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

Yaqing Li, Lisa Li, Marilee J. Stephens, Dwight Zenner, Katherine C. Murray, Ian Winship, Romana Vavrek, Glen B. Baker, Karim Fouad and David J. Bennett

Centre for Neuroscience, University of Alberta, Edmonton, Alberta, Canada

Running Title: AADC after spinal cord injury.

Keywords: Serotonin, 5-HTP, blood vessels, pericytes, muscle spasms, spasticity, motoneurons, aromatic acid decarboxylase, monoamine oxidase, spinal cord injury, 5-HT₂ receptor.

Corresponding Author:

Dr. David J. Bennett

5005-A Katz, Centre for Neuroscience,

Faculty of Rehabilitation Medicine,

University of Alberta

Edmonton, Alberta, T6G 2E1, Canada

Phone (780) 492 1516

Email: bennettd@ualberta.ca
ABSTRACT

Spinal cord transection leads to the elimination of the brainstem-derived monoamine fibres that normally synthesize most of the monoamines in the spinal cord, including serotonin (5-hydroxytryptamine, 5-HT) that is synthesized from tryptophan by the enzymes tryptophan hydroxylase (TPH, synthesizing 5-hydroxytryptophan, 5-HTTP) and aromatic L-amino acid decarboxylase (AADC, synthesizing 5-HT from 5-HTTP). Here we examine whether the spinal cord caudal to a transection remains able to manufacture and metabolize 5-HT. Immunolabelling for AADC reveals that, while most AADC is confined to brainstem-derived monoamine fibres in spinal cords from normal rats, caudal to a transection the AADC is primarily found in blood vessel endothelial cells and pericytes, as well as in a novel group of neurons (NeuN positive and GFAP negative), all of which strongly upregulate AADC with injury. However, immunolabelling for 5-HT reveals that there is no detectable endogenous 5-HT synthesis in any structure in the spinal cord caudal to a chronic transection, including in AADC containing vessels and neurons, consistent with a lack of TPH. In contrast, when we applied exogenous 5-HTTP (in vitro or in vivo), AADC-containing vessels and neurons synthesized 5-HT, which contributed to increased motoneuron activity and muscle spasms (long-lasting reflexes, LLRs), by acting on 5-HT$_2$ receptors (SB206553-sensitive) located on motoneurons (TTX-resistant). Blocking monoamine oxidase (MAO) markedly increased the sensitivity of the motoneurons (LLR) to 5-HTP, more than it increased the sensitivity of motoneurons to 5-HT, suggesting that 5-HT synthesized from AADC is largely metabolized in the AADC-containing neurons and vessels. In summary, after spinal cord injury AADC is upregulated in vessels, pericytes and neurons, but does not endogenously produce 5-HT, whereas when exogenous 5-HTTP is provided, then AADC does produce functional amounts of 5-HT, some of which is able to escape
metabolism by MAO, diffuse out of these AADC-containing cells, and ultimately act on 5-HT receptors on motoneurons.
INTRODUCTION

Descending brainstem-derived monoamines, such as serotonin (5-hydroxytryptamine, 5-HT) and noradrenaline (NA) play a powerful role in modulating the excitability of motoneurons in the spinal cord (Harvey et al. 2006; Heckman et al. 2005; Hochman et al. 2001; Jacobs and Azmitia 1992; Murray et al. 2010; Perrier and Delgado-Lezama 2005; Schmidt and Jordan 2000). 5-HT, particularly, increases motoneurons’ resting membrane potential and facilitates low-voltage gated persistent inward currents composed of sodium (Na PIC) and calcium (Ca PIC), allowing motoneurons to depolarize more easily and therefore generate appropriate muscle contractions (Harvey et al. 2006; Heckman et al. 2005). In normal rats, spinalmonoamines are primarily produced in the terminals of descending brainstem fibres (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 acid decarboxylase (AADC) that converts 5-HTP to 5-HT and L-DOPA to dopamine (DA); they also contain monoamine oxidase (MAO) that metabolises 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 monoamines synthesis, including TPH and TH (Clineschmidt et al. 1971; Magnusson 1973; Takeoka et al. 2010).

Despite the elimination of monoamine fibre 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; Mura et al. 2000; Ugrumov 2009), but remains uncertain after spinal cord injury (Jaeger et al. 1983). In the striatum (and arcuate nucleus) destruction of monoamine fibres (DA fibres; 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 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 spinal cord injury. 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 fibres 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. 1984; Jaeger et al. 1983). 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. 1984; Jaeger et al. 1983). 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 CSF (Karasawa 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 spinal cord injury, though 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). Peripheral 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 peripheral 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 are together 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; Kalaria 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-born 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 AADC and MAO enzymes in the 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 spinal cord injury, 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 paper.

While both peripheral and central AADC produces 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, peripherally synthesized 5-HT is mostly transported, stored in vesicles or metabolized before it gets into the circulation, and 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-HTP intrinsic to the spinal cord after transection, considering that most TPH is lost with injury, along with the associated descending 5-HT fibres (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 immunolabelling), and whether it is upregulated in different structures, including D-cells and blood vessels, to compensate for loss of brainstem 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 spinal cord injury, including the production of trace amines, like tryptamine, from dietary amino acids, like tryptophan (Berry 2004; Grandy 2007), as we examine in a later paper (Li and Bennett, in preparation).
METHODS

Adult female rats with spinal cord injury (SCI) were studied and compared to age-matched uninjured normal rats (3.5–5 months old). For the chronic SCI, adult rats were transected at the S2 sacral level at about 2 months of age, and experiments commenced after their affected muscles became spastic (1.5–3 months after injury), as detailed previously (Bennett et al. 1999; Bennett et al. 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 the whole sacrocaudal spinal cord that was removed from the chronic spinal or normal rats and maintained in vitro, while others were made with EMG recordings in the awake rats (Murray et al. 2010). Some rats were additionally studied only 1 to 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; Murray et al. 2011b). Briefly, all the 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 cerebrospinal fluid (mACSF). Spinal roots were removed, except the sacral S4 and caudal Ca1 ventral roots and the Ca1 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 24°C, with a flow rate >5 ml/min. A 45
minute 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 stimulation (single pulse, 0.1 ms, 0.02 mA, corresponding to 3 times afferent threshold, T) using a constant current stimulator (Isoflex, Israel). 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; Murray et al. 2011b). The stimulation was repeated five times at 10 second 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 (Axonscope 8, Axon Instruments). Ventral root reflexes were quantified using 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 where the response is mainly determined by the motoneuron CaPIC activity and not by stimulus-evoked synaptic inputs (Murray et al. 2011a; Murray et al. 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 fully act by the next recording session. Cumulative dose–response relations were
computed by increasing agonist doses at these 12 mins intervals (0.003, 0.01, 0.03, 0.1, etc µM 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 the 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) using 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 to 26–32 MΩ using a rotary beveller (Sutter BV-10). A stepper-motor micromanipulator (660, Kopf) 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 antidromic ventral root stimulation, and noting ventral horn location, input resistance and time constant (>6 ms for motoneurons) (Murray et al. 2010; Murray et al. 2011b). Data were collected with an Axoclamp 2b intracellular amplifier (Axon Instruments, Burlingame, CA) 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) modes.

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 about 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. The 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. The 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, NSD1015 and strychnine (all from Sigma-Aldrich), and SB206553, APV, bicuculline, 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 the 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 (electromyogram). Percutaneous EMG wires (50 μm stainless steel, Cooner wires, USA) 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 times 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 500–4000 ms interval to quantify spasms (long lasting reflex, LLR; using Axoscope, Axon Instr., and Matlab, Mathworks). EMG over 300 ms prior to stimulation was also averaged (background). Drugs were applied in vivo with intraperitoneal injection (i.p.), 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.

**Immunolabelling**

Rats were euthanized with Euthanyl (Bimeda-MTC; 700 mg/kg) and perfused intracardially with 100 ml saline containing sodium nitrite (1g/l, Fisher) and heparin (300 i.u./l, from 1000 units/ml stock, Leo Pharma) for 3-4 minutes, followed by 400 ml 4% paraformaldehyde (PFA; in
phosphate buffer at room temperature), over about 15 mins. 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. Spinal cord sections we mounted on slides and rinsed in Tris-buffered saline containing 0.5% TritonX-100 (TBS-TX). All subsequent antibody applications and rinses that followed them 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-GFAP (1:500, Millipore, MAB360), mouse anti-NeuN (1:500, Chemicon MAB377X, Alexa Fluor488 conjugated) and sheep anti-AADC (1:200, Millipore, AB119) in PBS-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 mins 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 TPS-TX, were applied on slides for 90 min at room temperature. To visualize the AADC with fluorescent labels, tyramide amplification was additionally performed (Invitrogen, TSA Kit #12), which included an Alexa Fluor 488 Tyramide following ABC amplification (Vector PK-6101). Alternatively, to view DAB labelling of AADC or 5-HT biotinylated donkey anti-sheep antibody (1:2000, Millipore, AP184B) or goat anti-rabbit 1:200, Vector ABC kit) was applied at room temperature for 2 hours in TBS-TX, followed by DAB-ABC amplification according to manufacturer guidelines (ABC, Vector PK-6101; DAB, Vector SK-4100). Image acquisition was with both conventional microscopy (for DAB) and confocal microscopy using 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 using 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 labelling of monoamine fibres in normal rats was used as a positive control for AADC and 5-HT, and loss of these fibres in chronic spinal rats a negative control.

Immunolabelling of vessels for 5-HT and AADC was quantified by counting the numbers of labelled vessels per 20 µm section and dividing by section area, to compute 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 immunolabelling and tissue from all rats were photographed at a fixed exposure to reduce bias from differing immunolabelling. 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. Immunolabelling 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 immunolabelling. D-cells were identified 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 mACSF containing sodium nitrite and heparin for 3-4 mins, to clear blood from the spinal cord, as described above for immunolabelling. 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 hr. 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, centrifuged to remove precipitated protein and the concentrations of 5-HT and its acid metabolite 5-HIAA in the supernatant were determined using high pressure liquid chromatography (HPLC) with electrochemical detection according to the procedures of Baker et al. (Baker et al. 1987).

Data analysis

Data were analyzed in Clampfit 8.0 (Axon Instruments) and Sigmaplot (Jandel Scientific) and expressed as mean ± standard deviation (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 instead used 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 (EC$_{50}$) 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, immunolabelling 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 labelling was seen mainly localized on descending monoamine fibres, which originate in the brainstem, consistent with AADC being a key enzyme in monoamine synthesis in normal cords. AADC-labelled fibres were seen descending in the white matter (WM, f in Fig 1A) and traversing into the grey 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 (v, Fig 1A). Occasionally, some blood vessels were weakly AADC-positive, and these vessels also had perivascular AADC-positive cells, which were likely pericytes (p, 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 (as detailed in a later figure).

In chronic spinal rats, AADC staining in monoamine fibres was eliminated caudal to the injury, in accordance with the severance of all descending fibres. However, a marked increase in AADC labelling showed up in almost all spinal vessels in both the WM and the GM (v, Fig 1B), and pericytes on these vessels were densely stained by AADC labeling (Fig 1B). These labelled 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 labelling under any conditions. To quantify the AADC in spinal microvessels, the number of AADC-labelled blood vessel branches per unit area was counted (see Methods for threshold criteria), and found to significantly increase 5-fold with injury (Fig 1D). All spinal regions, including GM, WM, Ventral Horn (VH), CC and Dorsal Horn (DH), showed this 5-fold increase in AADC-positive microvessels in chronic spinal rats, compared to normal (Fig 1C, D). The number of labelled pericytes (AADC-labelled) likewise increased 5-fold (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 the spinal cord injury strong AADC labelling appeared in cells scattered through the grey matter, especially located laterally and dorsally to the central canal at the boundary of the grey matter and white matter (n, Fig 1B, 1C, 1F). These AADC-positive cells were presumably neurons because of their morphology, as we verify later. 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 about 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, VH), 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 Figs 2I and 3C, described later), 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 CC decreased, rather than increased, with injury (Fig 1E), as detailed in a later section.

AADC labelling 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 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 monamine fibres, 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-HP after SCI**

To determine the functionality of the upregulated AADC after SCI, 5-HT immunolabelling was carried out in both normal and SCI rats with or without 5-HTP administration in vivo (i.p. 30 mg/kg, 25 mins before perfusion, DAB label). In normal spinal cords, without 5-HTP injection, only 5-HT fibres (brainstem-derived) were strongly labelled by the 5-HT antibody (f, Fig. 2C, 2G), though some diffuse background 5-HT labelling was present, as discussed below. Blood vessels were not 5-HT positive (v, white spaces), and 5-HT-positive neurons were not found (Fig 2C, 2G). After 5-HTP was injected in these normal animals, weak 5-HT labelling was visualized on a few blood vessels and associated with a few perivascular pericytes in spinal cord (v, p, Fig. 2D, 2H). 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 (i.p. 350 mg/kg/day) to deplete endogenous 5-HT. After two days pCPA depleted almost all endogenous 5-HT in sacral spinal cord, with only small amounts of 5-HT fibres seen near areas
where monoamine fibres 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 labelling found in a small fraction of the microvessels, pericytes and 5-HT fibres in both the WM and the GM (Fig. 2L), and no production of 5-HT elsewhere, including an absence in neurons and motoneurons (Fig 2L).

In untreated chronic spinal rats no 5-HT labelling was observed below the injury site (Fig. 2A). The tissue was so devoid of 5-HT (white) that it was indistinguishable from tissue processed identically for immunolabelling, 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. 2B, 2F), consistent with their strong AADC staining (Fig 1). Overall 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 based on their morphology and medial-lateral location, we suppose that they are the same neurons that were AADC-positive (Fig. 2F, Fig. 1), as confirmed 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).

**Diffusely distributed uptake of 5-HT in the 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 2G-H) and largely eliminated by 5-HT depletion (Fig 2K, with pCPA), suggesting that there was likewise some weak uptake of 5-HT in many structures, presumably from 5-HT escaping from 5-HT fibres. When we directly incubated isolated cords of chronic spinal rats in 5-HT itself (1 µM, for 1 hour, in vitro), there was likewise an increase in diffuse background 5-HT immunolabelling (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 the 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 mins), 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 pargyline, 1 µM each) together with the 1 µM 5-HT, then the HPLC analysis showed a 4-fold 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
collection 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 about 1 µM)(Vargova and Sykova 2008).

In contrast, the 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 (i.p. 30 mg/kg, 25 mins before perfusion), and
then conducted 5-HT immunolabelling. With this 5-HT injection, we found that 5-HT
immunolabelling 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 immunolabelling 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 hr). In these isolated and 5-HTP-treated cords intense 5-HT immunolabelling was visualized in vessels, pericytes and AADC neurons (Fig 2I), as in vivo. In contrast, incubation of isolated cords with 5-HT itself (1uM, 1 hour, in vitro) did not produce distinct 5-HT immunolabelling in vessels, pericytes and neurons, but only produced weak diffuse background 5-HT immunolabelling, 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 to in vivo 5-HTP injections, delivery of 5-HTP to the AADC-neurons appeared more effective, with cell bodies more intensely stained, 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, n and v), 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 immunolabelling 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 spinal cord injury,
Unlike in normal rats, in untreated normal rats, both 5-HT (red) and AADC (green) antibodies mostly labelled brainstem-derived monoamine fibres (f, Fig 3A), with 5-HT fibres making up a substantial fraction of the total monoamine fibres (AADC-labelled), while the remaining fibres 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 labelling revealed AADC-positive vessels and neurons (Fig 3B).

Application of 5-HTP (i.p. 30 mg/kg, in vivo 25 mins before perfusion) in these chronic spinal rats revealed that 5-HT synthesis (red) always co-localized with the enzyme AADC (green; Fig 3C). When we lowered the 5-HTP dose to a level near the minimum to have physiological effects (3 mg/kg, 25 mins prior to perfusion, as detailed later) we still saw both vessel and neuronal synthesis of 5-HT (Fig 2F, inset). This indicates that we can detect 5-HT with our immunolabelling with near minimal functional amounts of 5-HTP, and thus the lack of 5-HT labelling 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 labelling was sometimes weaker in neurons compared to vessels and pericytes, even though the AADC labelling was not (Fig 3C, Fig 4A). We suspect that the 25 mins 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 mins) in vitro with 5-HTP we saw more detailed
morphology labelled with 5-HT in AADC-neurons, as described above (Fig 2I).

On the contrary, pericytes stood out as a location where 5-HT fluorescence was always relatively
more intense than the AADC fluorescence in 5-HTP-injected chronic spinal rats (Figs 3C and
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 mins prior to perfusion, and 30 mins after carbidopa). After this treatment with carbidopa, only the AADC-neurons were 5-HT positive (n, Fig. 4B; not significantly
different in number than 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
immunolabelling staining pattern (Fig. 4B) remained similar compared to 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 labelling in the AADC neurons
became more pronounced (n, Fig.4B) compared to in chronic spinal rats treated with only 5-HTP
(n, 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 co-localized substantially with AADC (Fig. 4B arrow). 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 sacrocaudal cord from chronic spinal rats in vitro with 5-HTP (30 µM) together with leucine (3 mM, 30 mins 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 was hardly any AADC neurons labelled with 5-HT (significant reduction compared to without leucine), but microvessels and pericytes were still strongly labelled for 5-HT (Fig. 4C). Thus, transport of 5-HTP into AADC cells 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 cells.

**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 labelled AADC cells with 5-HT (after a 5-HTP injection) and double labelled with either NeuN (neuron specific) or GFAP (astrocyte specific). As shown in Fig 4D, the 5-HT-producing AADC cells co-labelled
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 (n in Fig 4E), indicating that they were not astrocytes. Additionally, vessels that synthesized 5-HT did not stain for GFAP, indicating the vessel AADC and 5-HT synthesis was not caused by astrocytes, even though astrocytes did surround vessels (arrow in Fig 4E).

**D-cells in the 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 (CC in Fig 5B-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-HP (arrow Fig 5B), allowing us to further characterize these cells with double 5-HT and NeuN or GFAP immunolabelling (for technical reasons we were never able to get the AADC antibody itself to work as a co-label with NeuN or GFAP).

Interestingly, we found that in these 5-HP injected rats (normal or injured), D-cells were not NeuN-positive (arrow 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 co-localize 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 grey matter and producing the new AADC-positive neurons that we observe after injury. Thus, we examined AADC labelling at just 1 to 2 days after spinal cord injury. 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 grey matter 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 which we quantified (Fig 6B). Application of 5-HTP dose-dependently increased the LLR up to doses of 3 mg/kg, with about 1 mg/kg producing 50% of maximal effect (EC$_{50}$; Fig 6B,D,G). Higher doses than 3mg/kg decreased the reflex, similar to what has been demonstrated for the action on 5-HT on spasms (Fig 6D-E, with excitation and
inhibition depending on 5-HT$_2$ and 5-HT$_1$ 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 mins before 5-HTP) to block vessel and peripheral AADC did not inhibit the action of 5-HTP (Fig 6C,E): neither the peak effect of 5-HTP (Fig 6F, 4-fold increase in LLR) nor the EC$_{50}$ (Fig 6G) were 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, with the centrally acting blocker NSD1015 significantly reduced the efficacy of 5-HTP and increased the EC$_{50}$ (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 to 15 mins 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 injections 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 due to stress, which we know can transiently increase the LLR (unpublished observations).

**Mechanisms of action of 5-HTP, including roles of AADC, 5-HT$_2$ 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 long-lasting reflexes (LLRs) from the ventral roots in response to a low-threshold stimulation (3XT) 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) 3-times higher than in pre-drug conditions (Fig 7B,D,F; significantly increase), and with an EC$_{50}$ of about 10 µM (Fig 7G). Again, as previously reported for 5-HT (Murray et al. 2011b), there was always an initial increase in LLR with 5-HTP (over about one order of magnitude in dose), after which further 5-HTP decreased the reflex, in accordance with the dual action of 5-HT$_2$ and 5-HT$_1$ receptors (Fig 7D). We only quantified the 5-HT$_2$ receptor mediated increase in the LLR. The AADC blocker NSD1015 completely inhibited 5-HTP’s effect, so that the LLR was not significantly different than in pre-5-HTP control conditions (in NSD1015, Fig 7C, 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 EC$_{50}$ again of about 10µM, not significantly different than without leucine (Fig 7G). This demonstrates the vessel AADC is sufficient alone to increase spasms, because only vessel AADC makes 5-HT in the presence of leucine, as described earlier (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-HT$_{2B/2C}$ receptor blocker SB206553 (10 µM, Fig 7E–F), which demonstrates that 5-HTP must be converted to 5-HT that in turn leaves the AADC-cells and acts on 5-HT$_{2B/2C}$ receptors. These are known to be the main receptors that increase the LLR with 5-HT, and in particular the 5-HT$_2$ receptors on motoneurons increase the LLR by increasing persistent inward currents (Murray et al. 2010; Murray et al. 2011a). The less selective 5-HT$_2$ receptor antagonist methysergide (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 EC$_{50}$ dose of about 10 nM (Murray et al. 2011a), our present finding that 5-HTP acts at an EC$_{50}$ dose 1000 times higher (10 µM), indicates that there is a 1000 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 blocking metabolism of 5-HT. Monoamine oxidase A (MAO-A) is the key enzyme that metabolises 5-HT into 5-HIAA and is located intracellular 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 a similar efficacy (peak response) to 5-HTP alone (Fig 7F), but the EC$_{50}$ of 5-HTP was markedly decreased to about 1 µM, and thus there is indeed a 10-fold metabolism (loss) of 5-HT after it is synthesized (Fig 7E,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 EC$_{50}$ 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 8-fold loss in 5-HT.
made from applied 5-HTP, and after the 5-HT leaves these cells there is an additional 2-fold 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-HT produced from 5-HTP acted directly on motoneurons. To
do this, we conducted intracellular recordings in motoneurons that were isolated from synaptic
input by applying TTX (2μM) (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 8B-C), and resting
membrane potential was depolarized (Fig 8B-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 8B,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-HT_{2B/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 EC_{50}
(53.7 ± 38.9 μM), compared to without synaptic blockade (15.1 ± 15.4 μM).
DISCUSSION

Spinal cord injury or neurodegenerative diseases like Parkinson’s disease (PD) result in a loss of monoamines, like 5-HT and DA, which 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 spinal cord injury, considering that the enzymes for synthesis of monoamines, including AADC, are normally mostly confined to monoamine fibres, and these fibres 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 spinal cord injury compensates for a loss of AADC in descending monoamines fibres after spinal cord injury. AADC in both spinal vessels and neurons are 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, though 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 (Hardebo et al. 1979a; Hardebo et al. 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-HP potency 10 fold), 4) the 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), though likely only a few percent of the 5-HT diffuses out of the cells in this way (100-fold less than applied 5-HP, 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 1000-fold losses in 5-HT, compared to applied 5-HP, before 5-HT reaches the motoneuron receptors, and thus about 15 µM 5-HP must be present in the spinal cord (in vitro) to affect motoneurons to produce the amount of 5-HT that affects motor function (about 15 nM EC₅₀).

Given these considerations of how 5-HT is synthesized from 5-HP after spinal cord injury, 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-HP would have to exceed 15 µM, and this never occurs, as 5-HP is generally not detected in serum, or at best detected in trace nM 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 about 1 mg/kg 5-HP systemically to affect motor function, which would raise systemic 5-HP concentrations to approximately 5 µM (0.001/220
MW, 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 immunolabelling showing a complete absence of 5-HT in the injured spinal, even though the same immunolabelling 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 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; b). Our observed increase in AADC with spinal cord injury is thus broadly consistent with the loss of 5-HT after injury, though again the function of this AADC remains uncertain. Possibly trace amines produced by AADC play a role after spinal cord injury, and we are investigating this in a companion paper (Li and Bennett, 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. 1984; Jaeger et al. 1983), but unexpectedly, we find that these cells largely disappear (or at least down regulate AADC) after spinal cord injury 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 spinal cord injury 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 previously reported in spinal neurons, especially in neonates (Gozal 2010), so it is not unexpected that neurons can produce AADC. Interestingly,
after DA neuron destruction (like in Parkinson’s diseases) a novel population of AADC-neurons also emerge in the striatum (Mura et al. 1995), like with what we see with spinal cord injury.

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 dopamine after nigral destruction in PD (Ikemoto 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 to not 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; Hardebo et al. 1979b; Hardebo and Owman 1980; Kalaria and Harik 1987; Spatz and McCarron 1998). However, our findings demonstrate that, at least after spinal cord injury, 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 reflex is not made more sensitive to 5-HTP by carbidopa (EC$_{50}$ 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 L-DOPA medication, in other neurological disorders like Parkinson’s disease.

5-HT is not endogenously synthesized in the 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 either from the inside (luminal) or outside (abluminal) of 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 brainstem-derived 5-HT fibres after spinal transection (Carlsson et al. 1964; Clineschmidt 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 spinal cord injury.

We are confident that our immunolabelling 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, 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 immunolabelling studies have detected small numbers of 5-HT fibres and even occasionally 5-HT-containing cell bodies below a chronic transection (Newton and Hamill 1989; 1988; Takeoka et al. 2009). We suggest that this was due to either: 1) non-selectivity of the antibodies detecting something other than 5-HT, or 2) fibres 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 affect 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 spinal cord injury (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 be 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 nitrite) 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 immunolabelling and 5-HT detected in our HPLC studies (see Results), though with the immunolabelling 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-HP is exogenously applied and the labelling of 5-HT on vessels is not an artifact of 5-HT in blood products. Our data from the vessel AADC blocker carbidopa confirms this, because rats treated with prior carbidopa produce no vessel 5-HT from exogenously injected 5-HP, unlike without carbidopa, and thus our observed vessel 5-HT is related only to AADC synthesis from exogenous 5-HP and not to contamination from blood products (platelets).

**MAO plays a major role in metabolizing 5-HT after injury.**

Monoamine oxidase (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 non-neuronal structures such as glia and blood vessel endothelial cells (Hardebo et al. 1980; Hardebo et al. 1979b; 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-HP on spinal cord function (LLR) verifies that MAO is functional after spinal cord injury. 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 (EC$_{50}$) 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-HTP 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 over 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), and 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 spinal cord injury. 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 (gray matter 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 fibres 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 to our immunolabelling, most this 5-HT will not reach motoneurons, but
instead get taken up by this diffuse uptake systems. 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 spinal cord injury exogenous 5-HT must be applied at about 10
times the dose expected from the known binding affinity of 5-HT to the relevant 5-HT receptors
(Murray et al. 2011a; Murray et al. 2011b). Indeed, this is consistent with our HPLC data that
demonstrates 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 spinal cord injury AADC and MAO enzymes in vessels and neurons maintain
the spinal cord’s ability synthesize and metabolize 5-HT from exogenously applied 5-HP,
despite the loss of monoamine fibres that normally provide the traditional role in amine synthesis
and metabolism. However, the balance of evidence indicates that endogenous 5-HP is not
available at adequate levels 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
5HT2 receptors become constitutively active after spinal cord injury (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 reflects 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).

ACKNOWLEDGEMENTS

Funding was provided by NIH (NS-047567 and NS-048170), the Canada Foundation for
Innovation, the Canadian Institutes of Health Research, the Alberta Heritage Foundation for
Medical Research and the University of Alberta. Special thanks to Leo Sanelli and Gail Rauw for
expert technical assistance.
Figure 1. AADC is upregulated in microvasculature and neurons after spinal cord injury.

A: Immunolabelling for antibody to AADC (black, DAB), in horizontal section of spinal cord of normal rat. AADC is seen mainly confined to monoamine fibres (f, seen on expanded scale in lower plot). 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 fibres disappear together with AADC labelling, but nearly all microvessels (v) in both WM and GM are now intensely labelled with AADC, with the darkest labelling in pericytes (p) adhered to the vessel wall. Neurons (n) are also labelled for AADC. Some D-cells (d) remain labelled. 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 labelled neurons (n) 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 to control, P < 0.05, n ≥ 5 rats per condition.

Figure 2. 5-HT synthesis from exogenous 5-HTP in chronic spinal rats. A: In untreated chronic spinal rats no 5-HT immunolabelling is seen in the spinal cord caudal to the lesion (5-HT labelled black with DAB, horizontal sections). B: Injection of 5-HTP (30 mg/kg IP, 25 mins prior to fixation) leads to synthesis of 5-HT in locations where we also see AADC, including on
the microvasculature (v), both in white and gray matter (WM, GM). However, there is also
diffuse 5-HT labelling throughout the spinal cord (darker than A). Expanded image in F below B.
C: In untreated normal rats, brainstem-derived monoamine fibres have 5-HT labelling, and there
is some diffuse background labelling of 5-HT. Expanded image in G below C. D: Injection of 5-
HTP in these normal rats causes a somewhat more intense diffuse 5-HT labelling, but very few
microvessels are labelled. Expanded image in H. E: Quantification of the number of 5-HT-
labelled 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 pCPA (350
mg/kg/day, 2 days prior), chronic spinal rat caudal to injury and chronic spinal rat rostral to
injury. * indicates significantly greater than normal rat, and # indicates significantly less than
chronic spinal caudal cord, P < 0.05, n ≥ 5 rats per condition.  F: Expanded image of chronic
spinal rat treated with 5-HTP (30 mg/kg), showing vessels (v), pericytes (p) and neurons (n)
labelled for 5-HT. The labelled neuron in the inset is from a different rat injected with a lower
dose of 5-HTP (3 mg/kg). G-H: Expanded images of normal rats showing 5-HT labelled fibres
with and without 5-HTP, and a relative lack of 5-HT-labelled vessels. I: Immunolabelling for 5-
HT in spinal cord of chronic spinal rat maintained in vitro and incubated in 5-HTP (30 µM for 1
hr), showing 5-HT synthesized in situ in vessels (v) and neurons (n). J: Lack of 5-HT
immunolabelling in chronic spinal rat injected with 5-HT (30 mg/kg, 25 min prior to fixation). K:
Relative lack of 5-HT immunolabelling in normal rat treated with pCPA, except in a few fibres
near the motor nucleus. L: Injection of 5-HTP in pCPA treated normal rats leads to 5-HT-
labelling in only a few fibres. n ≥ 5 for A – L.
Figure 3. 5-HT is not endogenously synthesized by AADC after injury. A: Double immunofluorescence labelling 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 fibres (f), some of which are 5-HT fibres (double stained orange). A few vessels are weakly AADC positive, but these do not synthesize 5-HT endogenously (not labelled red). B: Double labelling of untreated chronic spinal rats, showing 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 labelled 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.

Figure 4. Characterization of AADC neurons that appear after spinal cord injury.
A: Double labelling 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 labelled for AADC. Transverse section lateral to central canal. B: In chronic spinal rats pre-treated with carbidopa (50 mg/kg, 30 mins prior), and then given 5-HTP, AADC-labelled vessels no longer synthesize 5-HT, whereas AADC-labelled neurons continue to synthesize 5-HT. C: Quantification of the number of 5-HT-labelled 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 (L, in vitro 5-HT and leucine, in this case only), n = 5 to 8 rats per condition. D: Double labelling with 5-HT and NeuN in chronic spinal rat treated with 5-HTP (and carbidopa), showing AADC cells that synthesize 5-HT are indeed AADC neurons. E: Double labelling with 5-HT and 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).

Figure 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 fibres contain AADC and 5-HT (descending 5-HT fibres). Transverse sections shown, with dorsal at top of image, as also in B-D. B: Double labelling in chronic spinal rat treated with 5-HTP, showing a relative lack of D-cells labelled 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-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.

Figure 6. AADC-neurons and vessels both increase spasms in 5-HTP-treated rats.

A: Schematic of awake chronic spinal rat in holding tube, while recording tail muscle spasms with EMG, and evoking spasms with electrical stimulation of the tip of the tail (0.2 ms, 10 mA, single pulse). B: EMG response to stimulation, showing moderate long-lasting reflex (LLR) prior to 5-HTP, and very large LLR with 5-HTP injection (1 mg/kg). C: a block of peripheral and vessel AADC with carbidopa prior to 5-HTP (50 mg/kg, 30 mins prior, see Fig 4) does not prevent 5-HTP from similarly increasing spasms/LLRs (blue), showing that AADC neurons alone can produce enough 5-HT to augment spasms. D-E: Typical cumulative dose-response
relations for LLR in a chronic spinal rat without and with carbidopa. Increasing doses given at 15 mins intervals, and recording made just prior to each new dose (at 15 min post drug). F - G: Quantification of the peak effect of 5-HTP (peak of dose-response) and dose to produce 50% of peak effect (EC$_{50}$), showing that 5-HTP has similar effects on amplitude (efficacy) and potency, in rats without and with carbidopa (n = 5 and 6 rats, respectively). * significantly different from control; and # significantly different from control, but also significantly less than 5-HTP treated, P < 0.05, n > 5 per condition.

Figure 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-HT$_2$ receptors, though much of this 5-HT is metabolized by 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: Long-lasting reflex (LLR) recorded from VR after stimulation of DR (0.1 ms pulse, 3xT). 5-HTP application (10 µM) increases the LLR (red). C: Block of AADC with NSD1015 (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 EC$_{50}$ of about 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-HT$_2$ 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), leucine (3 mM, Leu), NSD1015, SB206553, clorgyline (MAOI). Efficacy normalized to control, so 100% indicates no change (SB206 and NSD1015). G: Summary of EC$_{50}$ 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 effect, # significantly different from control 5-HT effect, P < 0.05, n > 7 per condition.

Figure 8. 5-HTP directly increases motoneuron excitability in chronic spinal rats.

A: Schematic of setup for intracellular recording from motoneurons in spinal cords from chronic spinal rats, in vitro. B: Motoneuron current response (thick lower line) to slow voltage ramp (upper triangular plot), 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). As well the input conductance was decreased (leak current slope), as was the resting potential (at 0 nA, ticks).

Figure 9. Schematic of the action of AADC and MAO after spinal cord injury.

Exogenously applied 5-HTP (orange) is transported from the blood (top, red) into endothelial cells and eventually AADC neurons in the spinal cord (blue lower half of schematic), by facilitated diffusion of the L-transport system (L, green). AADC then converts 5-HTP to 5-HT (orange triangles), although a large part of the 5-HT is metabolized by MAO to 5-HIAA (in AADC containing cells). The remaining 5-HT diffuses (with difficulty) out of the cells, but at least half this is transported (perhaps by SERT) into cells diffusely located throughout the spinal cord.
cord and further consumed by MAO (small circular cell). The remaining 5-HT eventually reaches motoneurons, and activates 5-HT2 receptors that increase motoneuron excitability (PICs and spasms). See details in text. AADC also converts endogenous tryptophan to the trace amine tryptamine, which may play a role after injury, possibly with local effects on endothelial cells or pericytes, although tryptamine is also an excellent substrate for MAO.


Gozal EA. Trace amines as novel modulators or spinal motor function. In: *Biomedical Engineering*, Georgia Inst. of Tech., 2010, p. 262.


