT on spasms (Fig. 6, D and E, with excitation and inhibition depending on 5-HT2 and 5-HT3 receptors, respectively) (Murray et al. 2011b). The excitatory effect of 5-HTP lasted for over an hour for a single dose.
Prior application of carbidopa (50 mg/kg 30 min before 5-HTP) to block vessel and peripheral AADC did not inhibit the action of 5-HTP (Fig. 6, C and E): neither the peak effect of 5-HTP (Fig. 6F, 4-fold increase in LLR) nor the EC₅₀ (Fig. 6G) was significantly altered by carbidopa, even though we know that this dose of carbidopa dramatically reduces vessel AADC synthesis of 5-HT. This demonstrates that the AADC neurons can alone affect spinal cord function, as these neurons remain the sole source of 5-HT synthesis in carbidopa-treated rats (Fig. 4). In contrast, a complete blockade of all AADC

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Fig. 5. Characterization of AADC-containing D cells that line the central canal. A: double immunofluorescence for 5-HT (red) and AADC (green) in normal rat, showing that many AADC-containing cells line the central canal (CC) and have end feet that project into the canal (arrow). These D cells do not endogenously synthesize 5-HT (not red). In contrast, numerous fibers contain AADC and 5-HT (descending 5-HT fibers). Transverse sections shown, with dorsal at top, as also in B–D. B: double labeling in chronic spinal rat treated with 5-HTP, showing a relative lack of D cells labeled with AADC and that the few remaining cells are always located on the ventral aspect of the central canal (arrow). These cells make 5-HT when treated with 5-HTP but not without (latter not shown). C and D: D cells are NeuN negative (not neurons) and GFAP negative (not astrocytes) in both chronic spinal (shown) and normal (not shown) rats. Same rats as in Fig. 4.
with the centrally acting blocker NSD1015 significantly reduced the efficacy of 5-HTP and increased the EC50 (by 10-fold, to >30 mg/kg, data not shown; n = 5), demonstrating that conversion to 5-HT is necessary for the action of 5-HTP.

Carbidopa itself produced only a small transient increase in the LLR, in the first 10–15 min after injection (Fig. 6F), unlike the long duration and large effects of 5-HTP, suggesting that there is little endogenous AADC activity directly affecting motor function. Saline control injection had no significant long-term effect on the LLR (n = 8), although in some rats it increased the reflex for a few minutes (n = 2), likely because of stress, which we know can transiently increase the LLR (unpublished observations).

Mechanisms of action of 5-HTP, including roles of AADC, 5-HT2 receptors, and MAO. We further examined the action of AADC and 5-HTP in chronic spinal rats by recording ventral root activity in cords that were isolated and maintained in vitro (Fig. 7A), which, among other things, eliminated the interference of 5-HT synthesized in the peripheral system. We recorded LLRs from the ventral roots in response to a low-threshold stimulation (3 T) on dorsal roots (Fig. 7B). These in vitro LLRs are the counterpart of the LLRs recorded in vivo and thus are an indirect measure of muscle spasms (Murray et al. 2010). Application of 5-HTP dose-dependently increased the amplitude of LLRs, with a maximal response (efficacy) three times higher than in predrug conditions (Fig. 7, B, D, and F; significant increase), and with an EC50 of ~10 μM (Fig.
Again, as previously reported for 5-HT (Murray et al. 2011b), there was always an initial increase in LLR with
5-HTP (over 1 order of magnitude in dose), after which
further 5-HTP decreased the reflex, in accordance with the dual
action of 5-HT2 and 5-HT1 receptors (Fig. 7D). We only
quantified the 5-HT2 receptor-mediated increase in the LLR.
The AADC blocker NSD1015 completely inhibited 5-HTP's
effect, so that the LLR was not significantly different from
pre-5-HTP control conditions (in NSD1015, Fig. 7, C and D).
These data again verify that 5-HTP needs to be converted to
5-HT via AADC to enhance motor output, and this must be
occurring centrally (in vitro). Importantly, in the presence of
leucine to selectively block 5-HT synthesis in just AADC
neurons (see Fig. 4C), 5-HTP application still significantly
increased the LLR (Fig. 7F), with an EC50 again of ~10 μM,
not significantly different from without leucine (Fig. 7G). This
demonstrates that the vessel AADC is sufficient alone to
increase spasms, because only vessel AADC makes 5-HT in
the presence of leucine, as described above (Fig. 4C). Together
with our carbidopa in vivo data, we can conclude that both
vessels and AADC neurons produce functional 5-HT that
increases spasms upon 5-HTP application.

The increase in LLRs with 5-HTP was completely blocked
by the highly selective 5-HT2B/2C receptor blocker SB206553
(10 μM; Fig. 7, E and F), which demonstrates that 5-HTP must
be converted to 5-HT that in turn leaves the AADC cells and
acts on 5-HT2B/2C receptors. These are known to be the main
receptors that increase the LLR with 5-HT, and in particular
the 5-HT2 receptors on motoneurons increase the LLR by
increasing persistent inward currents (Murray et al. 2010,
2011a). The less selective 5-HT2 receptor antagonist methy-
sergide (10 μM) likewise blocked the action of 5-HTP (not
shown; n = 5/5).

Considering that we have previously reported 5-HT itself to
increase the LLR at a very low nanomolar EC50 dose of ~10
nM (Murray et al. 2011a), our present finding that 5-HTP acts
at an EC50 dose 1,000 times higher (10 μM) indicates that there
is a 1,000-fold loss of functional 5-HT during synthesis of
5-HT by AADC. We suspected that this could be partly due to
metabolism of 5-HT in AADC cells and thus tested this by

![Fig. 7. 5-HTP increases spasms in the isolated spinal cord of chronic spinal rats via synthesis of 5-HT with AADC and activation of 5-HT2 receptors, although much of this 5-HT is metabolized by monoamine oxidase (MAO). A: schematic of spinal cord caudal to an injury maintained in vitro, with stimulation of dorsal roots (DR) and recording from ventral roots (VR). B: LLR recorded from VR after stimulation of DR (0.1-ms pulse, 3 x afferent threshold (T)). 5-HTP application (10 μM) increases the LLR (red). C: block of AADC with NSD1015 (NSD, 300 μM 30 min prior) eliminates the response to subsequently applied 5-HTP (10 μM, blue). D: representative dose-response relations for 5-HTP application at increasing doses, with an EC50 of ~3 μM (red line). For comparison, application of 5-HT (green) increased the reflexes at much lower doses than 5-HTP. E: blocking MAO with clorgyline (1 μM) lowered the doses at which 5-HTP increased the reflex 10-fold (orange). Blocking the 5-HT2 receptor with SB206553 (10 μM) eliminated the response to 5-HTP (blue). F: summary of efficacy (peak effect) of 5-HTP and 5-HT on the LLR for all rats with the following conditions: control normal ACSF (red, left), leu-
cine (3 mM, Leu), NSD1015, SB206553 (SB206), and clorgyline (MAOI). Efficacy is normalized to control, so 100% indicates no change (SB206553 and NSD1015). G: summary of EC50 doses of 5-HTP or 5-HT for increasing the LLR, with the following conditions: ACSF, leucine, and clorgyline (MAOI). Note the logarithmic scale. *Significantly different from control 5-HTP ef-
fect. #significantly different from control 5-HT effect. P < 0.05, n > 7 rats/condition.

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blocking metabolism of 5-HT. Monoamine oxidase A (MAO-A) is the key enzyme that metabolizes 5-HT into 5-HIAA and is located intracellularly on the mitochondrial membrane (Kalaria and Harik 1987; Wang et al. 2013). When we applied clorgyline, a MAO-A inhibitor (Lena et al. 1995), 5-HTP still increased LLRs with an efficacy (peak response) similar to 5-HT alone (Fig. 7F), but the EC50 of 5-HTP was markedly decreased to ~1 μM, and thus there is indeed a 10-fold metabolism (loss) of 5-HT after it is synthesized (Fig. 7, E and G). Application of 5-HT itself increased the LLR similarly to 5-HTP, and again the MAO inhibitor did not affect the efficacy of this increase (peak amplitude, Fig. 7F) but did produce a significant reduction in the EC50 of exogenously applied 5-HT (by a factor of 2; Fig. 7G), although this effect was significantly smaller than that of 5-HTP. Thus, roughly speaking, in AADC cells (vessels and neurons) MAO causes an eightfold loss in 5-HT made from applied 5-HTP, and after the 5-HT leaves these cells there is an additional twofold loss to MAO metabolism before the 5-HT diffuses to 5-HT receptors where it increases the LLR (see Discussion for mechanism).

5-HT produced by AADC activates receptors on motoneurons. Next we wanted to confirm that 5-HTP-actuated directly on motoneurons. To do this, we conducted intracellular recordings in motoneurons that were isolated from synaptic input by application of TTX (2 μM) (Fig. 8A). Consistent with 5-HT’s excitatory action on motoneurons (Li et al. 2007; Murray et al. 2010), when 5-HTP was applied in all cases the motoneurons’ input conductance was decreased (slope of thin leak line in Fig. 8, B and C) and resting membrane potential was depolarized (Fig. 8, B and C; voltage at 0 nA, n = 3/3 rats tested). Furthermore, the persistent inward current (Ca PIC) onset voltage was decreased (zero slope region in current at Ca PIC label in Fig. 8, B and C) and the magnitude of Ca PIC was increased (at arrow in Fig. 8C; n = 3/3). All these effects are qualitatively similar to the effects of 5-HT (Li et al. 2007; Murray et al. 2010) and together suggest that applied 5-HTP is converted to 5-HT via AADC and then diffuses out of the AADC-containing cells to the motoneurons, where it acts on 5-HT2B/2C receptors. To confirm these results, we also recorded overall spontaneous motoneuron pool activity on ventral roots in the presence of synaptic blockers (50 μM APV, 50 μM bicuculline, 10 μM CNQX, 5 μM strychnine) to block indirect synaptic inputs to the motoneurons. Indeed, in this synaptic blockade, 5-HTP significantly increased spontaneous ventral root activity (264 ± 76% increase; n = 8, P < 0.05), though with a somewhat higher EC50 (53.7 ± 38.9 μM) compared to without synaptic blockade (15.1 ± 15.4 μM).

DISCUSSION

SCI and neurodegenerative diseases like PD result in a loss of monoamines, like 5-HT and DA, that are critical for motor function. One long-standing therapeutic strategy has thus been to replace monoamines by giving their precursors, such as 5-HTP or L-DOPA (Barbeau and Rossignol 1990; Bedard et al. 1979; Goldstein et al. 1982; Guertin 2009; Hayashi et al. 2010; Ikemoto 2004; Ikemoto et al. 1997; Lidbrink et al. 1974). However, the mechanism by which precursors are converted to monoamines has remained uncertain, especially with SCI, considering that the enzymes for synthesis of monoamines, including AADC, are normally mostly confined to monoamine fibers and these fibers are almost entirely lost with a complete spinal transection (Murray et al. 2010; Newton and Hamill 1988). Our results demonstrate that an upregulation of AADC in spinal cord blood vessels, pericytes, and neurons after SCI compensates for a loss of AADC in descending monoamine fibers after SCI. AADC in both spinal vessels and neurons is capable of generating 5-HT when the precursor 5-HTP is exogenously applied, thus explaining the therapeutic action of precursors like 5-HTP. In the brain of normal rats, AADC activity has previously been seen in vessel endothelial cells and pericytes, although in this case the monoamines produced from precursors by AADC are quickly metabolized by MAO (within minutes) (Wade and Katzman 1975) and thus do not accumulate substantially unless MAO is blocked (Harder et al. 1979a, 1979b), unlike what we see after injury.

Our results specifically demonstrate that exogenously applied 5-HTP acts by the following steps (also shown in the schematic in Fig. 9): 1) 5-HTP (and other amino acids like tryptophan) is taken up by the L-transport system (leucine sensitive) and other amino acid carrier systems into AADC-containing cells (vessels and neurons); 2) AADC synthesizes 5-HT (and likely tryptamine) inside the AADC-containing cells; 3) much of this synthesized 5-HT is then metabolized by MAO (MAO blockers enhance 5-HTP potency 10-fold); 4) the

![Fig. 8. 5-HTP directly increases motoneuron excitability in chronic spinal rats. A: schematic of setup for intracellular recording from motoneurons (MN) in spinal cords from chronic spinal rats in vitro. B: motoneuron current response (thick line, bottom) to slow voltage ramp (triangular plot, top) recorded in the presence of TTX to block synaptic inputs and thus isolate the motoneuron. At about ~55 mV a persistent inward calcium current (Ca PIC) was activated that caused the current to deviate downward (at arrow), relative to the extrapolated leak current (thin line). C: application of 5-HTP (100 μM) lowered the onset voltage of the Ca PIC (to near ~70 mV) and increased the size of the Ca PIC (arrow). Also, the input conductance was decreased (leak current slope), as was the resting potential (at 0 nA, ticks).](http://jn.physiology.org/doi/10.1152/jn.00508.2013)
remaining 5-HT somehow leaves the cells, which likely occurs by either simple diffusion or facilitated diffusion using the plasma membrane monoamine transporter (PMAT) (Berry et al. 2013; Engel and Wang 2005), although likely only a few percent of the 5-HT diffuses out of the cells in this way (100-fold less than applied 5-HTP, as occurs at the BBB) (Oldendorf 1971); 5) about half of this extracellular 5-HT is diffusely taken up by other cells and further metabolized by MAO (thus 2-fold loss); and 6) the remaining 5-HT reaches 5-HT2 receptors (SB206553 sensitive) on motoneurons (TTX resistant) and increases the motoneuron excitability and LLRs, as previously reported for 5-HT (Murray et al. 2010). Overall, there are 1,000-fold losses in 5-HT, compared with applied 5-HTP, before 5-HT reaches the motoneuron receptors, and thus ∼15 μM 5-HTP must be present in the spinal cord (in vitro) to affect motoneurons to produce the amount of 5-HT that affects motor function (∼15 nM EC50).

Given these considerations of how 5-HT is synthesized from 5-HTP after SCI, it is very unlikely that functional amounts of 5-HT can be endogenously produced by the spinal cord after injury. For useful 5-HT to be produced in the spinal cord, according to our in vitro data, circulating endogenous 5-HTP would have to exceed 15 μM, and this never occurs, as 5-HTP is generally not detected in serum, or at best is detected in trace nanomolar quantities (Coppi and Barchielli 1989; Engbaek and Magnusson 1978; Kema et al. 2000; Tyce and Creagan 1981). Furthermore, our in vivo data show that we must apply ∼1 mg/kg 5-HTP systemically to affect motor function, which would raise systemic 5-HTP concentrations to ∼5 μM (0.001/220 mol wt, assuming uniform distribution), consistent with our in vitro data (15 μM EC50), but again this is much higher than the trace nanomolar amounts of 5-HTP endogenously present in serum. These pharmacological data are consistent with our immunolabeling showing a complete absence of 5-HT in the injured spinal cord, even though the same immunolabeling methods can detect 5-HT produced by exogenously applied 5-HTP at near-minimal functional doses (as discussed further below).

While AADC is abundant outside of the spinal cord, especially in the liver, kidney, and gut (Berry 2004), this peripheral AADC is unlikely to produce 5-HT that affects spinal motor function, because only a very small fraction of serum 5-HT can cross the BBB (1–2%) (Oldendorf 1971), serum 5-HT concentrations are kept at low nanomolar levels by avid uptake into platelets (Kema et al. 2000; Paasonen 1965), and very little 5-HT is released into circulation by peripheral AADC sources. Again, this is consistent with the lack of functional endogenous 5-HT that we see after injury.

Finally, our data demonstrate that even if there is a small amount of endogenous 5-HT in the cord after injury that our methods did not detect, this 5-HT is likely to be taken up diffusely by many structures in the spinal cord, as occurs when we artificially raise 5-HT. We do not know the nature of this transport system, but we do know that the majority of the 5-HT transported by this system is metabolized by MAO, as discussed further below.

**AADC plasticity.** Previous studies have shown that expression of AADC is very labile in other brain regions, and in particular AADC is known to increase as monoamine levels decrease, as a compensatory mechanism (Berry 2004; Berry et al. 1996). This would allow more production of trace amines like tryptamine, phenylethylamine, or tyramine from readily available dietary amino acids, which in turn activate trace amine-associated receptors (TAARs) that augment the residual action of monoamines at their terminals by, for example, modulating monoamine transporters (Xie and Miller 2009a, 2009b). Our observed increase in AADC with SCI is thus broadly consistent with the loss of 5-HT after injury, although again the function of this AADC remains uncertain. Possibly trace amines produced by AADC play a role after SCI, and we are investigating this in a companion paper (Li and Bennett, manuscript in preparation).
Novel emergence of AADC in neurons with spinal cord injury. For decades we have known that AADC-containing D cells line the central canal (Jaeger et al. 1983, 1984), but, unexpectedly, we find that these cells largely disappear (or at least downregulate AADC) after SCI and thus do not likely contribute to much 5-HT synthesis. Instead, we find that a novel class of mediolateral neurons (NeuN positive and GFAP negative) newly express AADC after SCI and can alone produce enough 5-HT to affect motoneuron function (Fig. 9) when exogenous 5-HTP is applied, even in the presence of carbidopa to block vessel AADC. We find that these AADC neurons do not arise from D cells migrating from the central canal but instead appear to emerge by an upregulation of AADC in existing neurons, which starts early after injury and remains for as long as we have examined (months). Low-level AADC has been reported previously in spinal neurons, especially in neonates (Gozal 2010), so it is not unexpected that neurons can produce AADC. Interestingly, after DA neuron destruction (as in PD) a novel population of AADC neurons also emerge in the striatum (Mura et al. 1995), as we see with SCI.

The normal function of these AADC neurons is unknown. We find that these neurons often have processes that wrap blood vessels, and thus they may play a role in regulating vessel constriction and blood flow, together with the AADC in the vessel endothelial cells. Alternatively, in the striatum AADC neurons have been shown to be GABAergic (Mura et al. 2000) and also suggested to be pluripotent, eventually making DA after nigral destruction in PD (Ike moto 2004).

Vessels contribute to 5-HT synthesis from exogenous 5-HTP after spinal cord injury. It has likewise been known for decades that vessels contain AADC, but this was thought not to normally directly affect spinal function but on the contrary to act as part of the enzymatic BBB, converting excess precursors to monoamines so that MAO can metabolize them and remove them from the brain (Hardebo et al. 1979a, 1979b; Hardebo and Owman 1980; Kalaria and Harik 1987; Spatz and McCarron 1998). However, our findings demonstrate that, at least after SCI, AADC in vessels and associated pericytes contributes to synthesis of 5-HT that affects the spinal cord and specifically motoneuron function (as summarized in Fig. 9), thus completely reversing the role of this BBB enzyme from expelling 5-HT and its precursors to providing 5-HT to the spinal cord. The pericytes are especially interesting, in that they often contain relatively more 5-HT than AADC, suggesting that 5-HT synthesized from 5-HTP is somehow accumulated in these cells.

Over 90% of systemically injected 5-HTP is metabolized by AADC and MAO in the liver or more directly removed by kidney AADC, converting it to 5-HT that is expelled in the urine (Warsh and Stancer 1976). Carbidopa or other similar peripheral AADC blockers (that do not cross the BBB) thus dramatically increase circulating 5-HTP. This ultimately allows much more 5-HTP (or L-DOPA) to enter the spinal cord or brain (Hardebo et al. 1979b; Hardebo and Owman 1980; Warsh and Stancer 1976), where it can be acted on by AADC neurons (and not vessels blocked by carbidopa). However, despite carbidopa markedly increasing the availability of 5-HTP in the spinal cord, the spinal cord LLR is not made more sensitive to 5-HTP by carbidopa (EC50 unchanged), indicating that spinal AADC neurons alone cannot account for 5-HT synthesis without carbidopa. Instead, AADC in vessels must produce at least as much 5-HT as the AADC neurons. Thus, when carbidopa blocks vessel AADC, the loss of 5-HT synthesis is roughly offset by the increased synthesis of 5-HT by AADC neurons from increased 5-HTP delivery into the spinal cord. The importance of vessel AADC is confirmed by our leucine data, where reflexes remain influenced by 5-HTP even though leucine blocks uptake of 5-HT into AADC neurons. Our results raise the possibility that vessel AADC may also play a function in precursor treatment, like 1-DOPA medication, in other neurological disorders like PD.

5-HT is not endogenously synthesized in spinal cord after injury. Spinal microvessel and neuronal synthesis of 5-HT in the spinal cord only occurs when exogenous 5-HTP is applied. In microvessels, 5-HT is synthesized by AADC when 5-HTP is made available from either inside (luminal) or outside (abluminal) the vessel, as occurs when we systemically inject 5-HTP in vivo or bathe the cord in 5-HTP in vitro, respectively. However, with spinal cord transection there is no chance of the vessel getting endogenous 5-HTP from the spinal cord (abluminal), because 5-HTP and its synthesis enzyme (TPH) are largely eliminated together with the brain stem-derived 5-HT fibers after spinal transection (Carlsson et al. 1964; Clinesschmidt et al. 1971), and no endogenous spinal synthesis of 5-HT from endogenous tryptophan occurs in the chronic spinal rats (Fig. 2), which would require TPH as well as AADC. There is much confusion in the literature about the availability of 5-HTP in the blood because of technical difficulties, but it appears that there is little to no detectable 5-HTP that normally circulates in the blood (Coppi and Barchielli 1989; Engbaek and Magnusson 1978; Kema et al. 2000; Tyce and Creagan 1981). Thus the vessels are also unlikely to be able to obtain endogenous 5-HTP from the blood (luminal absorption), and this fits with our finding that vessels and other spinal cord structures are completely devoid of endogenous 5-HT after SCI.

We are confident that our immunolabeling method can detect even small functional amounts of 5-HT made endogenously by AADC in the spinal cord, because when we applied a very low dose of 5-HTP in vivo, near the minimum needed to see a functional increase in reflexes (and 10–30 times lower than standard doses used by us and others) (Bedard et al. 1979), we were still able to detect 5-HT synthesis in vessels and neurons. Thus our finding of a complete lack of detectable 5-HT in the cord caudal to a spinal transection indicates that there are not functional amounts of endogenous 5-HT made in the spinal cord, consistent with a lack of endogenous circulating 5-HTP (Engbaek and Magnusson 1978). Our lack of observed effect of carbidopa alone, the AADC blocker, provides additional support that endogenous 5-HTP has no functional effects, even though exogenous 5-HTP dramatically increases motoneuron function (and LLRs).

Unlike our present study and others (Carlsson et al. 1964), some previous immunolabeling studies have detected small numbers of 5-HT fibers and even occasionally 5-HT-containing cell bodies below a chronic transection (Newton and Hamill 1988, 1989; Takeoka et al. 2009). We suggest that this was due to either 1) nonselectivity of the antibodies detecting something other than 5-HT or 2) fibers associated with the autonomic system that have taken up 5-HT (Lincoln 1995). An interesting possibility related to point 1 is that some 5-HT antibodies may cross-react with tryptamine made by AADC.
from endogenous tryptophan. Regardless of the explanation for this discrepancy, there appears to be no endogenous 5-HT below a spinal transection that affects motor function, as our methods can detect minimal functional amounts of 5-HT and 5-HT receptor antagonists have no effect on motor function (Murray et al. 2010).

Previous HPLC studies have suggested that some 5-HT remains below a spinal transection (Hadjiconstantinou et al. 1984), but, at least in our rats, this 5-HT seems not to be functional. Also, careful examination of earlier HPLC studies of SCI (Hadjiconstantinou et al. 1984) reveals that in processing these spinal cords the authors did not clear the blood from the vessels, and so there must have been substantial 5-HT remaining in the platelets, which are a major store of 5-HT in the body (Paasonen 1965) and thus would have contaminated the results. In our experiments, we found it necessary to take extra precautions to dilate the vessels (with nitrate) and prevent coagulation (with heparin) during tissue perfusion and fixation, to clear the blood from vessels. Without these precautions, blood products were present that produced 5-HT immunolabeling and 5-HT detected in our HPLC studies (see results), although with the immunolabeling these were clearly distinguishable as being inside vessels as opposed to in vessel walls (endothelial cells). Thus we are sure that 5-HT can only be made by AADC in endothelial cells when 5-HTP is exogenously applied, and the labeling of 5-HT on vessels is not an artifact of 5-HT in blood products. Our data from the vessel AADC blocker carbidopa confirm this, because rats treated with prior carbidopa produce no vessel 5-HT from exogenously injected 5-HTP, unlike those without carbidopa, and thus our observed vessel 5-HT is related only to AADC synthesis from exogenous 5-HTP and not to contamination from blood products (platelets).

MAO plays a major role in metabolizing 5-HT after injury. MAO is the major enzyme that metabolizes biogenic amines like 5-HT and tryptamine (Berry 2004). While MAO is classically considered to function in monoamine terminals, there is plenty of evidence that MAO is diffusely located in the spinal cord (and throughout the body), including in nonneuronal structures such as glia and blood vessel endothelial cells (Hardebo et al. 1979b, 1980; Lang et al. 2004; Lena et al. 1995; Vitalis et al. 2002; Wang et al. 2013). Indeed, our data showing that MAO blockers have major influences on the actions of 5-HT and 5-HTP on spinal cord function (LLR) verify that MAO is functional after SCI. MAO acts entirely on intracellular substrates and specifically is tightly bound to the outer membrane of the mitochondria (Broadley 2010; Wang et al. 2013). Thus any observed action of MAO blockers must be on monoamines that are inside cells. Our finding that MAO blockers change the sensitivity (EC50) of the LLR to bath-applied 5-HT indicates that this 5-HT must be taken up by cells and metabolized by MAO, likely by MAO-A (Miller 2011), as summarized in Fig. 9.

Importantly, our results demonstrate that MAO inside AADC neurons and vessels must metabolize the majority of 5-HT made from 5-HTP, and it is partly this loss of 5-HT that makes endogenous 5-HT unlikely to affect motoneuron function, as we have discussed. Specifically, we found that MAO blockers increase the sensitivity of the LLR to 5-HTP by >10-fold, whereas they increase the sensitivity to exogenously applied 5-HT by 2-fold, indicating that much of the 5-HT manufactured by AADC is lost to MAO inside the AADC cells or vessel before the AADC can even escape the cells. The remaining 5-HT must diffuse (with difficulty) out of the cells (Berry et al. 1996); half of this is then lost to MAO in other cells, and only the remaining acts on 5-HT2 receptors to increase the LLR.

Diffuse amine transport after spinal cord injury. Our finding that the majority of exogenously applied 5-HT (or synthesized 5-HT, from 5-HTP) is metabolized by MAO also indicates that 5-HT is somehow taken up by cells in the spinal cord, in order for MAO to be able to metabolize it (MAO is intracellular). This indicates that there is a widespread transport system that takes up 5-HT, even after SCI. This transport idea is broadly consistent with our observations that in spinal cords from both normal rats and 5-HTP-treated chronic spinal rats there is a diffuse staining for 5-HT in almost all spinal structures, especially near sources of 5-HT (GM and specifically motor nucleus; extracellular 5-HT is washed away in tissue processing, and staining thus only represents intracellular 5-HT). This staining is much weaker than the staining of 5-HT fibers in normal rats or vessels in 5-HTP-treated injured rats but is, nevertheless, widespread and clearly exceeding 5-HT in untreated chronic spinal rats (not detectable staining, as in control sections without 5-HT antibody applied). Thus, even if there is an endogenous source of 5-HT or 5-HTP for the spinal cord after injury, which is undetectable by our immunolabeling, most of this 5-HT will not reach motoneurons but instead gets taken up by this diffuse uptake system. This may well further explain why very small, but detectable, amounts of 5-HT are sometimes reported after spinal transection when measured with HPLC or other biochemical methods (see above) (Hadjiconstantinou et al. 1984), and yet this endogenous 5-HT does not seem to affect motoneuron function (not sensitive to neutral antagonists) (Murray et al. 2010), as also discussed above. Furthermore, such a diffuse 5-HT uptake system that prevents 5-HT from affecting motoneurons may well explain why we have repeatedly found that after SCI exogenous 5-HT must be applied at ~10 times the dose expected from the known binding affinity of 5-HT to the relevant 5-HT receptors (Murray et al. 2011a; 2011b). Indeed, this is consistent with our HPLC data that demonstrate that 10 times more 5-HT accumulates in a spinal cord that is incubated in 5-HT than would be predicted if the applied 5-HT just stayed in the extracellular space (which is only 10% of the cord volume) (Vargova and Sykova 2008). The nature of the uptake system is unknown, though it may include PMAT (Engel and Wang 2005). Furthermore, while some studies indicate that the high-affinity serotonin transporter SERT is eliminated with injury (Hayashi et al. 2010; Kong et al. 2011), one study suggests a weak but diffuse SERT remaining in the spinal cord after injury (Husch et al. 2012), and this could contribute to 5-HT uptake.

Summary and possible roles of AADC. In summary, after SCI AADC and MAO enzymes in vessels and neurons maintain the spinal cord’s ability to synthesize and metabolize 5-HT from exogenously applied 5-HTP, despite the loss of monoamine fibers that normally play the traditional role in amine synthesis and metabolism. However, the balance of evidence indicates that endogenous 5-HTP is not available at levels adequate to have any effect on motor function. Thus AADC does not endogenously produce 5-HT that activates 5-HT2 receptors on motoneurons. In any case, the 5-HT2 receptors
become constitutively active after SCI (Murray et al. 2010) and thus no longer need 5-HT for their function. The AADC and MAO enzymes must at least serve to synthesize trace amines like tryptamine from dietary amino acids (Fig. 9) and subsequently metabolize them, respectively (Berry 2004), but we do not know the effects of these trace amines on motor function. Vessel MAO is thought to play a special function in preventing ingested tryptamine from entering the spinal cord, as tryptamine can be a component in certain foods (Broadley 2010). Given the high density of AADC in vessels and pericytes, we speculate that after injury AADC and associated trace amines help regulate blood vessel function, rather than motor function, and the changes in AADC with injury reflect a compensation for the loss of monoamines (Fig. 9), which normally regulate CNS vessel function (Broadley 2010; Lincoln 1995; Peppiatt et al. 2006; Rennels and Nelson 1975).

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AUTHOR CONTRIBUTIONS

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