

● INVITED REVIEW

Two-step production of monoamines in monoenzymatic cells in the spinal cord: a different control strategy of neurotransmitter supply?

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Abstract

Monoamine neurotransmitters play an important role in the modulation of sensory, motor and autonomic functions in the spinal cord. Although traditionally it is believed that in mammalian spinal cord, monoamine neurotransmitters mainly originate from the brain, accumulating evidence indicates that especially when the spinal cord is injured, they can also be produced in the spinal cord. In this review, I will present evidence for a possible pathway for two-step synthesis of dopamine and serotonin in the spinal cord. Published data from different sources and unpublished data from my own ongoing projects indicate that monoenzymatic cells expressing aromatic L-amino acid decarboxylase (AADC), tyrosine hydroxylase (TH) or tryptophan hydroxylase (TPH) are present in the spinal cord and that these TH and THP cells often lie in close proximity to AADC cells. Prompted by the above evidence, I hypothesize that dopamine and serotonin could be synthesized sequentially in two monoenzymatic cells in the spinal cord *via* a TH-AADC and a TPH-AADC cascade respectively. The monoamines synthesized through this pathway may compensate for lost neurotransmitters following spinal cord injury and also may play specific roles in the recovery of sensory, motor and autonomic functions.

Key Words: non-monoaminergic cell; monoamine precursor; 5-hydroxytryptophan; L-dopa; serotonin; dopamine; tyrosine hydroxylase; tryptophan hydroxylase; aromatic L-amino acid decarboxylase; spinal cord injury

Introduction

Monoamine neurotransmitters, including *e.g.*, dopamine (DA) and serotonin (5-HT), play an important role in the modulation of sensory, motor and autonomic functions in the spinal cord. These monoamines exert their effects by acting on different types of receptors in the spinal cord (Zhu et al., 2007; Sharples et al., 2014; Ghosh and Pearse, 2015; Zhang, 2016). Depending on the distribution pattern of monoamine receptors and monoaminergic innervation in different spinal segments, a specific monoamine neurotransmitter may exert different functions at different spinal locations. Traditionally it is believed that in mammalian spinal cord, these neurotransmitters originate almost exclusively from different regions of the brain. For instance, DA in the spinal cord is mainly supplied by the hypothalamic A11 projecting neurons (Björklund and Dunnett, 2007), 5-HT by the caudal brainstem raphe nuclei (Jacobs and Azmitia, 1992). Because of this anatomical arrangement, once the spinal cord is disconnected from the brain by trauma or disease, the content of these monoamines is dramatically reduced. Consequently, the function of the spinal cord is also largely interrupted. However, there is considerable evi-

dence that following complete transection of the spinal cord, small amounts of monoamine neurotransmitters can still be detected below the lesion (Schmidt and Jordan, 2000). In addition to causing adaptive plastic changes in monoamine receptors, these small amounts of monoamines may partly account for the increased activity of motoneurons and resultant clinical symptoms such as spasticity. The origin of these small amounts of monoamines has long been a cause for bewilderment. Earlier studies have indicated that one possible origin might be intraspinal monoaminergic neurons; however, their number is very small and they are not evenly distributed in the spinal cord (Mouchet et al., 1986; Newton and Hamill, 1988). Accumulating evidence from recent studies points toward a second possible origin – namely, aromatic L-amino acid decarboxylase (AADC) cells in the spinal cord.

The enzyme AADC is essential for the production of DA from its precursor L-dopa, of 5-HT from its precursor 5-hydroxytryptophan (5-HTP) and of some trace amines from related amino acids (Christenson et al., 1972). Our published data have shown numerous AADC-containing cells in different regions of the rat spinal cord (Zhang et al., 2012; Wienecke et al., 2014). Due to their monoenzymatic

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nature, AADC cells were not previously described as containing monoamines (Jaeger et al., 1983). However, recent findings by both my group and Dr. Bennett's group have shown that the ability of AADC cells to synthesize monoamine neurotransmitters is dramatically increased following spinal cord injury (SCI) (Li et al., 2014; Wienecke et al., 2014; Ren et al., 2016). For example, systemic application of 5-HTP or L-dopa (100 mg/kg body weight) following spinal cord transection caused almost 100% of the AADC cells below the lesion to express 5-HT or DA, whereas only ~10% of AADC cells in the control animals expressed 5-HT or DA given the same dose of precursor. However, such a phenotypic change of AADC cells was only detected when a monoamine precursor was given extrinsically. One may thus ask: Can AADC cells in the spinal cord synthesize monoamines without external monoamine precursor application and, if so, where in the spinal cord do the monoamine precursors originate? Obviously, if L-dopa and 5-HTP are to be produced in the spinal cord, there must be cells that contain tyrosine hydroxylase (TH) or tryptophan hydroxylase (TPH), the rate-limiting enzymes necessary for the hydroxylation of tyrosine to L-dopa and of tryptophan to 5-HTP. So, does the mammalian spinal cord contain such monoenzymatic neurons and, if so, what is the anatomical relationship between AADC cells and those containing TH or TPH?

Evidence for the Existence of Monoenzymatic Cells in the Spinal Cord and Possible Mechanism for the Production of Monoamines

Tyrosine hydroxylase cells have long been reported to exist in the rat cervical and sacral spinal cord (Mouchet et al., 1986). This result was confirmed recently by Hou et al. (2016) who demonstrated that in a normal adult rat, there are ~120 TH cells in the spinal segments L₆ to S₃ which are mostly located in the intermediate lateral parasymphathetic region, and that this number increases significantly following thoracic spinal cord transection, reaching more than 300 cells by the end of the 3rd post-injury week (The number of TH cells reported here is an extrapolation from Figure 4G of Hou et al., 2016 multiplied by 10 their cumulative TH cell count from every 10th section of spinal cord segment L₆ to S₃). They also showed that this region contained AADC cells, which is consistent with our published results (Wienecke et al., 2014). Hou et al. (2016) further demonstrated that about 4% of TH cells also expressed AADC – a figure that did not significantly change following spinal cord transection. My unpublished data from the lower lumbar and sacral spinal cord double-labeled with TH and AADC antibodies have confirmed this finding (Figure 1D1–D3). The TH cells were mainly found in the deep dorsal horn or intermediate region and majority of them did not express AADC although they were in close proximity to AADC cells. However, I have indeed detected a few AADC and TH double-labeled cells although they were not present in the histologic section presented in the D panels of Figure 1. As far as I know, there is no systematic study

concerning the existence of TPH cells in the mammalian spinal cord although it has been suggested that interneurons containing TPH exist in the cat spinal cord (Clineschmidt et al., 1971). My own unpublished studies have demonstrated TPH-immunopositive cells throughout the length of the rat spinal cord (data not shown) but with localization that differs from that of TH cells. In different spinal segments, the TPH-immunopositive cells were located in the superficial dorsal horn (most likely the inner layer of lamina II). Although this region is densely populated by AADC cells, no TPH-AADC double-labeled cells were found. Rather, THP cells and AADC cells are intermingled in this lamina, usually in close proximity to one another (Figure 1E1–E3).

With the demonstration that there are TH and TPH cells in the spinal cord that lie in close proximity to AADC cells, the next question that arises is: How are monoamines produced by these cells when each cell contains only a single enzyme for the production of monoamines? One way to provide monoamines from AADC cells in the spinal cord may be through a two-cell cascade production mechanism – *i.e.*, one group of monoenzymatic cells provides monoamine precursor and once the precursor is made, it is released to the extracellular space from which it is taken up by AADC cells and further converted to a final monoamine product. This hypothesis has been proposed by Ugrumov (2009) for synthesis of DA in the brain, where many monoenzymatic TH cells and AADC cells exist in assorted structures, including the arcuate nucleus and the striatum (Ugrumov et al., 2004; Kozina et al., 2016). These two different monoenzymatic cells could in some physiological and pathological conditions cooperatively provide DA (Kozina et al., 2016). Then one may ask a further question: does such a theory apply to the spinal cord? Given the existence of all necessary monoenzymatic, non-monoaminergic cells in the spinal cord, this seems possible. One piece of evidence supporting this hypothesis comes from a study by Hou et al. (2016). Using a high-performance liquid chromatography technique, they found that 3 weeks after spinal cord transection, more than 10% of the normal complement of DA remains in spinal cord segment L₆ to S₃. Although there were also some TH and AADC bienzymatic cells in this region, their small number suggests that they contribute little to the production of DA. In this connection, it should be stated that, using our sacral spinal cord transection model, we found DA-positive cells only following application of L-dopa (Ren et al., 2016). However, this is most likely due to our having studied only spinal segments S₄ to Ca₁, which lack TH cells. The production of monoamines through this two-cell cascade is believed to be more important for the supply of monoamine neurotransmitters following SCI because the spinal cord has lost its supraspinal supply. We have recently shown that the ability of AADC cells to synthesize 5-HT and DA from their respective precursors is dramatically increased and thus motor output is also dramatically increased following L-dopa or 5-HTP application (Wienecke et al., 2014; Ren et al., 2016). If the two-cell monoamine production hypothesis holds true, then the application of tryptophan or tyrosine should

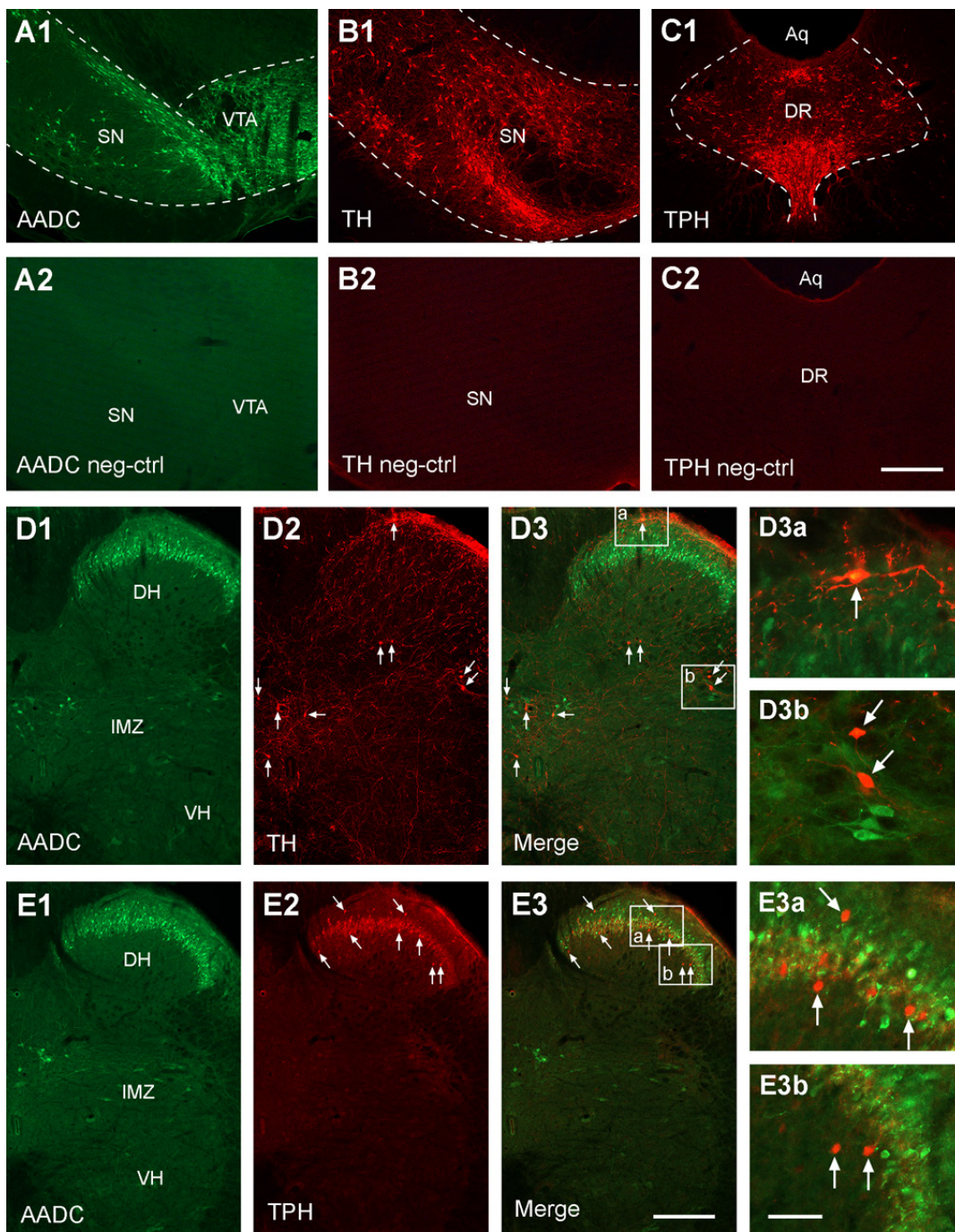


Figure 1 Distribution of TH- and TPH-immunopositive cells in a normal Sprague Dawley rat spinal cord and their relationships with AADC cells.

(A1–C2) Verification, through positive and negative control experiments, of the specificity of antibodies reported in this article. For each antibody, the immunostaining was performed on the adjacent midbrain sections. The results show that AADC, TH and TPH antibodies specifically labeled the structures expected to contain the respective enzymes in the midbrain (A1, B1 and C1). When the primary antibody was omitted the same structures showed no labeling (A2, B2 and C2). In the negative control sections, no effort was made to draw nuclear boundaries due to the difficulty identifying the different nuclei. (D1–D3) In an S2 transverse section double-immunolabeled with AADC and TH antibodies several TH-immunopositive cells (red color) are seen in the deep dorsal horn/intermediate zone (IMZ) and one in the superficial dorsal horn (arrows in D2). Although there are many AADC-immunopositive cells (green color) in the vicinity of TH cells (indicated by arrows in D3, D3a and D3b), no double-labeled cells were seen. (E1–E3) In another S2 transverse section double-immunostained with AADC and TPH antibodies numerous TPH-immunopositive cells were seen in the superficial dorsal horn (arrows in E2). Although these cells co-existed in the same region with AADC immunopositive cells no double-immunolabeled cells were detected (arrows in E3, E3a and E3b). Scale bar in C2, valid for A1–C2, 400 μ m; in E3, valid for D1–D3 and E1–E3, 250 μ m; in E3b, valid for D3a, b, E3a and b, 50 μ m. (Unpublished data from the author. Antibody information: Primary antibodies: rabbit anti-AADC, 1:500, Cat #: AB1569, Merck-Millipore; mouse anti-TH, 1:2000, Cat #: 22941, ImmunoStar; mouse anti-TPH, 1:1000, Cat #: T0678, Sigma-Aldrich. Secondary antibodies (all from Invitrogen): donkey anti-rabbit Alex Fluor 488, 1: 500, Cat #: A21206; donkey anti-mouse Alexa Fluor 594, 1:500, Cat #: A21202). Aq: Aqueduct; DH: dorsal horn; DR: dorsal raphe nucleus; Neg-ctrl: negative control; SN: substantia nigra; VH: ventral horn; VTA: ventral tegmental area.

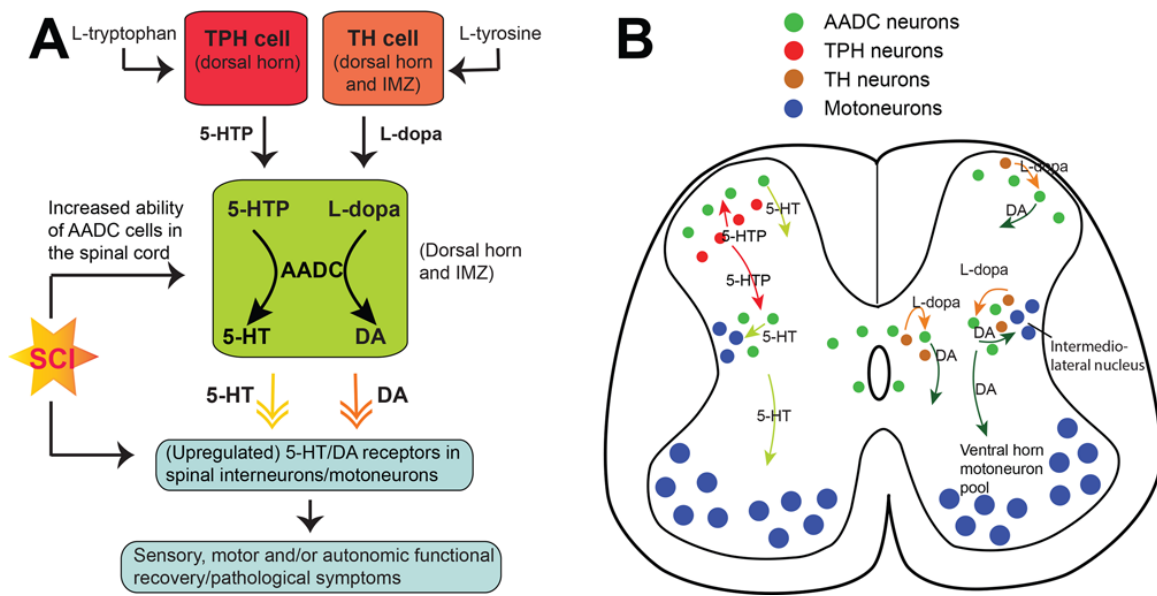


Figure 2 Two-cell monoamine synthesizing hypothesis in the spinal cord.

(A) Workflow of 5-HT and DA production and possible effects for spinal function after spinal cord injury. (B) Schematic drawing of a rat S2 spinal transverse section illustrating the locations and spatial relationships of different monoenzymatic cells and their roles in the production of 5-HT (left side) and DA (right side) in relation to the workflow in A. Left side: TPH cells are located in the superficial dorsal horn (red dots) from which 5-HTP can be synthesized if tryptophan is available. 5-HTP then diffuses to nearby AADC cells (green dots) where it is converted to 5-HT. Right side: TH cells (brown dots) are located in the superficial dorsal horn and deep dorsal horn/intermediate region where L-dopa can be synthesized if tyrosine is available. L-dopa then diffuses to nearby AADC cells where it can be transformed to DA. The arrows indicate presumed diffusion direction of neurotransmitter precursors and neurotransmitters to their targets, which include the motoneurons in ventral horn and the intermediolateral nucleus. 5-HT: Serotonin; 5-HTP: 5-hydroxytryptophan; AADC: aromatic L-amino acid decarboxylase; DA: dopamine; IMZ: intermediate zone; SCI: spinal cord injury; TH: tyrosine hydroxylase; TPH: tryptophan hydroxylase.

also increase motor output due to the production of 5-HT or DA. Paradoxically, it has been shown that the application of tryptophan exerts no effect on spinal reflexes in chronically spinalized cats (Shibuya and Anderson, 1968). Also, there seems to be no reports on the effects of tyrosine on motor output in SCI. These results might be explained by the detailed distribution of TPH and TH cells in the spinal cord. In **Figure 1**, it can be seen that TPH cells are mainly located in the superficial dorsal horn, making it likely that the application of tryptophan may preferentially act on sensory neurons. In addition, tryptophan can be converted to tryptamine directly by AADC cells, thus short-circuiting the production of 5-HT. TH cells are mainly located in the cervical and sacral regions. The cervical TH neurons may only represent caudal extensions of the medullary catecholaminergic cells (Mouchet et al., 1986). TH neurons in the sacral region lie mostly within autonomic nuclei and the superficial dorsal horn (Hou et al., 2016; see also **Figure 1D1–D3**).

Whilst monoamines synthesized by a two-cell cascade might be meaningful in instances of SCI, supraspinal inhibition of AADC cells might well suppress this synthetic route under normal physiological conditions (Wienecke et al., 2014). However, it appears that in some species, monoamines can be synthesized in the intact spinal cord *via* a route involving two different kinds of monoenzymatic cells. For example, in the rhesus monkey, diencephalospinal projecting neurons from the A11 group contain TH

but not AADC, indicating that DA in their spinal cords is synthesized locally by AADC cells from L-dopa produced and delivered by hypothalamic A11 neurons (Barraud et al., 2010). The presence of AADC cells in the spinal cord of the rhesus monkey (Barraud et al., 2010) supports this hypothesis. However, this finding in a non-human primate, may not apply to humans because human A11 spinal projection neurons have been shown to contain both TH and AADC (Kitahama et al., 1998). In any case, the synthesis of DA in monkey spinal cord involving one monoenzymatic neuron in the brain and another in the spinal cord is not equivalent to that involving two different monoenzymatic neurons in the spinal cord.

Functional Significance of Monoamines Produced from Monoenzymatic Cells

What is the functional significance of the two-cell cascade monoamine synthesis strategy in the spinal cord? First, this strategy would ensure that under normal circumstances monoamine activity in the spinal cord would be controlled by the brain, with the spinal cord itself producing only small, functionally insignificant amounts of monoamines. This intraspinal synthetic pathway would attain significance only following SCI, when the synthetic capacity of AADC cells is released from supraspinal inhibitory influences (Wienecke et al., 2014; Ren et al., 2016). In addition, although there are no data on the plastic changes of TPH, there is an increase

in the number of TH cells in lower lumbar and sacral spinal cord segments (Hou et al., 2016). Altogether, if there is tyrosine or tryptophan available in the regions where both AADC and TH or TPH exist, the final monoamine product could be synthesized. However, considering the small number of TH and TPH cells in the spinal cord the amount of monoamines synthesized by this pathway should be limited and far from saturated. Thus, extrinsically applied monoamines or monoamine precursors are still beneficial in different animal models of SCI (Barbeau and Rossignol, 1991; Fedirchuk et al., 1998; Landry and Guertin, 2004; Musienko et al., 2011). The efficacy of extrinsically applied monoamines or monoamine precursors would, however, depend on the time course of SCI since monoamine receptors are usually progressively upregulated in response to SCI (Kong et al., 2010; 2011; Ren et al., 2013) and the activity of AADC cells is also progressively increased (Wienecke et al., 2014). For example, it has been reported that motor activity in the spinal cord below a complete transection is progressively increased in response to the application of 5-HTP, with the effects starting at 5 days and reaching a plateau at 30 days post-injury (Bedard et al., 1979). In the case of acute SCI, the effect of monoamine precursors is very limited (Barbeau and Rossignol, 1991; Fedirchuk et al., 1998) because neural plasticity in monoamine receptors and monoenzymatic cells has not occurred in this time window. Second, because the monoenzymatic cells usually lack the machinery for monoamine transport and storage, the movement of monoamines or their precursors is largely by diffusion. Thus monoamine synthesis through a two-cell cascade in the spinal cord could provide a control mechanism that prevents excessive synthesis of monoamines, especially in pathological situations such as SCI in which most monoamine receptors are upregulated. Third, because AADC is a common enzyme for synthesis of DA, 5-HT and some trace amines, AADC cells can use L-dopa, 5-HTP and certain amino acids to provide different kinds of monoamines/trace amines depending on the actual precursor supply, thus providing a flexible and economical way to modulate spinal cord function. Finally, it provides a non-synaptic route for supplying monoamines to the spinal cord. That is, following a complete SCI, the supply of monoamines to the spinal cord by axons descending from the brain is interrupted; however, monoamine precursors including 5-HTP, L-dopa, tyrosine and tryptophan originating elsewhere – e.g., the endocrine system, food and drugs – can easily pass through the blood brain barrier and enter the spinal cord.

In both clinical practice and animal experiments monoaminergic precursors, monoamines or their receptor agonists/antagonists have been used to promote functional recovery after neurodegenerative diseases or neurotrauma. All these drugs have their advantages and drawbacks. The effects of monoamines are direct and supposed to act on all the subtypes of receptors. The downside is that they usually cannot pass through the blood brain barrier and thus need to be applied directly to the central nervous system, e.g., intrathecally. Monoamine precursors can be converted to mono-

amines and thus have the effects similar to monoamines. The advantage is that most of monoamine precursors can be systemically applied because they can pass the blood-brain barrier. However, it is difficult to control the location and amount of monoamine production following a precursor application and side effects, such as dyskinesia following L-dopa treatment in Parkinson's disease, are likely to occur. While agonists/antagonists act on specific monoamine receptor subtypes and thus exert specific effects on the specific receptors, their downside is that activation of single receptors is not likely to be effective because most physiological activities require orchestral activation of a number of receptors (Hayashi et al., 2010).

Finally, inhibition or activation of monoenzymatic cells alone may be useful in regulating recovery from neurodegenerative diseases or neurotrauma. For example, although the underlying mechanism requires further investigation, inhibition of AADC cells by benserazide hydrochloride can decrease motor activity recorded with electroneurogram *in vitro* (Wienecke et al., 2014).

Conclusions

To conclude, it is posited that, especially in cases of SCI, the monoamine neurotransmitters DA and 5-HT, which are normally received from supraspinal sources, may be synthesized wholly within the spinal cord by a two-step process involving two different populations of monoenzymatic cells (**Figure 2**), thus compensating for the lost source of monoamines. However, for each of these neurotransmitters, this synthetic machinery may be available only in certain sectors and at certain segmental levels and its effects may be exerted locally rather than at a distance. Whereas AADC cells occur widely in the central gray, intermediate zone and superficial dorsal horn, TH and TPH neurons have more restricted distributions. And because they provide the respective precursors for DA and 5-HT, it is the distribution of TH and TPH neurons that will determine which product of the postulated two-cell synthetic process will be made in a given region of the spinal cord. As examples of regional differences, DA seems to be produced mainly in the sacral region where it plays an important role in the regulation of autonomic function of pelvic visceral organs and of the regulation of the micturition reflex. By contrast 5-HT production *via* a two-cell cascade probably occurs mainly in the dorsal horn and thus would be expected to primarily affect sensory information processing. At this point, further physiological and anatomical experiments are needed to validate the two-cell cascade hypothesis. It should be stated that one should not overestimate the significance of the monoamines produced *via* this two-cell cascade. Neuroplasticity following SCI involves many physiological processes – e.g., up- or down-regulation and constitutive activation of monoamine receptors (Kong et al., 2010; Murray et al., 2010; Ren et al., 2013), up- or down-regulation of ion channels and transporters (Bouleguez et al., 2010; Boroujerdi et al., 2011), and sprouting of primary afferent fibers and/or spared corticospinal tract axons (Krenz and Weaver, 1998; Weidner et al., 2001). These

plastic changes may, on the one hand, lead to functional recovery and, on the other hand, to clinical complaints such as spasticity. What is needed is a better understanding of how to take maximal advantage of the beneficial aspects of neuroplasticity following neural trauma or disease.

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