TRACE AMINES AS NOVEL MODULATORS OF SPINAL MOTOR FUNCTION

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TRACE AMINES AS NOVEL MODULATORS OF SPINAL MOTOR FUNCTION

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LIST OF ABBREVIATIONS

CNS	central nervous system
MAO	monoamine oxidase
DA	dopamine
NA	noradrenaline
5-HT	serotonin or 5-hydroxytryptamine
AADC	aromatic-L-amino acid decarboxylase (DOPA decarboxylase)
TH	tyrosine hydroxylase
DBH	β-hydroxylase
PNMT	phenylethanolamine-N-methyltransferase
TAs	trace amines
PEA	β-phenylethylamine
CSF	cerebral spinal fluid
DAT	dopamine transporter
SERT	serotonin transporter
NET	noradrenaline transporter
LATs	L-type amino acid transporters
OCTs	organic cation transporters
TAARs	trace amine-associated receptors
HEK cells	human embryonic kidney cells
SLC	solute carrier
PKU	Phenylketonuria

GPCR	G protein-coupled receptor
IUPHAR	International Union of Pharmacology
CPG	central pattern generator
LLA	locomotor-like activity
NMDA	N-methyl-D-aspartate
L#	lumbar root #
V	ventral
L-dopa	levodopa
P#	postnatal day #
TibA	tibialis anterior muscle, ankle flexor
MGas	medial gastrocnemius muscle, ankle extensor
SemT	semitendinosus muscle, knee flexor / hip extensor
SemM	semimembranosus muscle, knee flexor / hip extensor
VasM	vastus medialis, knee extensor
RecF	rectus femoris muscle, knee extensor / hip flexor
AddM	adductor magnus muscle, hip adductor
Spinal MOD	Spinal Motor Output Detector
GUI	graphical user interface
i.p.	intraperitoneal
Moxd1	monooxygenase, DBH-like 1
aCSF	artificial cerebral spinal fluid
RMS	root mean square
AC	adenylate cyclase

cAMP	cyclic adenosine monophosphate
ATP	adenosine triphosphate
PKA	protein kinase A (cAMP-dependent protein kinase)
PLC	phospholipase C
PIP_2	phosphatidylinositol 4,5-biophosphate
DAG	diacyl glycerol
IP_3	inositol triphophate
CaMKII	calcium/calmodulin-dependent protein kinase type II
NOS	nitric oxide synthase
NO	nitric oxide
PKG	cGMP-dependent protein kinase
EMG	electromyographic

SUMMARY

Trace amines (TAs), tryptamine, tyramine, octopamine, and β-phenylethylamine, named for their low endogenous concentrations in mammals, are related to the classical monoamine transmitters, but have been understudied and thought of as false transmitters. They share structural, physiological, pharmacological, and metabolic similarities with the monoamines, including synthesis by the aromatic-L-amino acid decarboxylase (AADC) enzyme. In 2001, a new class of receptors preferentially activated by the TAs, termed trace amine-associated receptors (TAARs), was discovered establishing a mechanism for TA actions independent of classic monoaminergic mechanisms. While the TAs and some of their receptors are present in the mammalian central nervous system (CNS), their physiologic role remains uncertain. I hypothesized that the TAs are found intrinsically in the spinal cord, and that they are able to modulate spinal neural networks.

Using immunohistochemistry, numerous spinal neurons were identified that express AADC, the TAs (octopamine, tryptamine, and tyramine), and TAARs (TAAR1 and TAAR4). Similar results were seen for AADC and TAAR1 with *in situ* hybridization. The most consistent observation was for labeling D cells associated with the central canal and in motoneurons. Overall, these results provided evidence for the presence of an anatomical substrate onto which the TAs could have intrinsic biological actions in the spinal cord.

Using exogenous application of the TAs in the isolated spinal cord *in vitro*, and *in vivo* in the mid-thoracic chronically spinalized, I showed that the TAs could induce rhythmic locomotor-like activity. TA injection-induced hindlimb motor rhythms observed in chronic spinalized animals, supports TA spinal actions independent of the descending

monoaminergic systems. In the presence of NMDA, TA applications recruited a variety of rhythmic motor patterns in the isolated spinal cord. This ranged from locomotor activity indistinguishable from 5-HT/NMDA induced locomotion to complex patterns including, an episodic form of locomotion where there were locomotor bouts with intervening quiescent periods.

TA actions of pattern generating circuits: (i) had slower kinetics of activation than 5-HT and NA, (ii) were attenuated in the presence of monoamine transport inhibitors, and (iii) had increased intracellular labeling even when incubated in a nominally Na^+-free solution. Together these results suggest that the TAs required transport into neurons to exert their actions, and that transport occurred by Na^+-dependent monoamine transporters as well as additional Na^+-independent transporters.

Finally, I used the *in vitro* isolated spinal cord with attached hindlimbs to record electromyographic (EMG) activity from various hindlimb muscles: (i) to compare the relationship between the TAs and serotonin (5-HT) evoked motor coordination, and (ii) to examine the ability of the TAs to modulate ongoing 5-HT and NMDA locomotor-like activity. The TAs produced both the continuous and episodic patterns on muscles as observed in ventral root recordings, but EMG recordings provided more detailed insight into specific muscle actions. The TAs also generally increased both frequency and amplitude of ongoing 5-HT locomotor frequency, with tyramine and octopamine also particularly able to alter 5-HT motor coordination patterns.

CHAPTER 1

INTRODUCTION

1.1 The classical monoamine neurotransmitters

1.1.1 General background of the monoamines

The classical monoamine neurotransmitters are important central nervous system (CNS) neuromodulatory transmitters. Projections to the spinal cord from descending monoaminergic nuclei are involved in modulating spinal motor, autonomic, and sensory functions (Gerin et al. 1995). Altered monoamine transmitter levels are associated with many pathological disorders including dystonias, Parkinson's disease, schizophrenia, drug addiction, and mood disorders (Premont et al. 2001).

1.1.2 General monoamine transmission

Like all classic neurotransmitters, the monoamines are stored in vesicles at synaptic terminals and released into the synaptic cleft when the neuron is depolarized. On the postsynaptic side of the synapse, they bind G-protein coupled receptors, which lead to intracellular signaling pathways being activated. The monoamines that do not make it across the synaptic cleft are taken up by the presynaptic neurons through specific monoamine transporters. The monoamines are then repackaged into vesicles or are degraded by monoamine oxidases (**Figure 1.1**) (Premont et al. 2001).

1.1.3 Structure and synthesis of the monoamines

Monoamines have one amino group that is connected to an aromatic ring by a two-carbon chain ($-CH_2-CH_2-$). Two types of classical monoamines are catecholamines and

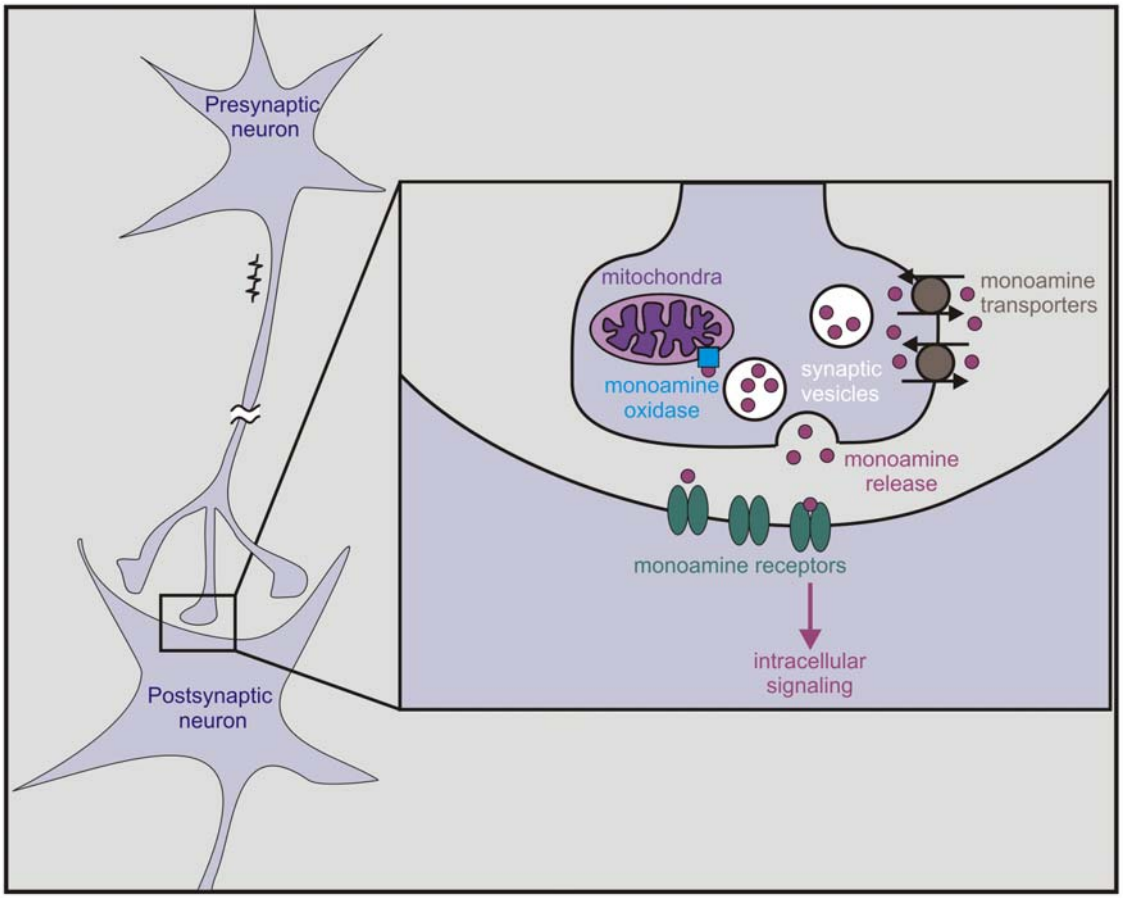

Figure 1.1: Monoamine synaptic terminal

Monoamines are released from synaptic vesicles when the neuron is depolarized. The monoamines either bind postsynaptic monoamine receptors, where they activate intracellular signaling, or are taking up by monoamine transporters. Once transported into the presynaptic neuron, the monoamines are either taken back up into the synaptic vesicles or are degraded by monoamine oxidases (MAOs).

indoleamines. Catecholamines, such as dopamine (DA), noradrenaline (NA), and adrenaline, contain a catechol group, which has two hydroxyl groups attached to a benzene ring. Indolamines, like serotonin (5-HT), contain an indole group, which has six-member benzene ring fused to a five-member nitrogen-containing pyrrole ring.

Both groups are synthesized from the precursor aromatic amino acids in neurons containing the appropriate synthesis enzymes, including aromatic-L-amino acid decarboxylase (AADC or DOPA decarboxylase), which is required for the synthesis of all of the monoamines (**Figure 1.2**). Catecholamines synthesis starts with tyrosine hydroxylase (TH). Then DA, NA, and adrenaline are formed by the cascade of AADC, dopamine-β-hydroxylase (DBH), and phenylethanolamine-N-methyltransferase (PNMT), respectively. 5-HT synthesis requires tryptophan hydroxylase followed by AADC.

1.1.4 Descending monoaminergic systems

Histofluorescence studies by Dahlstrom and Fuxe (1964) first identified populations of monoamine-containing nuclei, which have diffuse projections throughout the CNS. The nomenclature for these populations follows their discovery in chronological order. A cells are catecholaminergic. B cells are serotonergic (Dahlstrom and Fuxe 1965; 1964). The monoamines are synthesized in these nuclei, which are located in the brainstem, and are transported into the spinal cord, where the monoamines are widely distributed within the spinal cord.

DA projects to the spinal cord from the hypothalamus. DA labeling was identified in all laminae of the spinal cord. The labeling was concentrated in the dorsal horn and intermediolateral cell column. Also, there were varicosities in the motoneuronal cell groups (Holstege et al. 1996).

NA projects to the spinal cord from locus coeruleus and A5 and A7 groups in the pontine lateral tegmental. Locus coeruleus neurons project to the dorsal horn and intermediate zone. A5 and A7 axons project in the ventral and dorsolateral funiculi and terminate in the ventral horn and the intermediolateral cell column (Fritschy and Grzanna 1990).

There are 9 groups of 5-HT-containing neurons (B1-B9) (Tork 1990). 5-HT projects to the spinal cord from the raphe nuclei (medulary raphe pallidus (B1), raphe obscuris (B2), and raphe magnus (B3)) and are widespread, innervating all levels of the spinal cord (Rajaofetra et al. 1989; Schmidt and Jordan 2000). 5-HT fibers enter the ventral lumbar spinal cord around embryonic day 15, while the dorsal spinal cord is innervated around embryonic day 20 (Rajaofetra et al. 1989).

1.1.5 Monoamine oxidases

Monoamine oxidase (MAO) is an enzyme that catalyzes oxidative deamination of dietary amines, monoamines, and TAs and is found in the outer membrane of mitochondria. MAOs act rapidly and are very important in maintaining monoamine concentrations. Two different types of MAO, named A and B, have been characterized. They are encoded by different genes, and were originally defined by their sensitivity to MAO inhibitors. MAOA is selectively inhibited by clorgyline, and MAOB is selectively inhibited by deprenly and pargyline. MAOA has a high affinity for 5-HT and NA, while MAOB has a high affinity for PEA. DA and the other TAs are degraded by both. However, it should be noted that in rodent brains, DA is mainly degraded by MAOA, while MAOB plays a larger role in humans and primates (Bortolato et al. 2008; Shih et al. 1999).

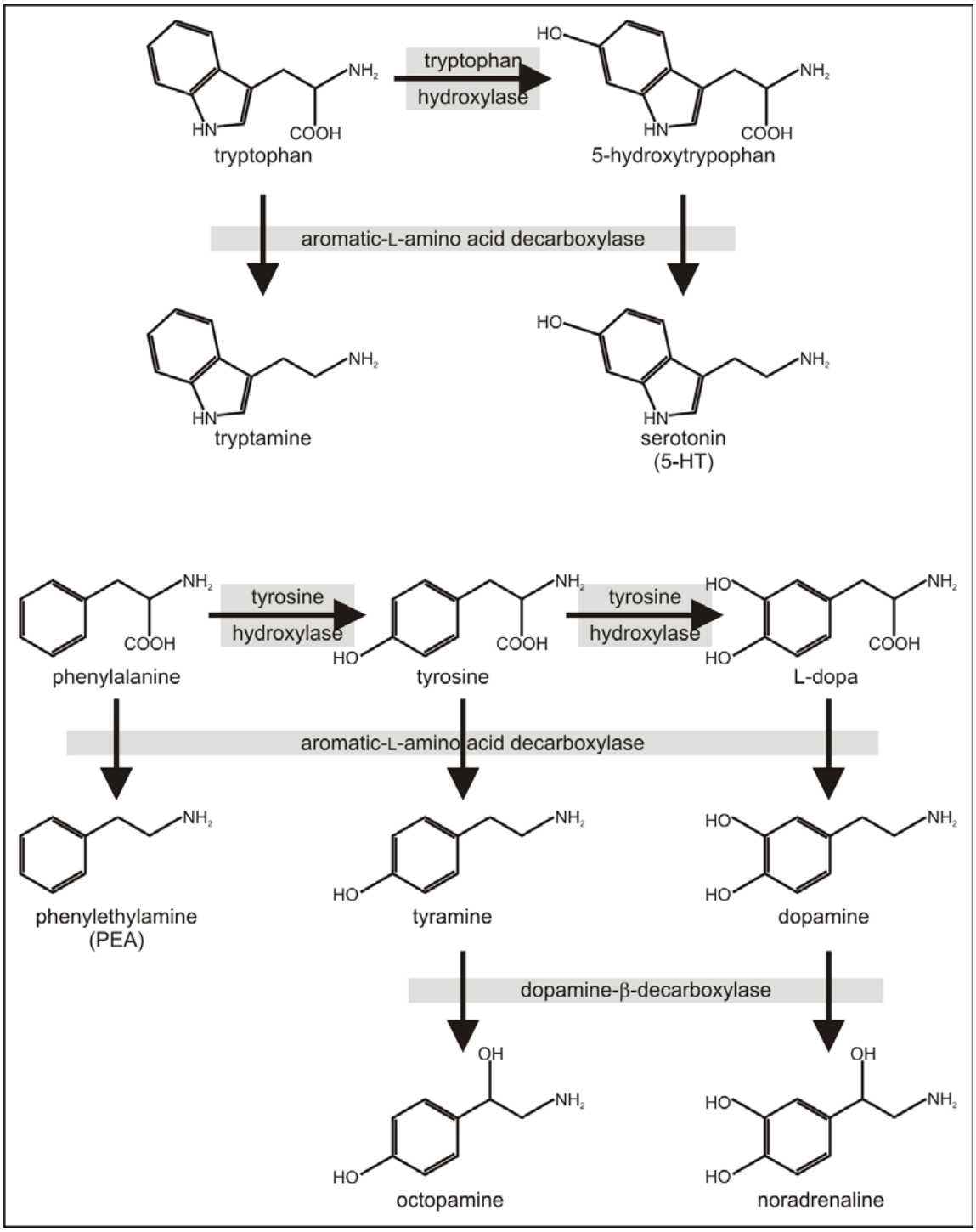

Figure 1.2: Biosynthesis of the monoamines and trace amines

The monoamines [5-HT, DA, and NA] and the TAs [tryptamine, PEA, tyramine, and octopamine] have similar structures and synthesis. Both the monoamines and TAs require aromatic-L-amino acid decarboxylase (AADC) for their synthesis from the aromatic amino acids [tryptophan, phenylalanine, and tyrosine]. Octopamine is the only TA that requires an additional enzyme, dopamine-β-hydroxylase, while all of the monoamines also require other enzymes including tyrosine hydroxylase, tryptophan hydroxylase, and/or dopamine- β-hydroxylase. In the periphery, phenylalanine is also converted to tyrosine by phenylalanine hydroxylase.

In the brain, MAOA was found primarily in catecholaminergic neurons, whereas MAOB is mainly expressed in serotinergic and histaminergic neurons. Both MAOs are also found in cerebral cortices, hippocampus, cerebellum, and spinal cord (Luque et al. 1995; Westlund et al. 1985). *In situ* hybridization demonstrated very strong expression of MAOA in motoneurons (Luque et al. 1995), while binding studies using selective MAOA and B-inhibitors suggest both MAOs are present in the spinal cord and central canal (Luque et al. 1995; Saura et al. 1992). Also, a subpopulation of sensory neurons in dorsal root ganglion were reported to express TH and MAO (Vega et al. 1991).

MAO inhibitors are used to treat a number of disorders, including depression, anxiety disorders, attention deficit disorder, Tourette's, Parkinson's disease, and Alzheimer's disease (Bortolato et al. 2008; Shih et al. 1999).

1.2 Trace amines

1.2.1 General background of the trace amines

The trace amines (TAs), named for their low endogenous concentrations in mammals, are a family of endogenous amines with structural, metabolic, physiological, and pharmacological similarities to classical monoamine neurotransmitters (Saavedra 1989). The TAs include tryptamine, tyramine, octopamine, and β-phenylethylamine (PEA). Like the classical monoamines, synthesis of the majority of the TAs is formed by the enzymatic decarboxylation of the precursor aromatic amino acids, phenylalanine, tyrosine, and tryptophan (**Figure 1.2**), by the enzyme AADC. Tyramine can be further enzymatically converted by DBH into octopamine. Metabolism of the TAs is primarily via MAOs (Shimazu and Miklya 2004). Like the classic monoamines, TAs are thought to play a role in human disorders such as schizophrenia, attention deficit disorder,

Parkinson's disease, and depression (Branchek and Blackburn 2003). Their presence has been documented in all vertebrate and invertebrate species studied, including humans (Axelrod et al. 1976; Berry 2004; Philips et al. 1978; Roeder 2005). While the role of TAs as neurotransmitters in the sympathetic nervous system of invertebrates is well characterized (Roeder 2005), their role in the mammalian CNS is still being characterized. Historically in mammals, TAs have been considered as metabolic by-products (Berry 2004) or "false transmitters" that displace monoamines from storage. Since these compounds were not thought of as active neuromodulators, there was a loss of interest in TAs that began in the late 1980's.

1.2.1.1 Discovery of the trace amines

PEA was first isolated from decomposing gelatin by Nencki, a contemporary of Louis Pasteur, in 1876. Subsequently, PEA was isolated from putrid egg whites, decomposing mackerel, ripe Emmanthaler cheese, and other fermentation products (Grandy 2007).

In 1906, Abelous and colleague demonstrated that extracts from putrefied horse meat dramatically increase arterial blood pressure. In 1909, Barger and Walpole, isolated and purified PEA and tyramine from putrefied horse meat. Barger and Dale demonstrated that PEA, tyramine, octopamine (which would be named later), and NA cause a rapid increase in arterial pressure when injected intravenously into the pithed cat, especially tyramine (Barger and Dale 1910; Barger and Walpole 1909; Grandy 2007).

Octopamine was first detected by Erspamer and Boretti in the salivary gland of *Octopus vulgaris*, hence the name (Erspamer and Boretti 1951). Subsequently, it was identified in the urine of several mammalian species, including humans and rats (Kakimoto and Armstrong 1962a; b).

1.2.1.2 Origin of the name "Trace Amines"

The title, "trace", was given to the TAs at the American College of Neuropsychopharmacology meeting in 1975 to describe their quantity (Boulton 1984; Usdin and Sandler 1976), which is several hundred-fold below those of the classical monoamines (Durden and Philips 1980; Usdin and Sandler 1976). It has since been suggested that it is a misleading title that diminishes their importance (Boulton 1984; Burchett and Hicks 2006; Dewhurst 1984).

As stated by Saavedra (1989): "On the basis of their low concentrations in mammalian brain, octopamine, β-phenylethylamine, tyramine, and tryptamine have been classified as "micro," "minor," or "trace" amines. The connotation 'trace' has perhaps been too restrictive, since the concentrations in brain are heterogeneous under physiological conditions and their turnover rates are very high" (Saavedra 1989).

1.2.1.3 Origin and understanding of the conventional view that the trace amines are "False Transmitters"

In the past, the TAs in the mammal have been considered as metabolic by-products (Berry 2004) or "false transmitters" that displace monoamines from storage, and act on transporters much like the amphetamines, and have no function of their own (Boulton et al. 1988; Saavedra 1989; Usdin and Sandler 1976). The first mention of "false neurochemical transmitter" in regard to the TAs came from Kopin et al. (1964). A "false neurochemical transmitter" is defined as, "substances normally not present in significant amounts in sympathetic nerves, which can be made to accumulate in the nerve endings and which can then be discharged by sympathetic nerve stimulation" (Kopin et al. 1964). He further refines the definition by adding that for it to be a neurotransmitter rather than a

"false transmitter," it must have the same physiological actions and pharmacological properties, in appropriate concentrations, as the natural transmitter" (Kopin 1968).

During the late 1950s and 1960s, there was a substantial amount of research regarding the effects of different enzymes and inhibitors (like MAO inhibitors) involved in the regulation of monoamines and TAs and their effects on sympathetic nerve endings and blood pressure (Bejrablaya et al. 1958; Burn and Rand 1958a; 1957; 1958b; Davey et al. 1963a; b; Day and Rand 1964; Kopin et al. 1964). In interpreting the results, Kopin made some assumptions that lead to the propagation of the theory that the TAs are "false transmitters" and not endogenous amines with their own actions. One was an important distinction between cause and effect. It was thought that "false transmitters" alter the available amount of NA by altering catecholamine synthesis, rather than alterations to catecholamine synthesis forming "false transmitters" (Kopin 1968). Second, Kopin's (1964 and 1968) definition of a "false transmitter" is very limited, and basically his definition made anything that was produced and accumulated in sympathetic nerves that was not NA a "false transmitter." By his definition, if an amine had actions that were different than NA, it must be "false transmitter".

Unfortunately, this view was the predominant view for well over 30 years, and many studies were interpreted based on the idea that the TAs were "false transmitters" that were released with or instead of monoamines and interfered with monoamine actions rather than having their own independent actions. These and other studies assume that there were no dedicated trace aminergic synapses so that the function of the TAs had to occur in other known monoamine-containing neural circuits (Boulton et al. 1988;

Saavedra 1989; Usdin and Sandler 1976). TAs have NOT been thought of as active neuromodulators in mammals (Borowsky et al. 2001).

1.2.2 Endogenous levels of the trace amines

Endogenous levels of TAs are several hundred-fold below those of the classical neurotransmitters DA, NA, and 5-HT, yet their rate of synthesis and circulating levels are similar to that of DA and NA (Durden and Philips 1980; Henry et al. 1988; Paterson et al. 1990). As a result, the TAs have a extremely rapid turnover rate, seen as an endogenous pool half-life of approximately 30 seconds (Durden and Philips 1980) suggesting that the TAs are exquisitely attuned to moment-to-moment fluctuations in substrate. The turnover rates and circulating levels of these neuroactive compounds may "be a more significant index of their importance than their endogenous concentrations" (Saavedra 1989). It should be considered that the estimates on the concentrations of the TA are based on the assumption of a uniform distribution (Dyck 1989), and since the TAs have a heterogeneous distribution with variations present in the distribution of individual TAs (Durden and Philips 1980; Paterson et al. 1990), they may not accurately reflect local concentrations, which are likely to be higher. The concentration of the TAs at the level of the single cell have yet to be determined (Berry 2007).

1.2.2.1 Synthesis of the trace amines

The TAs, PEA, tyramine, and tryptamine, are synthesized by the enzymatic decarboxylation of the aromatic amino acids, phenylalanine, tyrosine, and tryptophan (**Figure 1.2**), by the enzyme AADC (Dyck et al. 1983). Tyramine can be further enzymatically converted by DBH into octopamine. Conversion from the TAs to the monoamines does not appear to occur (Berry 2007).

The conversion from tyrosine to phenylalanine is via tyrosine hydroxylase in the CNS, but in extra-neuronal tissue it is primarily via phenylalanine hydroxylase (Berry 2007).

1.2.2.1.1 Dietary affects on the levels trace amines and aromatic amino acids

TAs and aromatic amino acids are found in many of the foods we eat. For example, tyramine is found in aged meat and cheese (Gardner et al. 1996), PEA is found in chocolate (Ghozlan et al. 2004), and the aromatic amino acids are found in all foods that have protein, such as meat. It has been shown that meals cause physiologic-size changes in aromatic amino acid concentrations (Fernstrom 1990). The raised levels of the aromatic amino acids in the brain affect the synthesis of neurotransmitters such as serotonin (Fernstrom and Wurtman 1971) and the catecholamines (Wurtman et al. 1974), and, presumably, the synthesis of the TAs. The enzyme composition of each neuron dictates what it will produce (Fernstrom 1990); for example, if a neuron takes up tryptophan and has AADC, but no tryptophan hydroxylase, it will produce tryptamine, but not 5-HT.

While diet-related central changes in aromatic amino acids were assumed to alter brain function by altering monoamine transmitter levels (Fernstrom 1990; 1977), the importance of diet to TA modulatory status may be significant, since diets rich in TAs and aromatic amino acids affect brain function (Branchek and Blackburn 2003) and can lead to excitability changes in locomotor circuits (Foldes and Costa 1975; Jackson 1975a; 1972; 1974; 1975b; Marsden and Curzon 1978; 1974; Thurmond et al. 1977).

Some studies saw increases in locomotion from tryptophan in MAO inhibitor-treated rats (Foldes and Costa 1975; Marsden and Curzon 1978), while others gave tryptophan

alone and saw decreases in locomotor activity (Tricklebank et al. 1978; Wurtman et al. 1980). It has been suggested that the differences could be due to increased relative levels of tryptamine (Foldes and Costa 1975; Wurtman et al. 1980). This is plausible since tryptophan caused a greater percent increase in brain tryptamine than brain 5-HT in MAO inhibitor-treated mice (Marsden and Curzon 1978; 1974) and since 5-HT accumulation rates due to tryptophan do not correlate with motility increases in MAO inhibitor-treated rats (Foldes and Costa 1975).

Phenylalanine and PEA both appear to have effects on the motor systems, possibly through PEA. Mice given phenylalanine supplements show increased motility (Thurmond et al. 1977), while rodents injected with PEA exhibit behaviors resembling those of amphetamines including locomotor activity (Jackson 1975a; b).

1.2.2.1.2 Aromatic-L-amino acid decarboxylase

Aromatic-L-amino acid decarboxylase (AADC) or dopa decarboxylase is the enzyme that decarboxylates aromatic amino acid, L-dopa, and L-5HTP into TAs and monoamines. It is not considered the rate-limiting enzyme for monoamines, except in Parkinson's disease patients with exogenous L-dopa; however, it is the rate-limiting enzyme for the synthesis of TAs (Berry et al. 1996; Dyck et al. 1983). Thus, events that regulate AADC activity should alter TA levels. Rapid changes in AADC activity, like phosphorylation (see below), can act to change the levels of the TAs and fine tuning their actions, thus allowing minute to minute regulation (Berry et al. 1996).

AADC can be regulated at both the level of protein activity and gene expression, which can lead to two phases of alteration in activity. Like the short term regulation of TH (Masserano et al. 1989), the short term changes in activity due to AADC are likely

mediated by phosphorylation, which is a reasonable mechanism for a rapid response. cAMP-dependent protein kinase (PKA) and cGMP-dependent protein kinase (PKG) both phosphorylate AADC *in vitro* (Duchemin et al. 2000; Duchemin et al. 2010). Long term regulation is due to changes in gene expression. There is excellent correlation between mRNA level and AADC activity (Coge et al. 1990). Two forms of AADC, neuronal and non-neuronal, have been identified. They are coded by a single gene, but use two distinct promoters followed by alternative splicing (Hahn et al. 1993; Ichinose et al. 1989).

In response to MAO inhibitors, AADC appears to have different short term and long term effects. Short term, AADC activity decreases following MAO inhibitors and l-DOPA (Cumming et al. 1995). Long term AADC has increased AADC mRNA (Berry et al. 1996).

In the developing rat embryo, AADC positive neurons are observed on day 12 of development in all cells of the notochord and in neuroepithelial cells of the ventral neural tube. AADC was enzymatically active, and these cells were tyrosine hydroxylase negative, which indicates that the monoamines are not being produced by these cells (Teitelman et al. 1983). One possibility is that the TAs are being produced.

There is also evidence that AADC levels in hypothalamus are under circadian controls (Ishida et al. 2002).

1.2.2.1.3 AADC positive neurons in the central nervous system and D cells

Histofluorescence studies by Dahlstrom and Fuxe (1964) first identified populations of monoamine-containing CNS neurons. It is known that monoaminergic neurons are profound modulators of CNS function with diffuse projections throughout the CNS. The

nomenclature for these populations follows their discovery in chronological order. A cells are catecholaminergic. B cells are serotonergic (Dahlstrom and Fuxe 1965; 1964). C cells discovered later by Hokefelt (1974) are adrenaline synthesizing neurons (Hokfelt et al. 1974). In the 1980s, Jaeger and colleagues identified a fourth class of neurons that contain AADC, but do not produce 5-HT, and lack tyrosine hydroxylase (cannot synthesize DA, NA, or adrenaline) (Jaeger et al. 1984a; Jaeger et al. 1983). Following the nomenclature, these cells were classified as D cells. They exist as at least 14 different cell clusters within various regions of the CNS (Jaeger et al. 1984a).

The first cluster of D cells, which are also the largest, are found in the spinal cord distributed along the central canal, primarily in lamina X (Jaeger et al. 1984a; Jaeger et al. 1983). These spinal cord containing AADC positive cells, called D1 cells, were identified by ultrastructural identification of synapses and secretory vesicles. D1 cells project at least one of their processes into the lumen of the central canal, which makes them part of a group of cerebrospinal fluid (CSF)-contacting neurons (Jaeger et al. 1983; Vigh et al. 2004). Spinal CSF-contacting nerve cells situated around the central canal are found from cyclostomes to mammals including monkeys (LaMotte 1988; Vigh et al. 2004). No neurotransmitter was identified for these neurons, and nothing is known about the function of spinal cord D1 cells. It remains a possibility that the D cells could produce the TAs (Jaeger et al. 1983). In fact, they are "ideally suited to the selective synthesis of TAs" (Berry 2004).

The other D cells neuron groups are found in nuclei in the hypothalamus, forebrain, midbrain, pons, and medulla. Interestingly, the most occur in the hypothalamus which is associated with the autonomic nervous system (Jaeger et al. 1984a). D neurons have

been demonstrated immunohistochemically in the brain of laboratory shrews (Karasawa et al. 1994), mice (Nagatsu et al. 1988), rats (Jaeger et al. 1984b; Jaeger et al. 1983; Nagatsu et al. 1988), and cats (Kitahama et al. 1988; Kitahama et al. 2007; Kitahama et al. 1990), and humans (Kitahama et al. 2009).

One intriguing hypothesis is that D1 cells function to monitor CSF-related events and relay the information into modulatory commands for the motor system. Urotensin II has been described as one of the most potent vasoconstrictor substances known to date (Watson and May 2004). Interestingly, in fish, urotensin II-immunoreactive CSF-contacting neurons were also demonstrated (Yulis and Lederis 1988a; b) with urotensin-immunoreactive fibers forming an ascending pathway via the ventrolateral funiculus and possibly also innervating motoneurons (Yulis and Lederis 1988a). Spinal motoneurons, in turn, have been reported to have the most intense urotensin II gene expression of all CNS neurons from frog to man (Coulouarn et al. 1998). Interestingly since all members of the TA family in human are expressed in kidney, and D1 cells may be part of a trace aminergic neuromodulatory system, a role in blood pressure regulation and electrolyte homeostasis is implicated (Borowsky et al. 2001).

It was recently reported that a loss of D-cells occurs in schizophrenic patients (Ikemoto et al. 2003).

1.2.2.2 Degradation of the trace amines

TAs are degraded via both MAOA and MAOB, except for PEA, which is preferentially degraded by MAOB (Philips and Boulton 1979; Yang and Neff 1973).

1.2.2.3 Concentrations and distribution of trace amines

There have been a number of techniques used to determine the amount of the different trace amines in the brain, cerebral spinal fluid, and plasma. Some of the techniques include measuring radioenzymatic assays with chromatographic separation (Danielson et al. 1977; Henry et al. 1988; Saavedra 1974), mass spectrometry with thin-layer chromatography, or gas chromatography (Durden and Davis 1993; Durden et al. 1974; Durden et al. 1973; Karoum et al. 1979; Philips et al. 1974a; b; Warsh et al. 1977; Wilner et al. 1974), and high-performance liquid chromatography (D'Andrea et al. 2003; D'Andrea et al. 2004; Yonekura et al. 1988).

The concentrations of TAs have been looked at in human, rat, rabbit, fowl, octopus, locust, and starfish. While the exact quantity of the TAs reported varies, they all agree that the TAs are heterogenously distribution in the brain. The TAs concentrations in neuronal tissue probably are in the range of 0.1-13 ng/g (Boulton and Juorio 1982; Durden and Davis 1993; Durden et al. 1973; Philips et al. 1974a; b). Adding MAO inhibitors increases the concentration by roughly 10-300% (Boulton 1976).

The highest concentrations of PEA were found in the caudate nucleus, olfactory tubercles, and hypothalamus. Smaller amounts were found in brainstem, cerebellum, and spinal cord (Juorio 1988). There are mixed reports on whether the concentration of PEA is higher in the spinal cord or brain (Boulton et al. 1977; Karoum et al. 1979).

The highest concentration of tyramine was found in the caudate nucleus, followed by hypothalamus and olfactory tubercles, and then finally followed by hippocampus, brainstem, cerebellum and the "rest" of the brain (Juorio 1979; 1977a; b; 1980). Tyramine was found in the spinal cord at higher concentrations that other brain regions

(Spector et al. 1963). Immunolabeling has confirmed a heterogeneous distribution of tyramine in the brain (Kitahama et al. 2005).

1.2.3 Transporters

1.2.3.1 Plasma membrane transporters

Plasma membrane transporters transfer substrates across the plasma membrane in or out of cells. There are a number of them discussed below that are capable of transporting the monoamines and/or the trace amines across the plasma membranes (**Figure 1.2**).

1.2.3.1.1 Monoamine transporters

Monoamine transporters control the extracellular concentrations of monoamines and maintain presynaptic function. They are localized to the presynaptic plasma membrane away from the synaptic cleft in the perisynaptic area and sometimes along axons and dendrites (Daws 2009), suggesting that the transmitters diffuse out of the synaptic cleft to be transported back into the terminal. The different monoamine transporters are dopamine transporters (DAT), 5-HT transporters (SERT), and NA transporters (NET). Monoamine transporters are expressed in neurons that contain their neurotransmitter (Torres et al. 2003). DAT, NET, and SERT are Na^+, Cl^--dependent transporters. Monoamine uptake involves sequential binding and co-transport of Na^+ and Cl^- ions due to an ion gradient (Torres et al. 2003). It has been shown that DAT and NET can transport both DA and NA (Giros et al. 1994).

Monoamine transporters are thought to be involved in psychiatric and neurological disorders. The monoamine transporters are important sites for therapeutic agents. Many drugs have been developed to target the monoamine transporters. There are specific

drugs for each of them. For example, selective serotonin reuptake inhibitors (SSRIs) target SERT and are used to treat depression. Interestingly, the more specific transport inhibitors are actually less clinically efficacious than those that block multiple transporters (Daws 2009). Monoamine transporters are also high affinity molecular targets for drugs of abuse like amphetamines, cocaine, and 3,4-methylenedioxy-metamphetamine "Ecstasy" (Jayanthi and Ramamoorthy 2005; Torres et al. 2003).

Xie et al. (2007) looked at the effects of co-expression of TAAR1 and the monoamine transporters in HEK (Human Embryonic Kidney) cells, a cell line commonly used in tissue culture to express and characterize proteins, including receptors (Thomas and Smart 2005). Monoamine transporters greatly facilitate transport of the TAs across the membrane (Xie et al. 2007). Further, when TAAR1 was co-expressed with monoamine transporters in HEK cells, PEA activation of TAAR1 inhibited uptake of the monoamines and increased the efflux of the monoamines, but this did not occur when only the monoamine transporters were expressed, suggesting an important modulatory role of TAAR1 in monoamine transporter function. Additionally, transport inhibitors blocked monoamine efflux in the co-expressed cells (Xie and Miller 2008).

Sotnikova et al. (2004) looked at the effects of PEA in wildtype, heterozygous, and DAT knockout mice (Sotnikova et al. 2004). PEA produced pronounced striatal DA release in wildtype mice without any detected in DAT knockout mice. Behaviorally, PEA produced a strong transient increase in locomotion in wildtype and heterozygous mice. However, in DAT knockout mice, whose phenotypes already features increases locomotor activity and stereotypy, PEA acted as a potent depressant. These data demonstrate that PEA also acts on targets independent of DAT (Sotnikova et al. 2004).

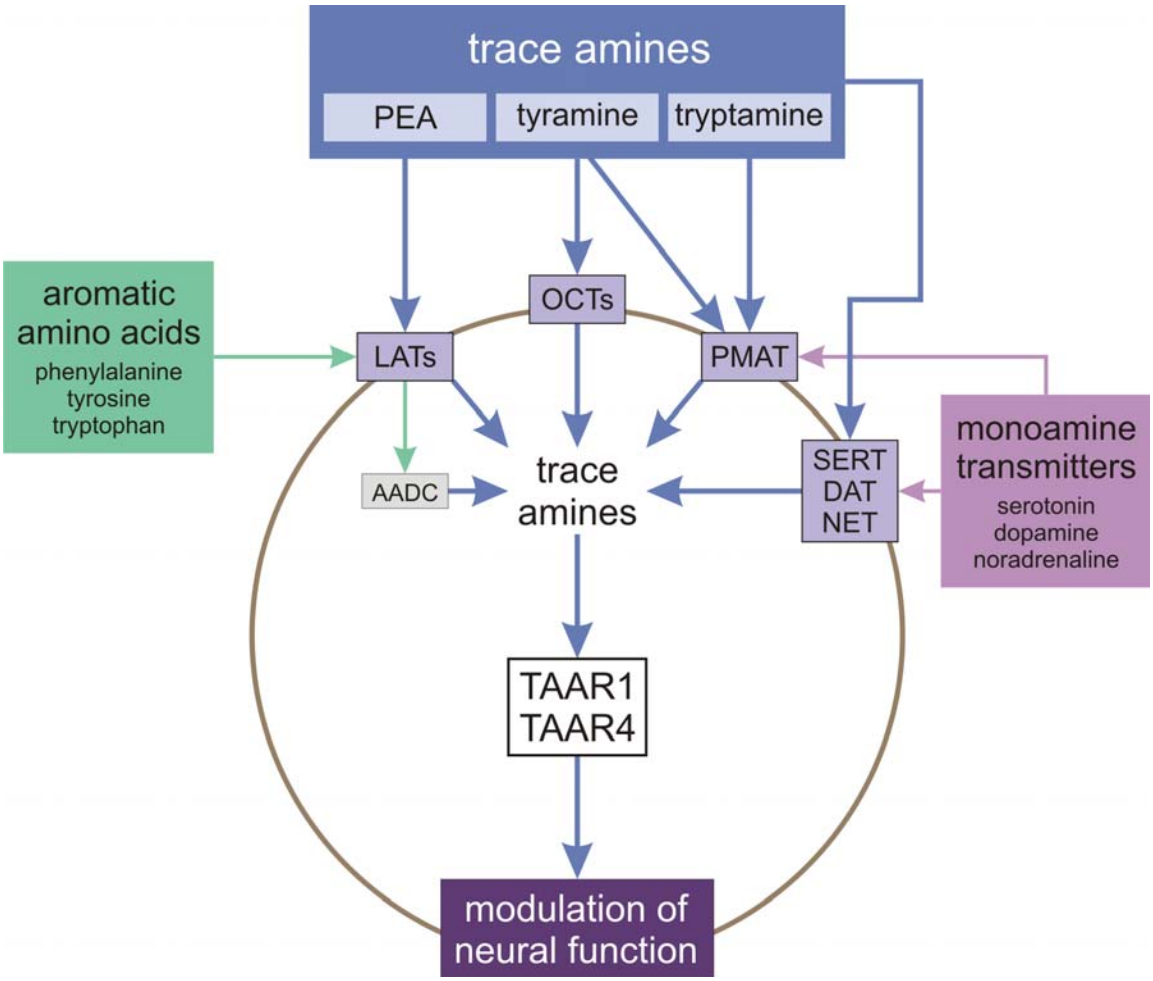

Figure 1.3: Transportation of the trace amines across the plasma membrane and their possible intracellular pathways

The TAs may be transported across the cell membrane via the Na^+-dependent monoamine transporters, SERT, NET, and DAT, and by the Na^+-independent transporters, L-amino acid transporters (LATs), organic cation transporters (OCTs), and the plasma membrane amine transporter (PMAT). Aromatic amino acids are transported across the plasma membrane via LATs, and are synthesized into TAs by AADC inside the cell. Additionally, the TAs could be having actions on the trace amine-associated receptors (TAARs), which are presumed to be located intracellularly. The transport of the TA, octopamine, has not been examined.

1.2.3.1.2 Organic cation transporters

Organic cation transporters (OCT) were first clone by Grundemann et al. (1994) and belong to a family of solute carrier (SLC) transporters, SLC22A (Grundemann et al. 1994; Jonker and Schinkel 2004). They are Na^+ and Cl^- independent transporters (Daws 2009). The family contains three subtypes OCT1, OCT1, and OCT3). OCT3 is the most densely expressed in the brain, followed by OCT1. OCT2 expression in the brain is low (Gorboulev et al. 1997; Grundemann et al. 1997). All three are found to be present in the spinal cord (Allen_Spinal_Cord_Atlas 2009). OCT3 is also known as extraneuronal monoamine transporter (Jonker and Schinkel 2004).

Tyramine is the best physiological substrate for OCT1 and OCT2 and the second best for OCT3. Histamine is the best physiological substrate in rat while it is NA in humans. While DA, NA, and 5-HT can be transported by OCT1 and OCT2 clearance is too low for them to be the primary molecule transported. The same is true for DA and 5-HT transport by OCT3 (Breidert et al. 1998; Grundemann et al. 1998; Schomig et al. 2006).

1.2.3.1.3 L-type amino acid transporters

System L amino acid transporters are plasma membrane amino acid transport system involved in the Na^+-independent transport of large neutral amino acids (Bodoy et al. 2005; Christensen 1990). It is essential in the penetration of amino acids through the blood-brain barrier (Christensen 1990). Four proteins have been identified: L-type amino acid transporter 1-4 (LAT1-4). LAT1 and LAT2 belong to the SLC7 family and are obligatory exchange, which means that they move their substrates in exchange for other substrates. LAT3 and LAT4 belong to the SLC43 family and are facilitative diffusion

transporters, which mean that they move one substrate down its concentration gradient (Bodoy et al. 2005).

LAT1 prefers large neutral amino acids with branched or aromatic side chains such as phenylalanine, tryptophan, leucine, isoleucine, methionine, histidine, tyrosine, and valine (Kanai et al. 1998). LAT2 exhibits broad substrate selectivity. It transports all L-isomers of neutral α-amino acids. Among those with the largest affinity are tyrosine, phenylalanine, and tryptophan (Segawa et al. 1999). Dopamine and tyramine did not inhibit LAT1 or LAT2-mediated transport (Morimoto et al. 2008; Uchino et al. 2002). LAT3 preferentially transports neutral amino acids, such as leucine, phenylalanine, isoleucine, and valine. LAT3 also weakly transports tyrosine and PEA, but not tyramine (Babu et al. 2003). LAT4 preferentially takes up neutral amino acids, phenylalanine, leucine, isoleucine, and methionine (Bodoy et al. 2005). LAT2-4 are found to be present in the spinal cord (Allen_Spinal_Cord_Atlas 2009).

1.2.3.1.4 Plasma membrane monoamine transporter

Plasma membrane monoamine transporter (PMAT) was first cloned and characterized by the Wang group. It is a Na^+-independent and membrane potential-sensitive transporter and is not homologous with SERT, DAT, and NET. PMAT is a part of the SLC29 family (Engel and Wang 2005; Engel et al. 2004). PMAT transports biogenic amines and the neurotoxin MPP^+. Transport affinity varies with the affinity order of tryptamine > serotonin > dopamine > tyramine with NA and adrenaline having very low (millimolar) affinity binding to PMAT (Engel and Wang 2005). Tyramine was capable of *cis* and *trans* transport and is likely to be a true PMAT substrate. While tracer flux studies show PMAT to be a powerful substrate for tryptamine transport, it is *trans*-inhibitory, so

whether tryptamine is a true PMAT substrate remains uncertain (Engel and Wang 2005). PMAT has similar properties to the OCTs including bidirectional transport and prototype OCT inhibitors, including cimetidine, and type II cations are also PMAT inhibitors suggesting that PMAT functions as a polyspecific organic cation transporter. PMAT is neuronally-expressed in widespread brain regions, is not found in astrocytes (Dahlin et al. 2007), and is widely expressed in spinal cord neurons (Allen_Spinal_Cord_Atlas 2009; Engel et al. 2004).

1.2.4 Storage and release of the trace amines

The TAs are detectable in synaptosomes (Baldessarini and Vogt 1972; Boulton and Baker 1975), which means that they are present in nerve terminals. PEA and tryptamine are unlikely to be stored or released from vesicles. The evidence for tyramine is more contradictory. Ultimately, it appears that while tyramine can be found in vesicles, it is unlikely to be released normally or via K^+-induced depolarization (Berry 2004; Burchett and Hicks 2006).

However, octopamine is likely stored in vesicles. Once tyramine is transported into a vesicle it is likely converted to octopamine by vesicular DBH. Octopamine can be released via K^+-induced depolarization and is found in noradrenergic terminals (Burchett and Hicks 2006).

1.2.5 Trace Amine actions in the spinal cord

Intravenous PEA enhanced the monosynaptic reflex in rats (Ono et al. 1991). In earlier work in the neonatal rat cord, tyramine directly depolarized motoneurons with an EC50 value comparable to DA (~50 μM) and depressed monosynaptic reflexes (Kitazawa et al. 1985). Tyramine has also been reported to have had antinociceptive actions (Reddy et al.

1980) and depress flexion and crossed-extension reflexes (Bowman et al. 1964). Much less is known about octopamine in mammalian systems. In spinal dorsal horn neurons, octopamine facilitated spiking activity in many neurons that were depressed by NA (Hicks and McLennan 1978).

1.2.6 Disorders in which the trace amines may be involved

Like the classic monoamines, TAs are thought to play a role in human disorders such as depression, schizophrenia, Phenylketonuria, attention deficit disorder, Parkinson's disease, migraines, addiction, and hypertension (Branchek and Blackburn 2003).

1.2.6.1 Phenylketonuria

Phenylketonuria (PKU) is an autosomal recessive deficiency of hepatic phenylalanine hydroxylase, which converts phenylalanine to tyrosine causing an accumulation of phenylalanine (Pietz 1998). This accumulation of phenylalanine causes elevated levels of PEA in the urine and plasma, since phenylalanine is converted to PEA by AADC. When left untreated, PKU is characterized by epilepsy, microcephaly, and mental retardation. Patients treated early by a phenylalanine-restricted diet are overall developmentally normal; but have motor-related neurological symptoms that include brisk deep tendon reflexes at lower limbs, tremor, clumsiness, and poor motor coordination (Pietz 1998).

PEA has similar pharmacological effects, namely hyperactivity, irritability and excitability, to symptoms seen in PKU patients (Ghozlan et al. 2004). Mice given phenylalanine supplements show increased motility (Thurmond et al. 1977), while rodents injected with PEA exhibit behaviors resembling those of amphetamines including locomotor activity and stereotypy (Boulton 1982; Jackson 1975a; 1972; 1974; 1975b; Paterson et al. 1990).

1.3 The trace amine-associated receptors

1.3.1 Discovery of the trace amine receptors

How the TAs were looked at needed revision when Borowsky et al. (2001) and Bunzow et al. (2001) independently identified a family of mammalian G protein-coupled receptors (GPCR) that displayed a high degree of homology to the classical monoamine receptors. Importantly, two of the TA receptors, now referred to as trace amine-associated receptors (TAARs) (Lindemann and Hoener 2005), were preferentially activated by TAs. TAAR1 has high affinity for PEA and tyramine (Borowsky et al. 2001; Bunzow et al. 2001), while TAAR4 has an affinity for PEA and tryptamine (Borowsky et al. 2001). This identification gives credence to earlier binding studies on the presence of high-affinity sites for tyramine, tryptamine, and PEA (Hauger et al. 1982; Kellar and Cascio 1982; Nguyen and Juorio 1989; Vaccari 1986; van Nguyen et al. 1989) and establishes a mechanism by which TAs can produce effects of their own, rather than the conventional view of interfering with the actions of classical neuromodulators. Also, it introduces the possibility of another major, currently uncharacterized, CNS aminergic transmitter system.

Borowsky et al. (2001) originally identified 15 distinct receptors from human and rodent tissue. Lindermann et al. (2005) set out to identify all TAAR receptors in human, chimpanzee, rat, and mouse. Their genome sequence screening identified 53 TAAR genes in the 4 species. They found 9 human (including 3 pseudogenes), 9 chimpanzee (including 6 pseudogenes), 19 in rat (including 2 pseudogenes), and 16 in mouse (including 1 pseudogene). The TAAR genes maps to a narrow region of a single chromosome in each of the species (Lindemann et al. 2005). In humans, this region is

associated with schizophrenia (Borowsky et al. 2001; Bunzow et al. 2001; Lindemann et al. 2005). Phylogenetic analysis indicated that the TAAR genes are a distinct family of receptors, not an extension of closely related GPCR receptors like the 5-HT receptors. Additionally, it indicated that the TAAR genes originated from a common ancestor (Lindemann et al. 2005), that they evolved after the invertebrate/vertebrate split (Borowsky et al. 2001), and that there were nine genes at the time of the primate/rodent split. These nine gene groups can be divided into three subgroups (TAAR1-4, TAAR5, and TAAR6-9) (Lindemann et al. 2005).

When using pharmacophore similarity analysis to examine the amino acids in the binding pocket for receptor-ligand interactions, the TAARs have a high level of ligand binding pocket similarity. This further agrees that the TAARs are more similar to each other than to other GPCRs, and that they can be divided into the same receptor subgroups as in the phylogenetic analysis. The pharmacophore analysis also predicts that the currently unidentified TAARs ligands must be small molecular weight compounds that are structurally similar to the TAs (Lindemann et al. 2005; Lindemann and Hoener 2005).

1.3.2 Nomenclature of the trace amine-associated receptors

Initially, there were many inconsistencies with how the TA receptors were named. Borowsky et al. (2001) used the abbreviation TA_1 for the TA receptors they found, while Bunzwo et al. (2001) used TAR. As more TA receptors were discovered, it became obvious that a uniform nomenclature was necessary. Since the majority of the TA receptors have unidentified ligands, Lindeman et al. proposed naming the receptor family trace amine-associated receptors (TAARs). The nomenclature is based on the order of the genes on the chromosome and the phylogentic relationships (Lindemann et al. 2005).

This nomenclature has been accepted by The Human Genome Organization (HUGO) Gene Nomenclature Committee (Maguire et al. 2009) and has been adopted in the literature. It is the nomenclature used here.

The International Committee on Standardized Genetic Nomenclature for Mice and Rat Genome and Nomenclature Committee further recommends the use of uppercase for human genes and lower case italics for rodent genes (Maguire et al. 2009).

Interestingly, although it acknowledges the use of the nomenclature TAAR, the International Union of Pharmacology (IUPHAR) officially calls TAAR1 TA_1, which abides by IUPHAR convention that no R be added to the abbreviated name for receptor proteins (Maguire et al. 2009).

1.3.3 Properties of the trace amine-associated receptors

1.3.3.1 Trace amine-associated receptor 1

1.3.3.1.1 Pharmacological characterization of trace amine-associated receptor 1

TAAR1 is most potently activated by tyramine and PEA, and displays a low affinity for tryptamine, octopamine, and DA in the rat and human (Borowsky et al. 2001; Bunzow et al. 2001). Numerous psychostimulant and hallucinogenic amphetamines, which have a very similar structure to tyramine and PEA, are also potent activators of rat TAAR1 (Bunzow et al. 2001). In the rhesus monkey, octopamine and methamphetamine where the most potent activators of TAAR1, but TAAR1 could also be activated by other TAs, monoamines, and amphetamines (Xie et al. 2007). Acetylcholine, nicotine, GABA, glutamate, morphine, and histamines do not activate rat TAAR1. However, none of the

antagonists for the biogenic amine receptors tested were able to antagonize rat TAAR1 (Bunzow et al. 2001).

1.3.3.1.2 Distribution and cellular location of trace amine-associated receptor 1

TAAR1 is localized to many regions of the CNS including the ventral horn of the spinal cord, olfactory bulb, cerebral cortex, amygdale, sustantia nigra, dorsal raphe, the locus coeruleus, and the ventral tegmental area. Many of which are major areas of monoaminergic cell groups (Borowsky et al. 2001; Bunzow et al. 2001; Lindemann et al. 2008; Xie et al. 2007). TAAR1 expression in the CNS is found in a variety of species including human (Borowsky et al. 2001), rhesus monkey (Xie et al. 2007), mouse (Borowsky et al. 2001), and rat (Bunzow et al. 2001).

Confocal images of HEK cells indicated that TAAR1 was found in the cytoplasm rather than at the cell surface (Bunzow et al. 2001; Miller et al. 2005), and was likely localized to intracellular membranes (Xie et al. 2008). Immunohistochemistry of different brain regions clearly shows TAAR1 cytoplasmic staining within the cell body and extending into the axon in rhesus monkey and mice (Xie et al. 2007). Two possibilities mechanisms by which the TAs have actions on an intracellular receptor are: first, transport into cells via transporters, LATs, OCT, PMAT, and monoamine transporters (see **section 1.2.3**) and second, synthesized in the cells where they are having actions (**Figure 1.2**).

TAAR1 expression was also found in stomach, kidney, lung, small intestines, liver, pancreas, prostate, skeletal muscle, spleen, and heart (Borowsky et al. 2001; Chiellini et al. 2007).

1.3.3.1.3 Facilitation of trace amine-associated receptor 1 by monoamine transporters

Given the probable intracellular location of TAAR1, access to TAAR1 agonists could be compromised in HEK cells. Since tyramine and PEA are taken up into cells by the DAT (Miller et al. 2005; Sitte et al. 1998), Miller et al. (2005) found that by co-expressing rhesus monkey TAAR1 and human DAT, PEA and tyramine significantly changed cAMP production compared to TAAR1 alone (Miller et al. 2005). There are neurons that express TAAR1, or DAT, or co-express TAAR1 and DAT in the substantia nigra of both rhesus monkey and mice (Xie et al. 2007). Further, co-expression of TAAR1 with the any of the monoamine transporters (DAT, SERT, and NET) enhanced the activation of TAAR1 by TAs, monoamines, and methamphetamine. Blocking with monoamine transport inhibitors reduced the effect on TAAR1 activation (Xie et al. 2007).

Further, when TAAR1 was co-expressed with monoamine transporters in HEK cells, PEA activation of TAAR1 inhibited uptake of the monoamines and increased the efflux of the monoamines, but this did not occur when only the monoamine transporters were expressed, suggesting an important modulatory role of TAAR1 in monoamine transporter function. Additionally, transport inhibitors blocked monoamine efflux in the co-expressed cells (Xie and Miller 2008). Control experiments show that D1 receptors do not have the same effect as TAAR1 (Xie et al. 2007). D2 autoreceptors have opposite effects as TAARs possibly providing a presynaptic balance in the control of transporter function (Xie and Miller 2009; Xie et al. 2007).

1.3.3.1.4 Trace amine-associated receptor 1 knockout mice

Wolinsky et al (2006) provided the first conclusive evidence for a role of TAAR1 in CNS function by demonstrating behavioral changes in TAAR1 knockout mice. Here, the TAAR1 receptor agonist amphetamine, in the absence of its action on TAAR1, showed

an enhanced psychomotor-stimulating effect, temporally correlated with larger increases in striatal DA and NA release, and 2.6 fold increase in the proportion of striatal high-affinity D2 receptors. TAAR1 therefore appears to play a depressant modulatory role in catecholaminergic function (Wolinsky et al. 2007). Lindemann et al (2008) also studied TAAR1 knockout mice and corroborated several of the above findings. They also found that these mice display enhanced amphetamine-triggered increases in locomotor activity and an elevated spontaneous firing rate of dopaminergic neurons in the ventral tegmental area. Tyramine specifically decreased the spike frequency of these neurons in wild-type but not in TAAR1 knockout mice (Lindemann et al. 2008). Xie and Miller (2008) also examined TAAR1 knockout mice. PEA significantly inhibited uptake and induced efflux of DA, NA, and 5-HT in transfected cells and brain synaptosome of rhesus monkeys and wild-type mice, but not in synaptosomes of TAAR1 knockout mice. The PEA effect was blocked by transporter inhibitors demonstrating that TAAR1 signaling is required for PEA to alter monoamine transporter function. They also showed these effects to be independent of monoamine autoreceptors (Xie and Miller 2008).

1.3.3.2 Trace amine-associated receptor 4

TAAR4, which was previously called TA_2 (Lindemann and Hoener 2005) is activated by PEA and tryptamine. TAAR4 has "poor surface expression" and "subcellular localization" (Borowsky et al. 2001), which suggests that it too may be localized intracellularly.

1.4 Spinal cord anatomy

The spinal cord is an integral part of the central nervous system. It is involved in the transfer of information from the brain to the rest of the body, as well as integration and

coordination of sensory, motor, and autonomic functions. The spinal cord is protected by the vertebral column and surrounded by the meninges. The meninges are three membranes that cover the spinal cord. The outer layer is the dura mater, the middle layer is the arachnoid mater, and the innermost layer is the pia mater. CSF is a clear fluid in the subarachnoid space and the ventricular system (central canal in the spinal cord) that also protects the spinal cord and maintains the electrolytic environment.

The spinal cord is segmentally organized into cervical, thoracic, lumbar, and sacral segments. Spinal circuits controlling hindlimb function dominate in caudal thoracic and lumbar segments levels. In humans, there are eight cervical, twelve thoracic, five lumbar, and five sacral segments. In the rat, there are eight cervical, thirteen thoracic, six lumbar, and four sacral segments. The spinal cord is divided into white and gray matter. The white matter is located on the outside of the gray matter and consists of myelinated axons tracts that relay information to and from the brain and between spinal segments. The gray matter, which is in the center of the cord and is shaped like a butterfly, consists of neuronal cell bodies and can be separated into dorsal and ventral horns. The gray matter can be divided by cytological characteristics of the nerve cells and their cytoarchitectonic aggregation into ten anatomical layers called laminae or Rexed's laminae (I-X) (Rexed 1954; 1952).

Sensory neurons enter the spinal cord via the dorsal roots and synapse onto spinal neurons largely within the dorsal horn, while the ventral horn contains neural elements associated with motor output. These neurons orchestrate coordinated motor activity by projecting to motoneurons in lamina IX whose axons exit via ventral roots to innervate skeletal muscle at the neuromuscular junction (Hochman 2007). Each muscles in

innervated by a pool of motoneurons that form longitudinal columns that may extend up to three spinal segments in the rat (Nicolopoulos-Stournaras and Iles 1983). Among the largest neurons in the CNS, motoneurons receive large amounts of synaptic input through their extensive dendritic arbors which they are able to integrate. Motoneurons are considered the "final common pathway" for motor processing as they are the final step in integrating all of the spinal cord systems, and their output creates the appropriate muscle contraction for the intended behavior (Brownstone 2006; Hochman 2007).

1.5 Locomotion studies

1.5.1 Central pattern generator

The entire neural networks required for generating coordinated locomotor activity resides in the spinal cord (Grillner 1981; Kiehn and Butt 2003) and has been found in almost all vertebrate species studied (Hultborn and Nielsen 2007). These neuronal networks have been called the central pattern generators (CPGs) and consist of sets of interneurons that organized themselves into networks that control motor output through motoneurons. Currently, the exact structure and location of the CPGs in mammals remains unknown, but many models exist trying to explain how the CPGs might work. These CPGs are present at birth (Cazalets et al. 1992; Kiehn 2006; Kudo and Yamada 1987; Smith and Feldman 1987), and are not fully mature until a few weeks postnatal (Westerga and Gramsbergen 1990).

Locomotion is defined as the act of moving from one place to another, in order to accomplish this goal, different vertebrate species use different strategies. For example, lamprey and hagfish are today's representatives of early vertebrates. They employ a left-right alternation of each segment, leading to a wave of motor activity along the body.

This strategy is also used by most fish and amphibian larva. Fish that have fins, which are the precursors to limbs, use them to steer. More complicated vertebrates with limbs, such as cats and rodents, must coordinate the limbs and the trunk to achieve smooth muscle movements and postural control. It is thought that separate CPGs are present to help generate specific motor patterns whether it is simple walking or more complicated gait patterns such as trotting and galloping. At its most basic level on a single limb, the CPG can be thought of as alternation between flexors and extensors (Grillner and Jessell 2009).

1.5.1.1 Models of the central pattern generator

1.5.1.1.1 Half-Center Model

Graham Brown showed that rhythmic alternation of flexor and extensor muscles in the cat hindlimb is a central mechanism during experiments on decerebrate cats who were acutely spinalized and deafferented. In order to explain the alternation between flexor and extensor activation in the limb, Brown proposed a simple neuronal organization, the "half-center" model. Basically, two half-centers, one for flexors and one for extensors, receive reciprocal inhibition, so that only one of the two half centers would be active at a time. The switch from one to the other would happen due to fatigue, leading to an alternation of flexor and extensor muscles. Before Brown and until Lundberg resurrected the idea of the half-center in the 1960s, the prevailing idea was that locomotor movements were due to a series of reflexes (Hultborn and Nielsen 2007; Stuart and Hultborn 2008). Today, the idea of the half-center is still part of most models of the CPG.

1.5.1.1.2 Unit Burst Generator

One limitation of the "half-center" model is that it assumes strict alternation of flexor and extensor activity. However, it has been determined that activation of flexor and extensor muscles is more complex. Instead, there are differences in the onset and offset of different muscles, which are activated at the appropriate time (McCrea and Rybak 2008). The unit burst generators model of the CPG assumes that there are multiple unit burst generators (or CPGs) that control the synergistic muscles at one joint (i.e.- knee flexors or ankle extensors). Each unit burst generator is capable of bursting alone. Interconnections between the unit burst generators decide the relative phasing between different muscle groups and the timing during locomotion (Grillner 1981).

1.5.1.1.3 Other CPG Models

In order to account for more complex locomotor activity, models are becoming increasingly more complex. They include more interneurons and more connections within the circuitry to account for experimental findings. For example, some muscles, like semitendinosus, can generate two bursts per cycle; extra interneurons that provide a connection from both "half-centers" to both the extensor and flexor motoneurons populations can be added to the "half-center" model to correct for this (McCrea and Rybak 2008; Perret and Cabelguen 1980; Perret et al. 1988). The "half-center" model also does not incorporate sensory feedback, and so extra interneurons, like Ia interneurons, can provide motoneurons with sensory input (McCrea and Rybak 2008; Orlovskiĭ et al. 1999).

A number of models have proposed more than one level of organization to account for independent changes that cannot be explained single-level models (Burke et al. 2001;

Koshland and Smith 1989; Kriellaars et al. 1994; Perret and Cabelguen 1980; Perret et al. 1988; Rybak et al. 2006a; Rybak et al. 2006b). In particular, there appear to be independent changes in motoneurons recruitment and rhythm generation which can be separately affected by sensory input (Burke et al. 2001; Kriellaars et al. 1994).

Studies of deletions, which are spontaneous reductions or absences of one or more expected rhythmic bursts of activity, provided clues into CPG organization (Lafreniere-Roula and McCrea 2005). Deletions have been observed during fictive locomotion, treadmill locomotion, and scratch in cats (Duysens 1977; Lafreniere-Roula and McCrea 2005), as well as in the turtle scratch reflex (Stein 2005; Stein and Grossman 1980). Deletions are considered errors or failures in the rhythmic activity (Rybak et al. 2006a). There are different types of deletions including resetting and non-resetting deletions or complete deletions and partial deletions. In a non-resetting deletion, despite the absence of a burst, the next burst reemerges at an integer number of the missing locomotor period at the time that would have been expected had the deletion not occurred, suggesting that the CPG "remembers" and maintains the pattern even when the motoneurons are silent. This pattern is not consistent with a simple "half-center" model (Lafreniere-Roula and McCrea 2005).

Rybak et al. (2006a) proposed a model that combines features of both the half-center and the unit burst generator organizations into a two-level locomotor CPG model. The model has a two-level architecture, where the first level is a half-center rhythm generator, which acts as the "clock." The second level is an intermediate pattern formation network that coordinates the activation of the motoneuron populations to which it projects. This allows separate control of the two levels. Therefore, sensory signals or perturbations can

affect one of the two levels without affecting the other. This model was designed to have a great amount of flexibility and to be able to explain more complicated motoneuron activities, including double bursts and deletions. For example, non-resetting deletions could occur due to changes in excitability to the pattern formation level and not the rhythm generator level. It should be noted that while the model is quite extensive, it currently only models one limb (Rybak et al. 2006a).

1.5.2 The neonatal rodent preparation

The *in vitro* isolated spinal cord preparation in the neonatal rat was first developed by Otsuka and Konishi (1974) to allow for the precise control over the extracellular medium. They recorded from ventral roots using silver electrodes or glass suction electrodes, which they found to be stable. They stimulated dorsal roots and were able to record monosynaptic and polysynaptic reflexes (Otsuka and Konishi 1974). The preparation was used to study respiration (Smith and Feldman 1987; Suzue 1984). Then by keeping the hindlimbs attached or using suction electrodes on ventral roots, it was demonstrated that locomotor patterning could be produced by chemical activation or stimulation of sensory pathways (Smith and Feldman 1987).

The isolated intact neonatal rodent spinal cord can produce a coordinated hindlimb motor output that spatiotemporally resembles locomotion in the intact hindlimb (Atsuta et al. 1988; Cazalets et al. 1990; Kiehn and Kjaerulff 1996). This activity is referred to as locomotor-like activity (LLA), and the motor output recorded from lumbar roots L2 and L5 ventral roots largely corresponds to activity in flexors and extensors respectively (Kiehn and Kjaerulff 1998). The muscles patterns in the neonatal rodent are similar to

locomotor patterns in the adult (Cowley and Schmidt 1995; Hayes et al. 2009; Juvin et al. 2007; Kiehn and Kjaerulff 1996).

1.5.3 Locomotor studies in the neonatal rodent

1.5.3.1 Pharmacological activation

Various neurochemicals have been used to initiate or induce rhythmic activity in the neonatal rodent spinal cord, including 5-HT (Cazalets et al. 1990; Cazalets et al. 1992; Kiehn and Kjaerulff 1996), NA (Kiehn et al. 1999; Sqalli-Houssaini and Cazalets 2000), DA (Kiehn and Kjaerulff 1996), γ-aminobutyric acid N-methyl-D-aspartate (NMDA) (Cazalets et al. 1990; Kudo and Yamada 1987), glutamate (Cazalets et al. 1992), acetylcholine (Cowley and Schmidt 1994a), Potassium (Bracci et al. 1998), and arginine vasopressin (Pearson et al. 2003).

There are differences in the rhythmic patterns produced by the different neurochemical applications. For example, DA induces rhythmic activity that is slower than 5-HT as well as differences in phase (Kiehn and Kjaerulff 1996), and NA produces a slow alternating pattern with clear right-left alternation, but not between flexors and extensors (Sqalli-Houssaini and Cazalets 2000).

NMDA and 5-HT have been used widely both alone and in combination. In combination, they are a very reliable method for inducing long lasting LLA (Kiehn et al. 1999; Kjaerulff and Kiehn 1996; Sqalli-Houssaini et al. 1993).

1.5.3.2 Electrical activation

It has been shown that electrical stimulation techniques can activate the hindlimb locomotor CPG in the neonatal rodent spinal cord (Bonnot et al. 1998; Gabbay et al.

2002; Gordon and Whelan 2006; Lev-Tov et al. 2000; Lev-Tov and Pinco 1992; Marchetti et al. 2001). Different areas can induce LLA, include the brainstem (Atsuta et al. 1990; 1988; Zaporozhets et al. 2004), the ventrolateral funiculus (Magnuson and Trinder 1997), dorsal roots (Marchetti et al. 2001; Whelan et al. 2000), sacrocaudal afferents (Lev-Tov et al. 2000; Strauss and Lev-Tov 2003), and cauda equina (Gordon and Whelan 2006; Whelan et al. 2000) .

1.5.3.3 Lesioning studies

Lesion and activity studies have mapped the core neuronal elements of the locomotor CPG to the ventral half of the spinal cord (Kjaerulff and Kiehn, 1996; Cowley and Schmidt, 1997)

The ability to generate locomotor-like rhythms in the spinal cord is distributed through the lumbar enlargement; however, the more rostral segments (L1-L2 in rodents) have a greater ability to generate rhythmic outputs in isolation than caudal segments (Cazalets et al. 1996).

1.5.3.4 The use of molecular genetics to understand CPGs

Molecularly identification and genetic manipulation of different classes of neurons in the ventral half of the neural tube during development is starting to give insight into the roles of these neurons during locomotion. Distinct dorsoventral gradients produced by expression of transcription factors trigger differentiation in the developing cord. In the ventral cord, motoneurons and four ventral (V) interneuron populations (V0-V3) are derived (Goulding 2009; Jessell 2000). All five classes appear to influence the CPG. Efforts are being made to correlate the embryonic cell type with previously identified adult cell types (Goulding 2009).

V0 interneurons are locally projecting intersegmental commissural interneurons (Moran-Rivard et al. 2001) that project to contralateral motoneurons and are responsible for left-right alternation during locomotion (Lanuza et al. 2004). There are both excitatory and inhibitory subtypes (Goulding 2009).

V1 inhibitory interneurons are a heterogeneous population of inhibitory interneurons that project ipilaterally to motoneurons. They are located in region VII, which receives projections from muscle afferents. Two known types of adult interneurons are derived from V1 intereneurons: Renshaw cells (9%) and Ia inhibitory interneurons (13%) (Alvarez et al. 2005). V1 intereneurons affect the frequency of the locomotor CPG, specifically affecting the higher frequencies. Three different genetic approaches were used to either inactivate or delete V1 interneurons, leading to an inability to burst or step at higher frequencies (Gosgnach et al. 2006).

V2 interneurons are comprised of V2a excitatory and V2b inhibitory interneurons, both of which project ipsilaterally and to motoneurons (Al-Mosawie et al. 2007). V2a interneurons play an important role in the controlling of left-right alternation. After V2a neurons are ablated, there is an uncoupling of the left and right sides of the spinal cord and a loss of left–right alternation. The V2a interneurons contact commissural interneurons, including V0 interneurons (Crone et al. 2008). Not much is known about the function of V2b inhibitory interneurons (Goulding 2009).

V3 interneurons are excitatory neurons that project primarily across the midline. Roughly 15% of the neurons project ipsilaterally, including some that have axonal processes to both sides of the spinal cord. V3 interneurons contact motoneurons, Ia

inhibitory interneurons, Renshaw cells, and V2 intereneurons. V3 help establish stable and balanced locomotor rhythm (Zhang et al. 2008).

1.6 Neuromodulation

Neurotransmission is accepted to be the primary means of communication between neurons and traditionally thought of as fast (milliseconds), point-to-point (neuron to neuron), and simple (either excitatory or inhibitory). However, neural communication is not quite as simple as this. Instead, there are many other ways that neurons can communicate that differ from classical neurotransmission. One common way is through neuromodulation. As a general guideline rather than a firm definition, neurotransmission is mediated by ionotropic receptors, which are ligand-gated ion channels responsible for the fast communication and neuromodulation is mediated by metabotropic receptors, which activate second-messenger systems (Katz 1999; Katz 1995).

Neuromodulation is a substance that alters the cellular and synaptic properties of a neuron and alters the subsequent neurotransmission, so that even with the same input, the output will be different (Katz 1999; Katz 1995; Katz and Frost 1996). Thus, it allows the circuit to become more flexible (Katz 1995). Three different types of neuromodulation are extrinsic neuromodulation, intrinsic neuromodulation, and biochemical integration.

Extrinsic neuromodulation is usually thought to originate from sources extrinsic to the circuit being affected and therefore not a part of the circuit itself (Katz 1995). It is considered the 'conventional' form of neuromodulation typified by neuronal systems modulating the activity of other 'hard-wired' circuits (Katz 1995). A classic example would be the monoaminergic nuclei, such as the raphe nuclei, substantia nigra, and locus coeruleus, which project fibers throughout the brain and spinal cord producing potent

modulation of spinal sensory, autonomic, and motor activity (Hochman et al. 2001; Millan 2002; Schmidt and Jordan 2000).

By contrast, with intrinsic neuromodulation, neuromodulatory substances can also be released by neurons that are intrinsic to a circuit, and can affect other neurons and synapses within the same circuit. One functional consequence of intrinsic neuromodulation is that it operates whenever the circuit is active because it is an integral part of the circuit and as such its actions are proportional to the amount of activity within the circuit itself (Katz and Frost 1996). As stated by Katz and Frost (1996) "'intrinsic neuromodulation is not as widely recognized as extrinsic neuromodulation, but we believe that it might be just as pervasive" (Katz and Frost 1996).

Biochemical integration modulates the neuron in which it acts. During biochemical integration, the biochemical intracellular signals (e.g. second messengers like cAMP) are integrated to affect the cellular and synaptic properties of the neuron, often across different time scales (Katz and Clemens 2001).

1.7 The significance of an exquisitely regulated trace aminergic system

The TAs have turnover rates similar to DA and NA (Durden and Philips 1980; Paterson et al. 1990) which is "a more significant index of their importance than their endogenous concentrations" (Saavedra 1989). High turnover rates are demonstrated by blocking amine degradation with MAO inhibitors resulting in significant accumulation of TAs (Berry 2004). In addition, it has been shown that AADC activity is physiologically-modifiable and has dramatic influence over TA concentrations but not the classic monoamines levels (Berry 2004). Thus, endogenous levels of TA are much easier to control than the classical monoamines and represent a modulatory system whose

transmitter concentration can be altered several fold in a very short time (Berry 2004). If the primary role for the TAs was via activation of the TAARs to modulate circuits activated by the classical monoamines, then control of the rate of synthesis by changing AADC activity could have profound modulatory actions on these circuits. Simply changing the endogenous level of the TAs, could act as a switch to change the output, say as a "modulatory switch," that could amplify/reinforce the circuitry. This mechanism could easily apply anywhere there was AADC expression.

CHAPTER 2

GENERAL METHODS

2.1 Electrophysiology

2.1.1 The neonatal rat isolated spinal cord preparation

The spinal cord contains all of the necessary circuitry at birth to produce complex motor outputs including locomotor patterning (Grillner 1981). Over the last two decades, the *in vitro* isolated spinal cord preparation in the neonatal rodent has become the dominant animal model for studying the spinal mechanisms controlling the operation of the mammalian locomotor CPGs. The *in vitro* isolated rodent spinal cord preparation can produce a coordinated hindlimb motor output that spatiotemporally resembles locomotion in the intact hindlimb (Atsuta et al. 1988; Cazalets et al. 1990; Kiehn and Kjaerulff 1996) as well as the adult (Cowley and Schmidt 1995; Hayes et al. 2009; Juvin et al. 2007; Kiehn and Kjaerulff 1996).

Many different neurochemicals have been used to induce locomotor-like activity patterns. Locomotor-like activity (LLA) can be characterized as left/right and ipsilateral alternation of bursts between flexors and extensors (Kiehn and Kjaerulff 1998; Sqalli-Houssaini et al. 1993). Locomotor-inducing neurochemicals include serotonin (5-HT), noradrenaline (NA), dopamine (DA), excitatory amino acids, acetylcholine, N-methyl-D-aspartate (NMDA), glutamate, and acetylcholine (Barbeau and Rossignol 1991; Cazalets et al. 1990; Cazalets et al. 1992; Cowley and Schmidt 1994a; Kiehn and Kjaerulff 1996; Kiehn et al. 1999; Kudo and Yamada 1987; Sqalli-Houssaini and Cazalets 2000).

The isolated *in vitro* spinal cord preparation has become an important model due to its many advantages. The extracellular medium can be controlled easily (Otsuka and Konishi 1974), and the spinal cord remains intact, which means that more of the spinal cord circuitry is intact than in a slice preparation (Suzue 1984). With no blood brain barrier, it is possible to make all drugs accessible to the spinal cord, which means that instead of just studying the precursors like L-DOPA, drugs like dopamine could be tested on the spinal cord (Cazalets et al. 1992). This easy access allows for manipulation of the system through pharmacology, stimulation, dissection of the spinal cord, and transgenics. Further, the surgery and electrode placement is relatively fast and easy. Due to the small size of the neonatal spinal cord, passive diffusion of oxygen and the control medium maintain viability of the spinal cord to keep it alive for extended periods of time (Smith and Feldman 1987). It also allows for intracellular recordings (Fulton and Walton 1986; Jahr and Yoshioka 1986). Use of the *in vitro* neonatal rodent preparation has increased our understanding of the spinal motor system.

In chapter 4, I used the isolated neonatal rat spinal cord maintained *in vitro* to study trace amine (TA) modulatory actions on the spinal cord and then compared them to the classical monoamine transmitters. To do this, the spinal cord of postnatal (P) day 0-5 neonatal Sprague-Dawley rats was isolated and motor activity was monitored using glass suction electrodes attached to ventral lumbar roots, typically bilaterally on L2 and L5 (**Figure 2.1**). Although the ventral roots contain both flexor and extensor motor axons, L2 ventral root activity primarily indicates activity in flexors, while L5 ventral root activity primarily indicates activity in extensors (Kiehn and Kjaerulff 1998). Activity was induced with the TAs [tyramine, octopamine, PEA, and tryptamine] and the

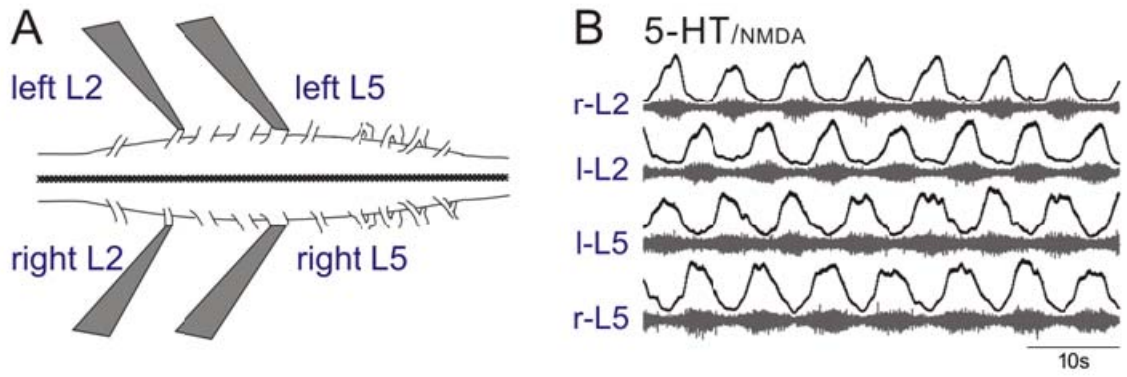

Figure 2.1: *In vitro* neonatal rat isolated spinal cord experimental setup
A. All of the electrophysiological experiments conducted in Chapter 4 use the isolated neonatal rat spinal cord maintained *in vitro*. For studies on locomotor-like motor rhythms, suction electrodes are placed on lumbar L2 and L5 ventral roots bilaterally to monitor population motoneuron flexor and extensor activity, respectively. Drugs are applied to the bath. **B.** Recording of locomotor-like activity showing alternation between right and left L2 flexors, with each flexor rhythm alternating with the L5 extensor rhythm on the same side. For most of the result subsequently displayed, raw electroneurograms are displayed below their rectified filtered waveforms.

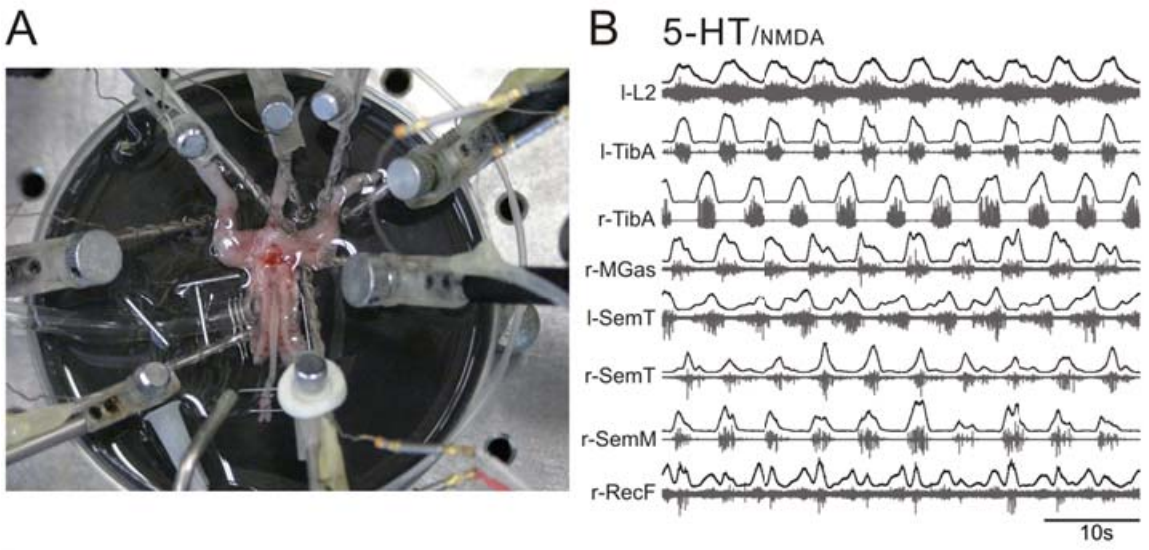

Figure 2.2: *In vitro* neonatal rat isolated spinal cord with attached hindlimbs experimental setup
A. All of the electrophysiological experiments conducted in Chapter 5 use the isolated neonatal rat spinal cord with attached hindlimbs maintained *in vitro*. For studies on the output of muscle coordination, glass suction electrodes were placed on the left lumbar L2 ventral root and typically 7 muscles of the hindlimb. Drugs are applied to the bath. **B.** Electromyographically recording of locomotor-like activity showing coordination of the muscles.

monoamines [5-HT, dopamine, and noradrenaline] both alone and in the presence of NMDA. Locomotor-like activity was analyzed using custom MATLAB software that I wrote, and is described below in **Section 2.3**.

2.1.2 The neonatal isolated spinal cord with attached hindlimbs

While ventral root recordings offer a simple way to monitor the spinal motor output, they do not give the whole story. Ventral root activity may not reflect recruitment of individual muscles (Cowley and Schmidt 1994b). Anatomical studies have shown that ventral roots are actually quite heterogeneous, containing axons projecting to both flexors and extensors (Nicolopoulos-Stournaras and Iles 1983). Important changes in motor coordination between flexor and extensor activity may be lost due to the heterogeneity of these roots. It has been long known that muscle recruitment by spinal cord circuitry is more complicated than just simple flexor/extensor alternation. These distinct complex patterns and sequential activation of muscles have more recently been demonstrated in the *in vitro* spinal cord (Grillner 1981; Kiehn and Kjaerulff 1996). Distinct motor patterns of recruited muscles were observed when induced by different transmitters such as 5-HT and dopamine as well as pharmacological and electrical stimulation, suggesting that the spinal locomotor network is flexible and that extrinsic modulation can modify the network coordination (Kiehn and Kjaerulff 1996; Klein et al. 2010).

In chapter 5, I used the *in vitro* isolated spinal cord of P day 0-5 neonatal Sprague-Dawley rats with attached hindlimbs to record muscle activation electromyographically. I studied the patterns produced by the TAs and NMDA compared to 5-HT and NMDA, as well as the ability of the TAs to modulate ongoing 5-HT and NMDA LLA. To do this, the spinal cord of neonatal Sprague-Dawley rats were isolated with the hindlimbs still

intact and with the dorsal roots cut. Dorsal roots were severed to eliminate the sensory feedback and simplify the spinal cord output to reflect the changes in spinal cord circuitry due to the trace amines. Modulatory actions of the TAs can be inferred to reflect the actions on motor circuits.

Glass suction electrodes were then placed on the left lumbar L2 ventral root and up to 7 muscles of the hindlimb (**Figure 2.2**). Recordings were acquired from the following muscles: tibialis anterior (TibA; ankle flexor), medial gastrocnemius (MGas; ankle extensor), semitendinosus (SemT; knee flexor / hip extensor), semimembranosus (SemM; knee flexor / hip extensor), vastus medialis (VasM; knee extensor), rectus femoris (RecF; knee extensor / hip flexor), and adductor magnus (AddM; hip adductor). Some of the muscles (e.g. TibA) were often recorded bilaterally. The left lumbar L2 ventral root was always recorded for two reasons: comparison of ventral root activity profile in relation to that obtained in the isolated spinal cord preparation reported above, and its reliable recruitment allowed a common comparison between experiments where muscle EMG recruitment was variable. Rhythmic motor activity was evoked by two different pharmacological methods. First, tryptamine, tyramine, octopamine, PEA, or 5-HT was co-applied with NMDA. Second, 5-HT and NMDA were co-applied to get ongoing locomotor-like activity onto which tryptamine, tyramine, octopamine, and PEA were added. Locomotor-like activity was analyzed using custom MATLAB software I wrote especially for this project, described below in **Section 2.3**.

2.2 Terminology for bursting patterns

In both chapter 4 and chapter 5, three main types of locomotor-like patterning were encountered. First, there is locomotor-like bursting with a regular frequency. Second,

the same locomotor-like pattern is produced, but **significantly** slower. Third, there are multiple types of episodic bursting patterns produced; however, each is characterized by bouts of rhythmic bursting interrupted regularly by quiescent periods (**Figure 2.3**). The figure below is intended to clarify the types of patterns and the terminology used to describe the bursting.

2.3 SpinalMOD: A MATLAB Graphic User Interface for Burst Detection

SpinalMOD (**Spinal** **M**otor **O**utput **D**etector) is a MATLAB Graphical User Interface (GUI) I wrote for the analysis of locomotor-like activity. It detects the onset and offset of the bursts being analyzed and calculates the bursts statistics, such as period, burst duration, and phase. It is designed with a simple GUI so that it can be operated easily by users who are not familiar with programming languages (**Figure 2.4**).

2.3.1 GUI menu structure

When SpinalMOD is first opened, there are a series of menus in the top left hand corner. There are three first level menus, namely “File”, “Edit”, and “Settings”. The “File” menu offers options to open data, save analysis, open figures into a pop-up window for closer examination, and for exporting analysis. The “Edit” menu offers the ability to undo and redo changes in variables. The “Settings” menu offers options to change the file name and set the sample frequency of the data being analyzed. Once an option has been selected, most options have a pop-up window that guides the user through the desired action.

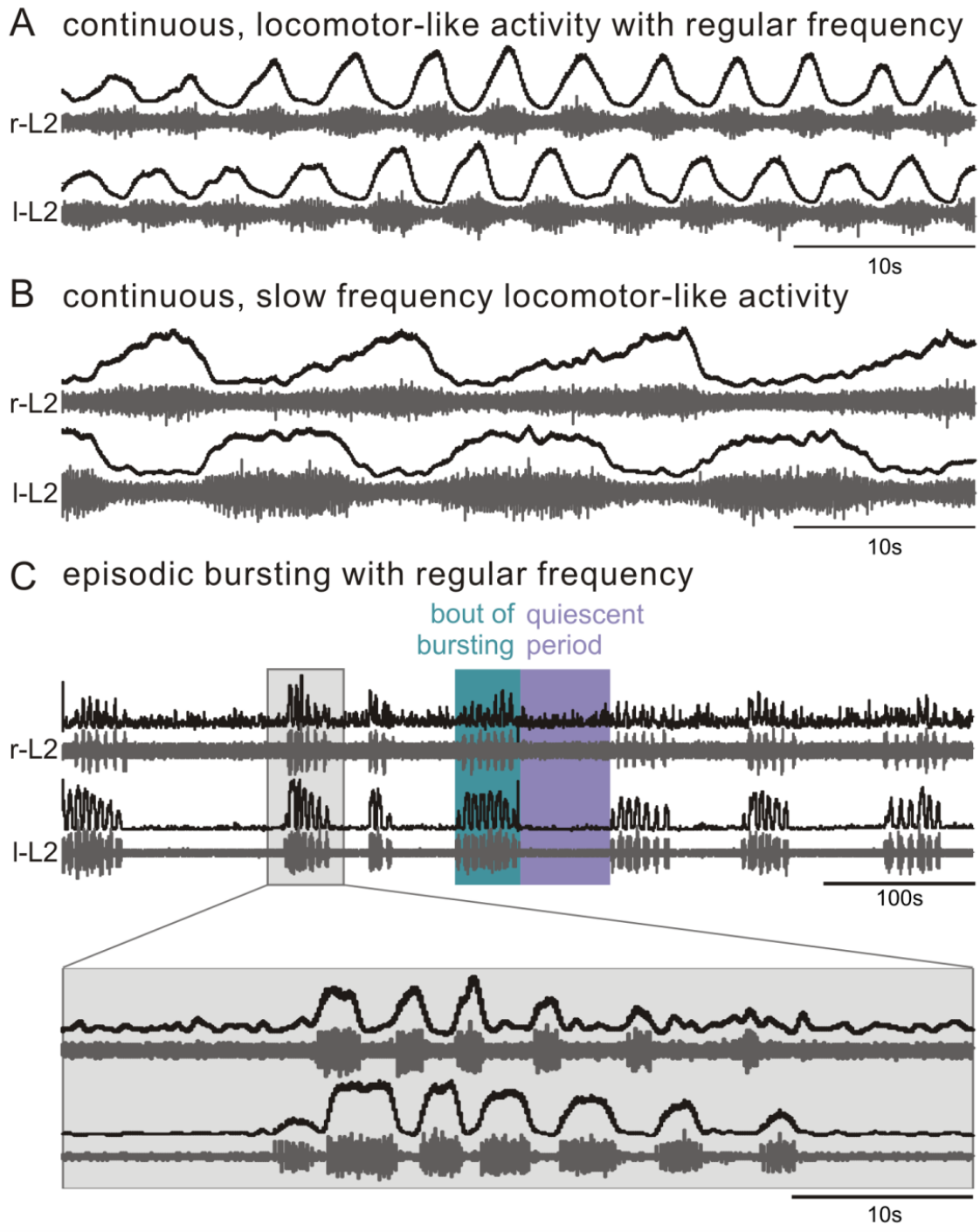

Figure 2.3: Terminology for the different types of trace amine induced bursting
There are three different broad types of bursting for the TAs. **A.** The first type is continuous locomotor-like bursting that has a regular frequency. The frequency of 5-HT/NMDA and TA/NMDA locomotor-like activity is statistically indistinguishable. **B.** The second type is continuous locomotor-like bursting that has a significantly slower burst frequency. **C.** The third type is episodic bursting, which is characterized by bouts of bursting with epochs of comparatively quiescent periods. The bursting in the bouts is typically locomotor-like. The lower box in C shows a bout of bursting on the same time scale as seen in A&B.

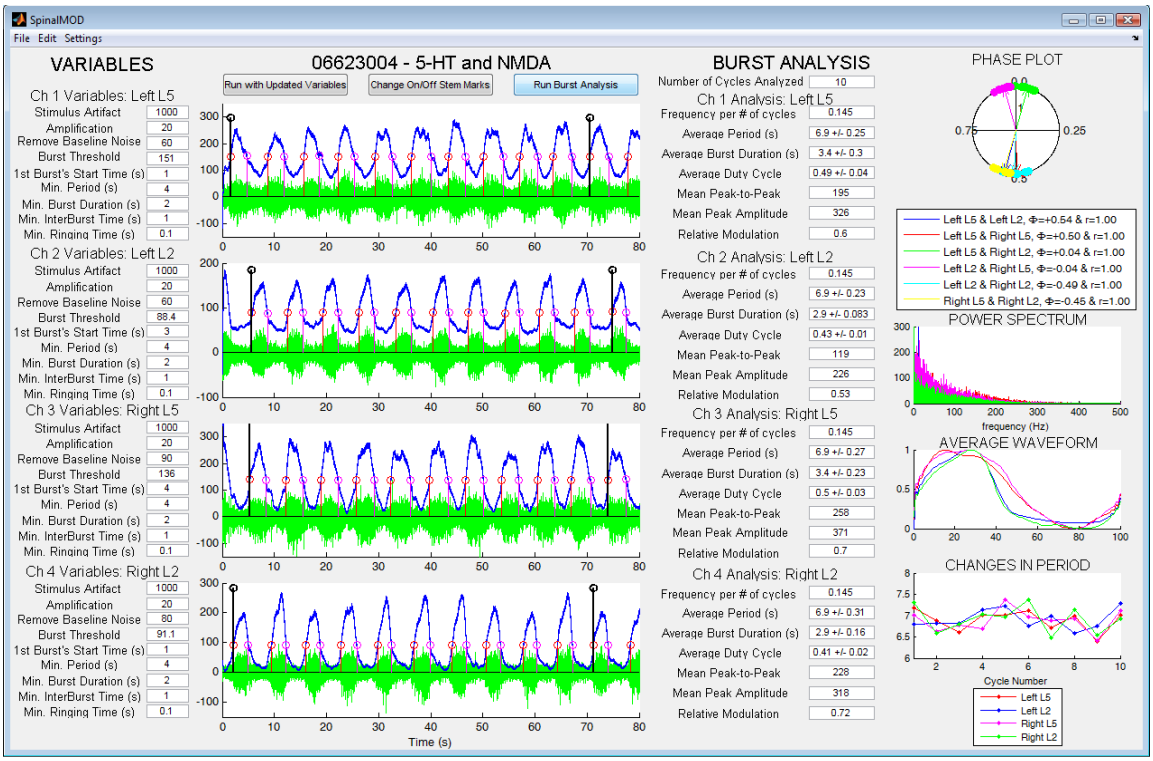

Figure 2.4: SpinalMOD Graphical User Interface

The graphical user interface (GUI) is designed to be easy to use. The input variables are listed to the left under VARIABLES for all four channels. To change the variable, the user simply clicks inside the white box and changes the value. The waveforms for the data are show in the middle. Raw data is in green. Filtered data is in blue. Onset stems are in red, and offset stems are in magenta. If the stems are not in the correct location, the user changes the variables and selects "Run with Updated Variables." If the algorithm fails to find the correct onset and offsets for the bursts, the stems can be manually changed with the "Change On/Off Stem Marks." To run the burst analysis, the user would push the "Run Burst Analysis" button, and select first the number of bursts to be analyzed and then the first burst to be analyze. Black stems mark the beginning and end of the analyzed period. Once analyzed, the values calculated are shown in the Burst Analysis section to the right of the GUI.

2.3.2 Running the burst detection

To get started analyzing data, the user selects the file to be analyzed from the location it has been saved. Currently, two types of files can be opened for analysis. The first file format is the .abf, which was recorded using pCLAMP acquisition software (v8-9, Molecular Devices; Union City, CA). This data is inputted into SpinalMOD via a custom script written by Michael Sorensen, called readabf.m. The second file format is the .mat, which is the form data that has already been analyzed in MATLAB is saved.

When new data is being entered, the user can select how many seconds of data to open, which channels are to be analyzed, and the name of the channels to be analyzed. The GUI can analyze four channels of data at one time. For each channel, the raw data is rectified and processed through a low-pass Chebyshev filter. The burst threshold is calculated by taking the mean of the filtered data. Values above the threshold are tested to determine the onset and offset of each burst. An algorithm (found in **Appendix C**) uses the input variables on the left side of the GUI. A series of if statements determines the onset and offset of a burst by evaluating whether specific criteria are met. The if statements start at the beginning of the rectified and filtered array and evaluate every point above threshold to determine if it fits the criteria to be first a burst onset and second a burst offset. The onset of each burst crosses the threshold on an upward slope, and the offset of each burst crosses the threshold on a downward slope.

The important variables for detecting the bursts are: 1st Burst's Start Time (s), Min. Period (s), Min. Burst Duration (s), Min. InterBurst Time (s), and Min. Ringing Time (s) (**Figure 2.4 and 2.5**). 1st Burst's Start Time (s) is the time in seconds that the user wants the GUI to start searching for the first burst onset to be detected and later analyzed. Min.

Period (s) is the minimum period in seconds between the onset of each burst. Min. Burst Duration (s) is the minimum time in seconds of the burst duration. Min. InterBurstTime (s) is the minimum time in seconds between the offset of one burst and the onset of the next burst. Min. ringing time (s) is a variable to prevent early detection of the burst offset. Since the algorithm examines the array point by point, this variable is the minimum time in seconds after the offset has been detected that there cannot be another offset detected (**Figure 2.5**).

Once the data has been opened and has gone through the first calculation of onset and offset, the raw data, filtered data, onsets, and offsets are then graphed for the user. The user can change the input variables and select the "Run with Updated Variables" button, repeating the burst analysis to correct the burst analysis output. If the algorithm is unable to detect the onset and offset correctly, typically due to noise or a poor signal to noise ratio, the user has the option to manually place the burst onset and offset markers.

2.3.3 Running the burst analysis

Once the burst markers correctly mark the beginning and end of each burst, the user can select the "Run Burst Analysis" button, and the burst characteristics will be calculated. The burst analysis calculates frequency, average period, average burst duration, average duty cycle, mean peak-to-peak amplitude, mean peak amplitude, relative modulation amplitude, the phase between the different channels, the power spectrum, and the average waveform (Kjaerulff and Kiehn 1996). To examine the data and analysis further, pop-up windows open graphs for the waveforms, phase, power spectrum, average waveform, changes in period, changes in burst duration, changes in duty cycle, and changes in peak amplitude.

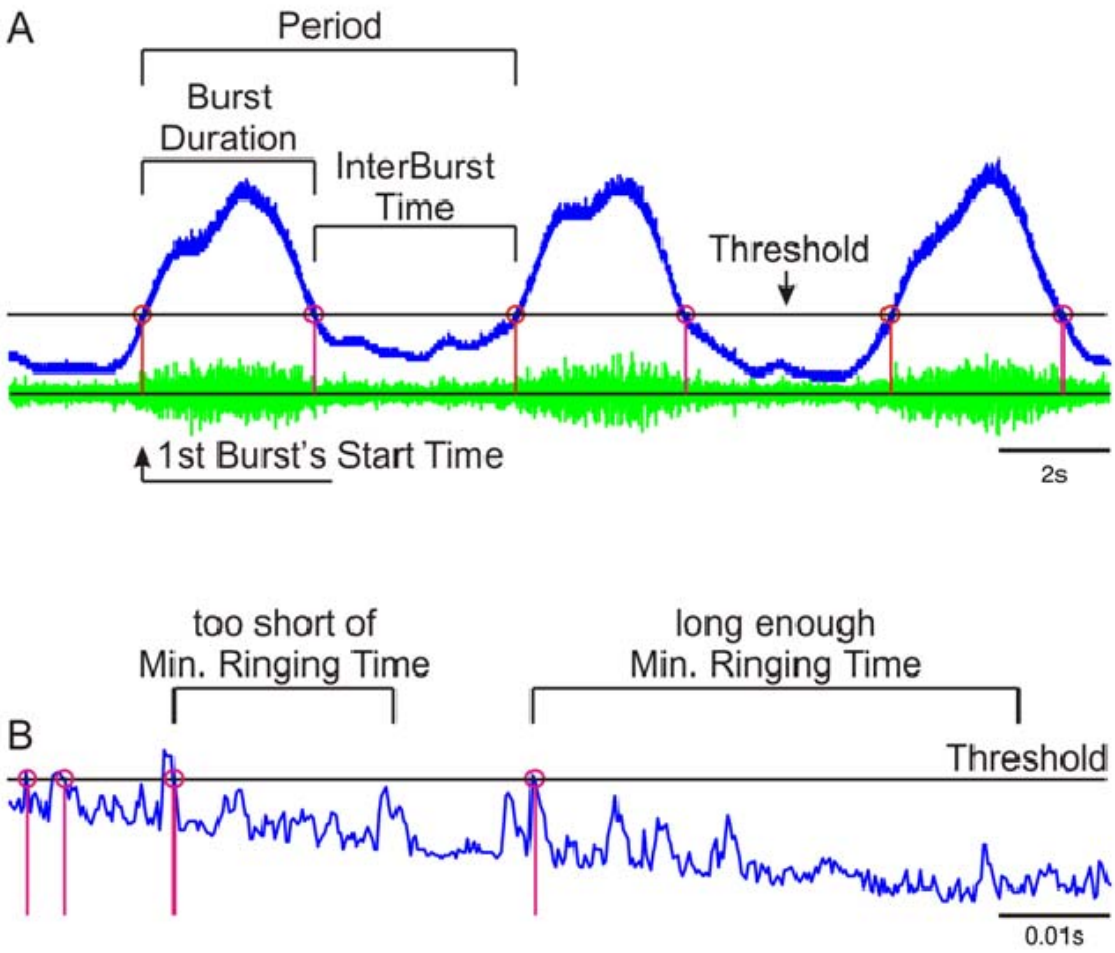

Figure 2.5: SpinalMOD terminology

The important variables to calculate the burst onset and offset are 1st Burst's Start Time (s), Min. Period (s), Min. Burst Duration (s), Min. InterBurst Time (s), and Min. Ringing Time (s). **A.** A graphical representation of 1st Burst's Start Time, Period, Burst Duration, and InterBurst Time are shown. **B.** The min. ringing time variable was implemented to prevent early detection of the end of the burst. While many points may fit the other criteria for being the end of the burst, ringing time helps to insure that the last of these points is selected. Shown here are 4 points that fit the other criteria for the end of the burst. If the time of the Min. Ringing Time is not long enough (left) then the wrong point is marked as the end of the burst. When it is long enough (right), the correct point is marked. B is a zoom in of the offset for the first burst in A.

2.4 Contributions to this dissertation

While I did the majority of the work for this dissertation, I would like to acknowledge the people who helped and whose work is included in this dissertation. The technical work for the *in situ* hybridization work for AADC and TAAR1 was carried out by Dr. Hong Zhu. The technical work for all of the immunohistochemistry and DiI was carried out by Michael A. Sawchuck. Michael A. Sawchuck also helped me to create the confocal images while I was processing the immunohistochemistry work. Their work was conducted in consultation with Dr. Shawn Hochman and me.

I was responsible for all electrophysiology and *in vivo* experiments, the analysis, the statistics, and the writing of the dissertation. Dr. Shawn Hochman acted as an advisory role.

I wrote the whole SpinalMOD program, with some help on the filtering and getting started on using GUIs from Kate Williams Meacham. SpinalMOD runs a custom script written by Michael Sorensen, called readabf.m, that takes data recorded using pCLAMP acquisition software (.abf) and converts it to be inputted into MATLAB.

CHAPTER 3

THE LOCALIZATIONS OF AADC, THE TRACE AMINES, AND TAARS PROVIDE AN ANATOMICAL SUBSTRATE FOR THE TRACE AMINES IN THE MAMMALIAN SPINAL CORD

3.1 Abstract

Like the conventional monoamine transmitters, the trace amines (TAs), tryptamine, tyramine, octopamine, and β-phenylethylamine (PEA), are synthesized from the same precursor amino acids via aromatic amino acid decarboxylase (AADC) and metabolized via monoamine oxidases (MAOs). While these TAs are present in the mammalian CNS, their physiologic role remains uncertain. Using *in situ* hybridization and immunohistochemistry, widespread AADC labeling was observed in many spinal neurons, including motoneurons and D cells surrounding the central canal. Tyramine, tryptamine, and octopamine also had widespread labeling in similar areas consistent with AADC producing them. The trace amine-associated receptor 1 (TAAR1) and 4 (TAAR4), which are activated by TAs, had similar labeling patterns, in total providing the substrate for the TAs to have intrinsic biological actions.

3.2 Introduction

The classical monoamine neurotransmitters, dopamine (DA), noradrenaline (NA), and serotonin (5-HT), play an important role in modulating spinal cord sensory and motor function (Clarac et al. 2004; Hochman et al. 2001; Jacobs and Fornal 1993; Millan 2002; Rekling et al. 2000; Schmidt and Jordan 2000). They are not made in the spinal cord, but in descending monoaminergic neurons that project to the spinal cord (Gerin et al. 1995).

These transmitters are closely related to another group of endogenous monoamines called trace amines (TAs), which may also act as neuromodulatory transmitters (Berry 2004; Burchett and Hicks 2006; Lindemann and Hoener 2005). The TAs, tryptamine, tyramine, octopamine, and β-phenylethylamine (PEA), have structural, metabolic, physiologic, and pharmacologic similarities to the classical MA transmitters (Saavedra 1989). Both the monoamines and the TAs are synthesized from the same precursor amino acids. However, while both require the synthesis enzyme aromatic-L-amino acid decarboxylase (AADC), unlike the classical monoamines, AADC is the only enzyme required to produce most of the TAs; octopamine (like NA) further requires dopamine-β-hydroxylase (DBH). Conversion from the TAs to the monoamines does not appear to occur (Berry 2007).

While traditionally viewed as metabolic by-products or false transmitters in vertebrates (Berry 2004; Boulton 1976; Grandy 2007; Kopin et al. 1965), the recent discovery of trace amine-associated receptors (TAARs) establishes a mechanism by which TAs can produce effects of their own (Borowsky et al. 2001; Bunzow et al. 2001; Hauger et al. 1982; Kellar and Cascio 1982; Lindemann and Hoener 2005; Nguyen and Juorio 1989; Vaccari 1986; van Nguyen et al. 1989). Tyramine and PEA activate TAAR1, while PEA and tryptamine activate TAAR4 (Borowsky et al. 2001). The presence of other TAARs in rat spinal cord, and whether the TAs act on them remains to be determined (however see (Liberles and Buck 2006)).

A trace aminergic neuronal system has yet to be identified. Candidate cells include D cells, which contain the essential synthesis enzyme AADC, but not tyrosine hydroxylase (TH), DBH, or phenylethanolamine N-methyltransferase (PNMT), and hence are able

to produce the TAs, but not the classical monoamines (Berry 2004; Jaeger et al. 1984a; Jaeger et al. 1984b; Jaeger et al. 1983; Nagatsu et al. 1988). D1 cells are associated with the lumen of the central canal and were the first non-monoaminergic AADC positive cells to be identified in the spinal cord (Jaeger et al. 1984b; Jaeger et al. 1983). It has also been reported that there are more cells in the gray and white matter, but nothing is known of their properties (Nagatsu et al. 1988). In the presence of the precursor amino acids, phenylalanine, tyrosine, and tryptophan, AADC containing cells should be able to synthesize the TAs, PEA, tyramine, and tryptamine, respectively. TAAR1 mRNA is also found in the spinal cord (Borowsky et al. 2001), but no studies have looked at TAAR1 or TAAR4 spinal protein expression or function.

I hypothesized that the TAs are an intrinsic amine system in the spinal cord, and therefore, that the spinal cord would contain the AADC, TAs, and TAARs. To test this hypothesis, immunohistochemistry was used to identify spinal neurons that express AADC, tyramine, tryptamine, octopamine, and the TAARs, and *in situ* hybridization was used to identify AADC and TAAR1. Overall, AADC, tyramine, tryptamine, octopamine, and the TAARs were found to be present in the spinal cord. Some of these results have been presented in abstract form (Gieseker et al. 2004; Gozal et al. June 15, 2007; Gozal et al. 2010; 2007b; Gozal et al. 2006).

3.3 Methods

All experimental procedures complied with the NIH guidelines for animal care and the Emory Institutional Animal Care and Use Committee. Sprague-Dawley rats postnatal (P) day 0-5, 14, and adult were used.

3.3.1 *In situ* hybridization

Complete adult rat spinal cords were dissected out and the whole cords were stored in RNAlater (Qiagen, Valencia, CA) at -80° until use. Total RNA was extracted from the mouse spinal cord by using Qiagen RNeasy Mini kits (Qiagen, Valencia, CA). Five microgram of total RNA was subject to cDNA synthesis with oligo-dT15 primer and SuperScript II Reverse transcriptase (Invitrogen, Carlsbad, CA) for one hour at 42° C. The reverse transcriptase was inactivated, and RNA was degraded by heating at 95° C for 5 min. Of the 20 µl of cDNA obtained from the synthesis reaction 5 µl were directly added to the PCR reaction using a PCR Mastermix kit (Eppendorf, Hamburg, Germany) containing 1 µM gene-specific primers. The primer used in this study was designed by the Invitrogen-OligoPerfect™ Designer program (Invitrogen, Carlsbad, CA). Non-radioactive single-stranded digoxigenin cRNA probes were used for in situ hybridization using methodology reported previously (Zhu et al. 2007). Briefly, single stranded, digoxigenin-labeled antisense and sense probes are transcribed in vitro using T7 and Sp6 RNA polymerase (Promega). The probe sequence for rat dopa decarboxylase (AADC) is 523-927bp (GenBank U31884), 404 bp product. The probe sequence used for TAAR1 is 400bp long (GeneBank#AF380186). Hybridization is carried out at 68 °C overnight with 3µg/ml digoxigenin-labeled antisense cRNA probe. Sense probes were used at identical concentrations and development reaction as a negative control. Sections were washed with concentrated standard saline citrate (SSC) and then incubated with anti-digoxigenin-AP, Fab fragments (1:5000, Roche) in blocking buffer overnight at 4C. The color development reaction was carried out in the dark and neutralized with color stop buffer (10 mM Tris, pH 5, 1 mM EDTA). Slides were then dehydrated through a series of

alcohol washes, coverslipped with Vectamount (Vector Labs) and viewed on a Nikon E800 light microscope. Images are digitized with Nikon ACT-1 software.

3.3.2 Thoracic transection of the spinal cord

Rodents were anesthetized with isoflurane via inhalation. Following dorsal laminectomy to expose lower-thoracic segments of the cord, one section of the cord between T8-T12 was removed using surgical microdissection scissors. Gel foam was placed in the site of transection to maintain the gap between rostral and caudal cord. The incision was closed with sutures. Rodents recovered for 1-3 weeks before immunohistochemistry or lipophilic dye labeling of axonal tracts. This is a sufficient time for degeneration of descending monoaminergic axon terminals (Commissiong and Toffano 1989).

3.3.3 Immunohistochemistry

Sprague-Dawley rats were anesthetized with urethane (1.5 mg/kg), perfused with 1:3 volume/body weight of prefix (0.9%NaCl, 0.1%$NaNO_2$, 10units/1m heparin) followed by equal volume/body weight of Lana's fixative (4% paraformaldehyde, 0.2% picric acid, 0.16 M PO_3); pH 6.9. In a small subset of experiments, the isolated cords of P2 littermates were incubated in regular aCSF either with or without the TAs, octopamine, tyramine, and tryptamine (all at 100 μM) for 2 hours and then processed. In many of the experiments, Fluorogold, which does not cross the blood brain barrier, was injected intraperitoneal (i.p.) 24 hours prior to sacrifice to retrogradely label most spinal motoneurons (Ambalavanar and Morris 1989; Merchenthaler 1991). The spinal cords were then isolated and post-fixed for 1 hour in Lana's fixative than cryoprotected in 10% sucrose, 0.1M PO_3 until sectioned into 10 um thick sections on a cryostat and processed

for immunohistochemistry. All incubations and washes were performed in 0.1M PO_3-buffered saline containing 0.3% triton X-100 (PBS-T). Tissue was washed overnight in PBS-T at 4°C followed by incubation in primary antibody for 48-72 hours. Slides were then washed three times for 30 minutes and incubated in secondary antibody. The following antibodies combinations were used:

Table 3.1: Antibodies used for immunohistochemistry expression

Primary Antibody	Secondary Antibody	Tertiary
Rabbit anti-tyramine 1:1000 (Chemicon)	Biotin anti-rabbit 1:250 (Jackson Immunoresearch)	Extravidin Cy3 1:1000 (Sigma)
Rabbit anti-TAAR1 1:1000 (Lifespan Biosciences)		
Rabbit anti-DDC 1:00 (Biomol Sciences)	cy3 anti-rabbit 1:250 (Jackson Immunoresearch)	
Rabbit anti-tyramine 1:100 or 1:1000 (Chemicon)		
Rabbit anti-tryptamine 1:50 (Chemicon)		
Rabbit anti-octopamine 1:500 (Chemicon)		
Rabbit anti-TAAR1 1:1000 (Lifespan Biosciences)		
Rabbit anti-TAAR4 1:5000 (Novice Biochemicals)		
Sheep anti-DDC 1:100 (Biomol Sciences)	FITC anti-sheep 1:100 (Jackson Immunoresearch)	
Mouse anti-NeuN 1:50 (Chemicon)	FITC anti-mouse 1:100 (Jackson Immunoresearch)	

In all experiments, omission controls were used for the primary antibodies. Others have provided pre-absorption controls abolishing staining for AADC (Ishida et al. 2002), tryptamine (Dabadie et al. 1990), and octopamine (Karhunen et al. 1993), but there is only evidence for displaced tyramine binding (Geffard et al. 1984). Thus, tyramine pre-absorption controls were performed. Appropriate antibody concentrations (1:100 and 1:1000) were absorbed with 1mg of antigen (Cell Sciences) for 1 hour prior to

incubation. This abolished staining for tyramine in 6 of 7 animals. Images were photographed with a Nikon (Tokyo, Japan) digital camera through a Nikon E800 microscope or using an Olympus FV1000 inverted confocal microscope. Images were processed using Corel Draw (Corel, Ottawa, Ontario, Canada).

3.3.4 Lipophilic dye labeling

One week after midthoracic spinalization the P14 rats were anesthetized, and the cords were isolated and preserved in 2% paraformaldehyde fixative. Cords were then suspended in agarose gel and labeled with the carbocyanine dye DiI. Crystals of DiI were placed at the cut surface of various ventral funiculus regions. The dye was allowed to diffuse to identify grey matter projections sites. The cord was then imaged via fluorescent microscopy using the Neurolucida.

3.4 Results

3.4.1 AADC and the trace amines were widely expressed in adult rodent spinal cords

In situ hybridization (**Figure 3.1**) and immunohistochemistry (**Figure 3.2**) carried out in adult rat lumbar spinal cord showed that AADC was detected with widespread labeling throughout the spinal cord. AADC expression was most notable around the central canal, the ventral funiculus, and in ventral neurons, including motoneurons. There was some nuclear binding for AADC in the motoneurons, which has been previously reported for AADC (Mann and Bell 1991). AADC labeling around the central canal was consistent with labeling of D cells as reported previously (Jaeger et al. 1983). There was also clear

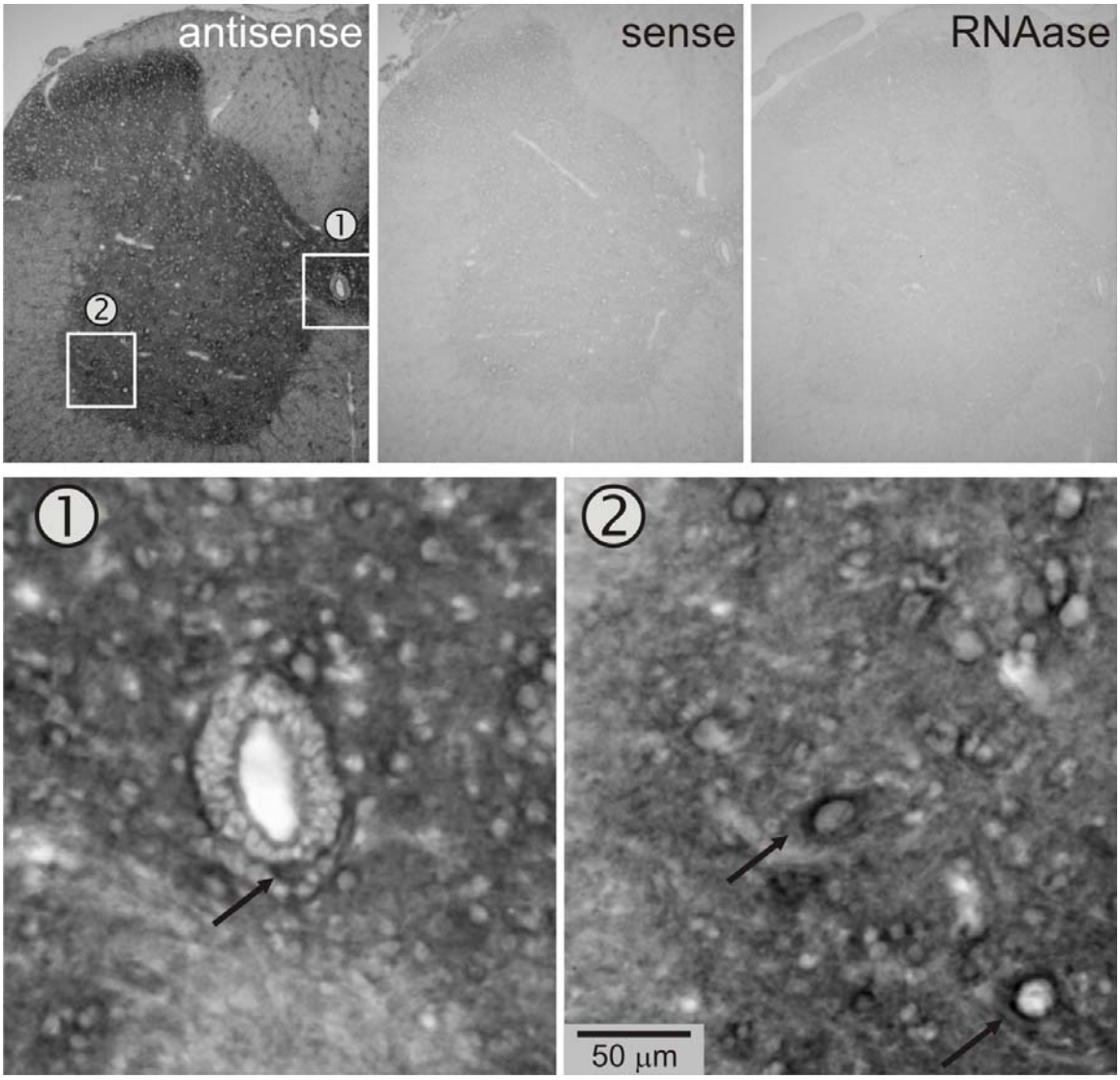

Figure 3.1: *In situ* hybridization reveals AADC expression throughout the spinal gray matter
Top row. Sense and RNAase treatment confirms specificity of antisense probe. Lower row provides magnification of boxed regions in top panel. Left. Central canal regions with arrow pointing to putative D cell. Right. AADC labeling in putative motoneurons at arrows.

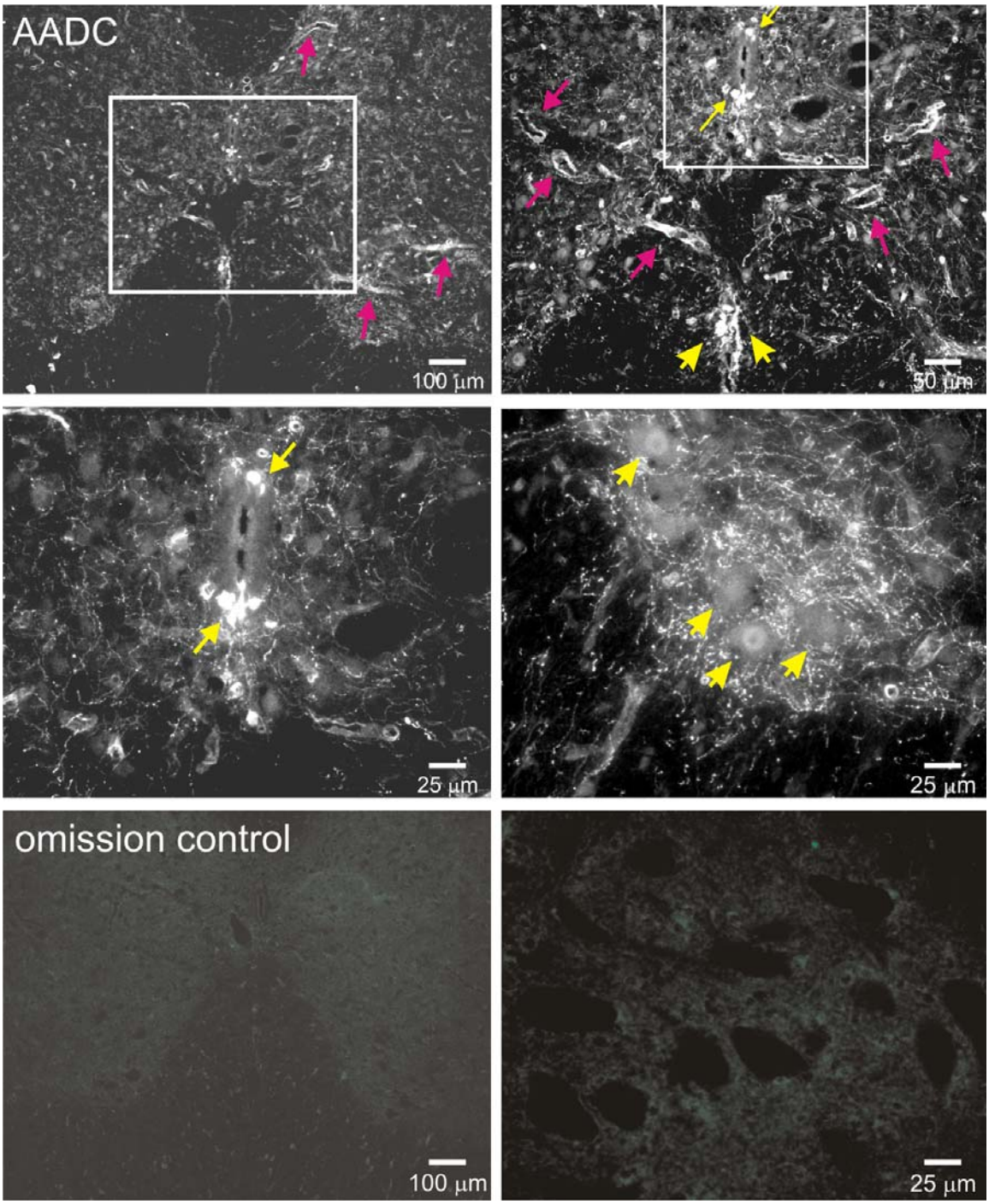

Figure 3.2: Immunohistochemistry reveals AADC labeling throughout the spinal gray matter
Intense AADC labeling was found in blood vessels (pink arrows) and in the D cells (yellow arrows). Top row at right show a magnification of the ventromedial cord to highlight the vessel labeling, the D cells, and the AADC$^+$ labeling in the ventral white matter (arrowheads). Middle left panel is a further blow-up to show the AADC$^+$ cells at the central canal. Note that some AADC$^+$ neurons were also found in the dorsal central canal. Middle right panel show AADC$^+$ terminal arborizations surrounding motoneurons. Most of these arborizations are presumably from descending monoaminergic systems. Note however that motoneurons also appear to be weakly AADC$^+$ (arrowheads). Bottom row are the omission controls for ventromedial cord (left) and motoneurons (right).

AADC immunolabeling surrounding blood vessels (**Figure 3.2**, top) where AADC activity is known to be high (Hardebo et al. 1979; Nagatsu et al. 1988). It is known that the amino acid precursors can be transported across blood vessels into the CNS (Daniel et al. 1976); therefore, one possible explanation for the presence of AADC in blood vessels is the synthesis of TAs as they cross the blood brain barrier.

To determine the extent to which AADC labeling was related to expression in descending axons fibers, I performed a complete mid-thoracic spinalization of an animal at P4, and waited 3 weeks prior to sacrifice. After spinalization, TH labeling was abolished (not shown). There was a near-complete loss of AADC fiber labeling in the spinal cord consistent with most expression arising from descending aminergic systems. However, AADC labeling remained in the ventral funiculus, around the central canal, including D cells, as well as associated with blood vessels, and in ventral neurons, including motoneurons (**Figure 3.3B**). Interestingly, even in these cells, AADC labeling intensity appeared reduced. It is noteworthy that, unlike the *in situ* data, with widespread AADC expression, AADC immunolabeling post-spinalization is much more restricted. The most obvious explanation for these differences is that intrinsic AADC expression is altered by events related to spinalization. The absence of TH labeling in lumbar cord suggests that after spinalization the only amines that can be intrinsically produced in these spinal regions are the TAs.

To confirm the presence of AADC and absence of TH and DBH after spinalization, I examined expression profiles created previously in the lab by microarray analysis using the mouse U74Av2 Affymetrix gene chip (Cui et al. 2006). I found that AADC cDNA was present in the whole spinal cord and in medial and lateral motor column

motoneurons both in control mice and in mice 3 weeks after thoracic spinal cord transection, thus confirming the above immunolabeling results. Also, I found that TH and DBH cDNA were absent both in control mice and mice 3 weeks after cord transection. Thus, all of the immunolabeling for TH I observed in the control spinal cord, and the TH and DBH labeling found by others (MacDermid et al. 2004) is due to expression in descending projection systems.

Next, immunolabeling was undertaken to compare octopamine, tryptamine, and tyramine expression in the ventral horn of the adult rat spinal cord (**Figure 3.4**). Octopamine and tryptamine immunolabeling were observed throughout the neuropil of the ventral horn, and for both, expression appeared to be in a subpopulation of motoneurons. In comparison, tyramine expression is weak or absent in motoneurons, but had unique punctate labeling surrounding motoneurons. Immunolabeling for PEA was not done because there is not a specific antibody commercially available for PEA.

3.4.2 AADC and the trace amines were widely expressed in neonatal rat spinal cords

Next, immunolabeling studies of AADC and TA expression were conducted in the neonatal spinal cord to match the age at which electrophysiological studies were undertaken in the subsequent chapters (**Figure 3.5 and 3.6**). AADC labeling was similar to that found in the adult with widespread labeling. Labeled spinal neurons were most notable adjacent and ventral to the central canal, the ventral funiculus, and in ventral neurons, including motoneurons (**Figure 3.5A**). As observed in the adult, but more strikingly, $AADC^{+}$ neurons associated with the central canal projected ventrally in a stream of cells with subsequent termination of putative axonal projections in the most

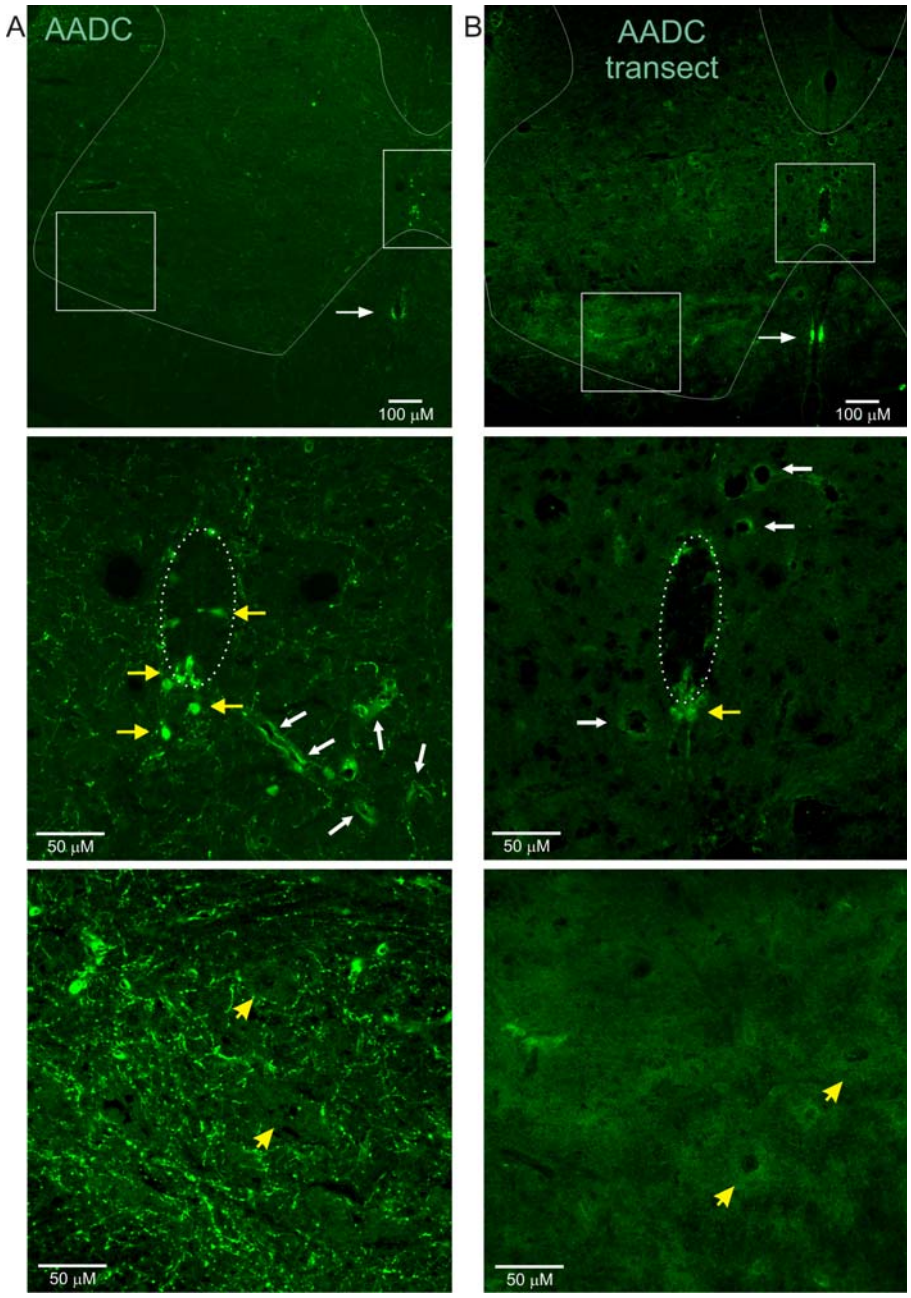

Figure 3.3: AADC labeling in the adult lumbar spinal cord before and after transection

A. Labeling of AADC in the ventral spinal cord of the control P25 adult rat. Top. Arrow identifies $AADC^+$ tract at the midline of the ventral funiculus white matter. Boxes identify the central canal and motoneuron regions, which are magnified below. Middle. Epithelial cell layer surrounding central canal (outlined) includes $AADC^+$ neurons as well as additional neurons emanating from ventral aspect of canal (e.g. yellow arrows). Some blood vessels also appear to be $AADC^+$ (white arrows). Punctate labeling for AADC is associated with axonal arborizations. Bottom. $AADC^+$ labeling surrounding motoneurons (yellow arrowheads). **B.** AADC labeling 3 weeks after midthoracic spinalization in a littermate of A. Same format as in A. Note that $AADC^+$ puncta were lost while neuronal labeling associates with the epithelial region surrounding the central canal and around motoneurons, while white matter labeling in the ventral funiculus remain. Also, note that motoneurons appear to be weakly $AADC^+$. Again, structures surrounding blood vessels also appear to be $AADC^+$. All images are confocal images taken at optical section thickness of 3.52 µm, top panel; 0.94 µm, middle and bottom panels.

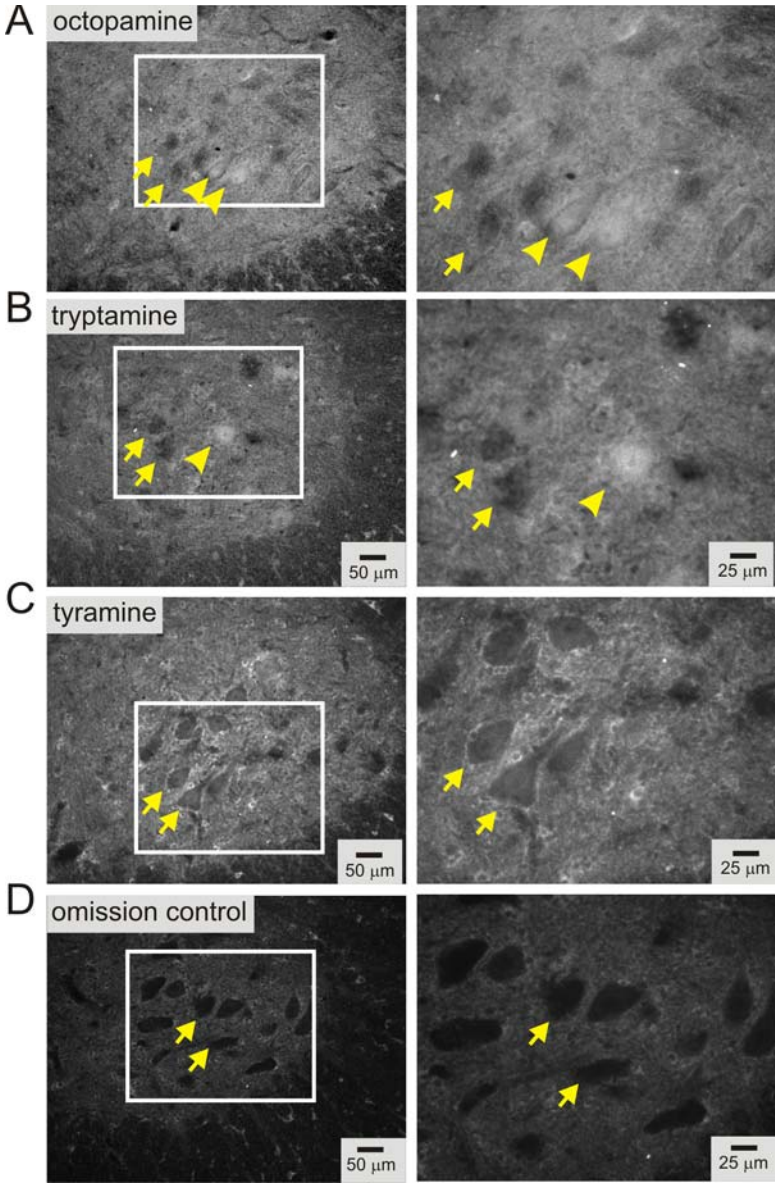

Figure 3.4: Trace amine labeling in the adult rat spinal cord

A. Octopamine immunolabeling was seen throughout the neuropil of the ventral horn. Interestingly some motoneurons appear to be octopamine^{+} (arrowheads) and other are octopamine^{-} (arrows). **B.** Like octopamine, tryptamine immunolabeling was diffuse and weak in the ventral horn neuropil and a subpopulation of motoneurons appear to be tryptamine^{+} (arrowhead) and others were tryptamine^{-} (arrows). **C.** Tyramine immunolabeling was weak or absent from motoneurons (arrows) but, unlike tryptamine and octopamine, tyramine labeling was more punctate and appears to surround motoneurons. **D.** Omission of primary antibody to show background labeling generated with the fluorescent antibody used to label the TAs.

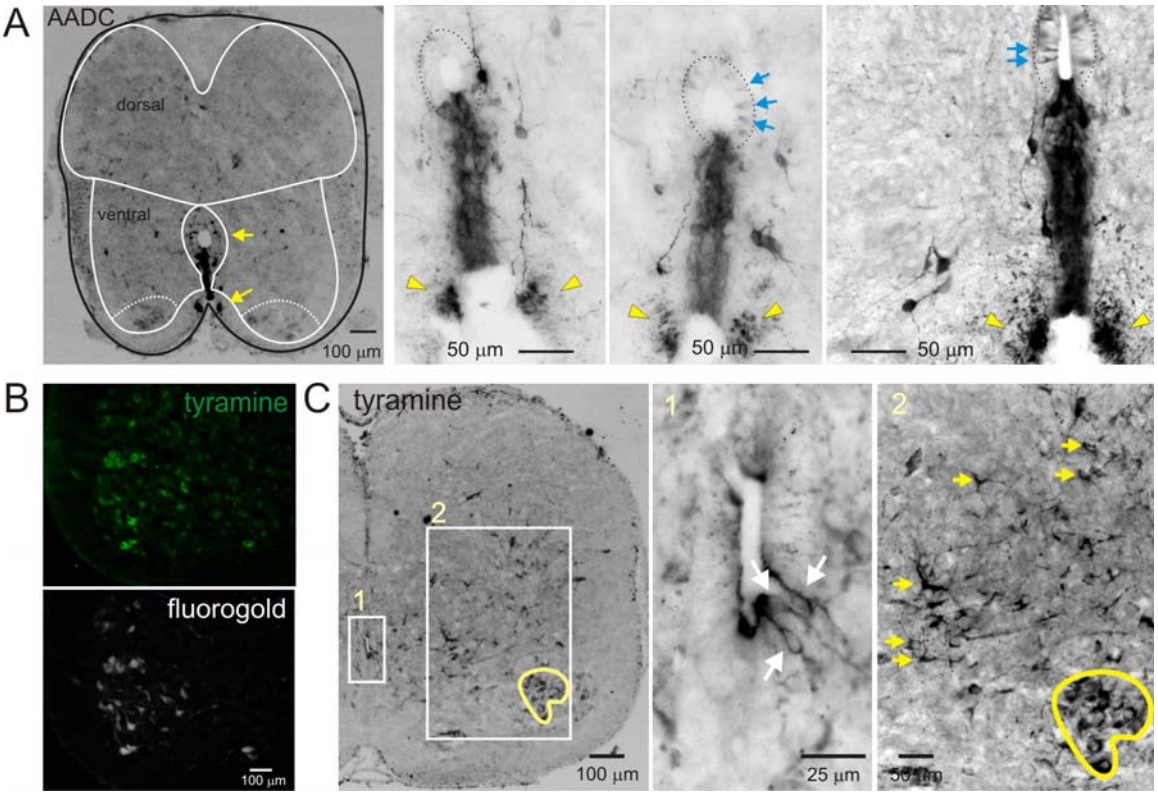

Figure 3.5: Distribution of AADC and tyramine in the neonatal spinal cord

A. Left. Low power of AADC immunolabeling in spinal cord. Superimposed on this section is an outline of the spinal cord (black) with interior white lines approximately demarcating dorsal and ventral gray matter and central canal region. Note that strongest labeling was associated with D cells intermingled with epithelial cells surrounding the central canal (top arrow). Also, note the associated vertical row of cellular labeling projecting ventrally, and bilateral white matter labeling in the ventral funiculus (bottom arrow). There was also weak AADC immunolabeling in putative motoneurons (in lamina IX below dotted lines). Right. Three panels showing higher power images from separate sections illustrating the diversity of AADC labeling in the ventral medial grey matter region. Epithelial cell layer surrounding central canal is outlined. Common to all was the vertical stream of projections with intermingled cells ventral to the central canal. These appear to end in a white matter tract in the ventral funiculus (arrowheads). **B.** Tyramine immunolabeling in motoneurons retrogradely labeled with Fluorogold. The day before sacrifice the rat pup was injected i.p. with fluorogold to retrogradely label the motoneurons. **C.** Tyramine labeling was found in a subset of neurons primarily in the ventral half of the cord. The central region (1) and laminae V-IX (2) are shown magnified. Note the tyramine labeled cells intermingled with epithelial cells lining the central canal in middle panel (arrows) and labeled neurons in the central gray matter (arrows) and in putative motoneurons (circled region) in the right panel.

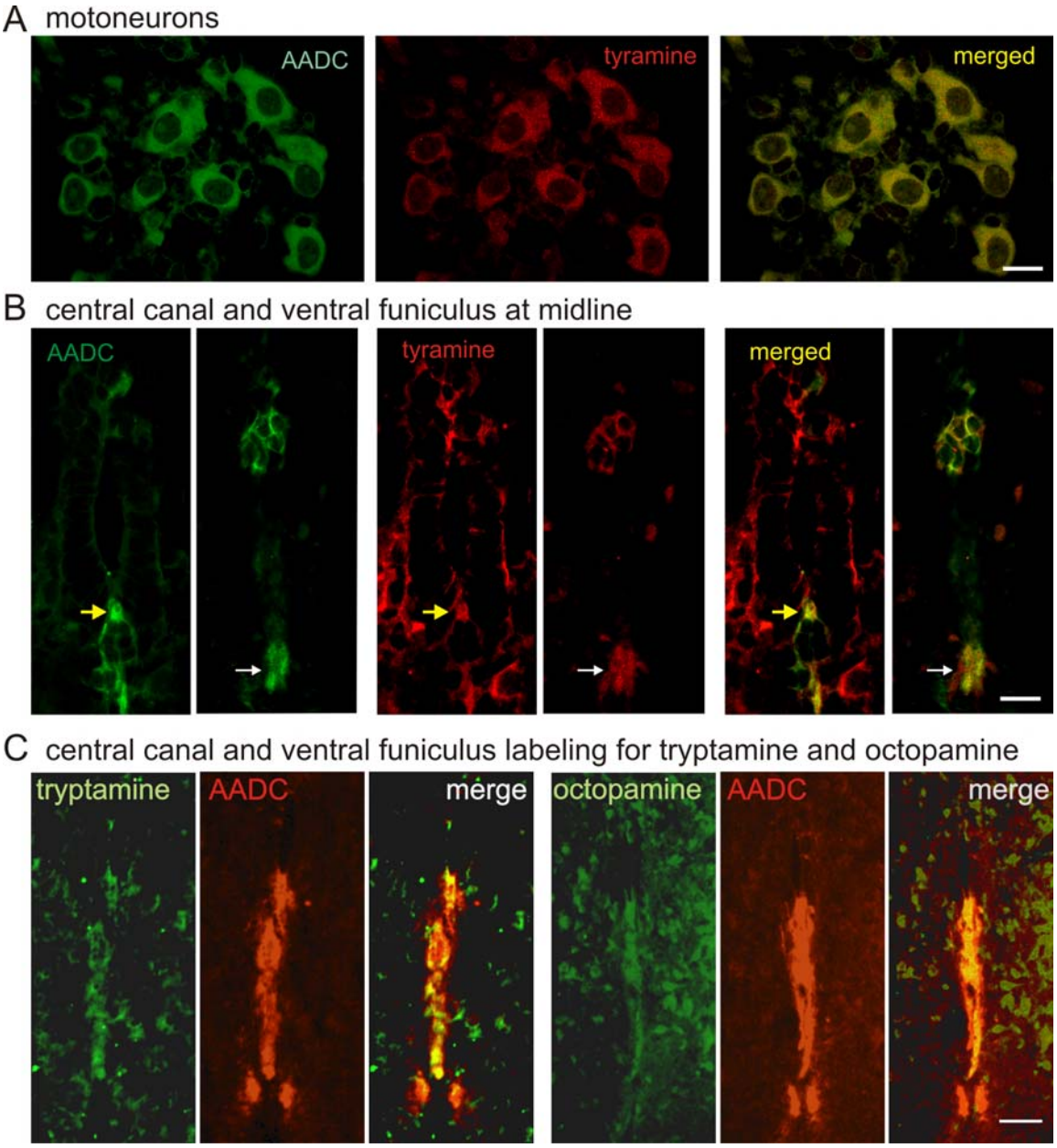

Figure 3.6: Co-expression of tyramine and AADC in neuron subpopulations of the neonatal rat lumbar spinal cord

A. AADC and tyramine immunolabeling in putative motoneurons. While AADC produces more uniform cytoplasmic labeling (left) tyramine labeling includes larger puncta, which were not co-labeled in merged image at right. **B.** AADC and tyramine labeling in D cells associated with the central canal (yellow arrows; left panels for each pair) and its ventral cellular projection stream appearing to terminate at a midline tract in the ventral funiculus (right panels in each pair). White arrows identify ventral funiculus. Note D cells, ventral midline cells and ventral funiculus are co-labeled. All images in A and B are high power confocal images with an optical section of 0.4 µm. **C.** Evidence for tryptamine and octopamine co-expression with AADC. Images were obtained from 10 µm sections. Confocal microscopy was not used for this panel. Scale bar; 20 µm in A and B, 50 µm in C.

medial portion of the ventral funiculus (**Figure 3.5A**, right). In addition, $AADC^{+}$ neurons were found often in the lateral regions near the central canal as well as in the dorsal horn, although more rarely.

Tyramine immunolabeling was characterized by enormous variability between different animals, but with consistent motoneuronal labeling. The identity of motoneurons was confirmed by prior retrograde labeling of motoneurons (**Figure 3.5B**). The most common pattern seen across animals was a widespread diffuse labeling. This is consistent with the widespread distribution of the TA synthesis enzyme AADC. In a subset of animals tested, tyramine preferentially labeled ventral horn interneurons. **Figure 3.5C** shows tyramine expression in an animal with particularly extensive interneuronal labeling in the ventral horn. Widespread labeling was also seen for tryptamine and octopamine. I presume that the variability in different animals reflects the exquisite sensitivity of the TAs to shifts in network activity, substrate availability, transport efficacy, synthesis, and degradation.

While there was clear co-labeling of AADC and tyramine in spinal motoneurons, the co-labeling revealed differences; tyramine labeling also included larger puncta (**Figure 3.6A**). While not shown, comparable punctate labeling was also seen for octopamine and tryptamine. Tyramine, tryptamine, and octopamine were co-expressed with AADC in central canal D cells, in the ventral stream of cells at the midline, and in the ventral funiculus (**Figure 3.6B and C**). These results are consistent with the notion that D cells and related midline neurons are tyraminergic and tryptaminergic. With no DBH found in spinal cord neurons, octopamine should not be produced in the spinal cord yet significant labeling was observed. As octopamine can be co-released with NA (Saavedra 1989) and

subsequently taken up into neurons, this may account for the observed expression. Additionally or alternately, a recently identified enzyme, monooxygenase, DBH-like 1(Moxd1) may be converting tyramine to octopamine in spinal neurons.

As mentioned above for tyramine, there was enormous variability for expression of all three TAs examine; the D cell and other midline neurons were not always clearly immunopositive for all the TAs.

3.4.3 Central canal cells project to the ventral funiculus

To determine whether cells at the central canal could have axonal projections to the ventral funiculus, DiI crystals were applied to various ventral funicular regions in the fixed lumbar spinal cords of a P14 rat one week after thoracic spinalization. When dye placement contacted the midline tract, central canal-associated cells were retrogradely labeled, confirming that cells consistent with the location of D cells can project to the ventral funicular white matter tract (**Figure 3.7**), as expected based on the AADC labeling.

3.4.4 The trace amines were transported into neurons

To test whether the TAs are selectively taken up by neurons, isolated spinal cords were preincubated in the TAs in animals injected with Fluorogold the day before to retrogradely label motoneurons (Merchenthaler 1991). Immunostaining showed that Fluorogold labeled motoneurons were TA^+ (**Figure 3.8**). Further, tyramine, tryptamine, and octopamine uptake appears to be selective to neurons, not glia as staining for the neuron specific marker, NeuN, and the TAs showed that virtually all ventral spinal cord neurons were labeled (**Figure 3.8**). The selectivity to neurons argues against global non-

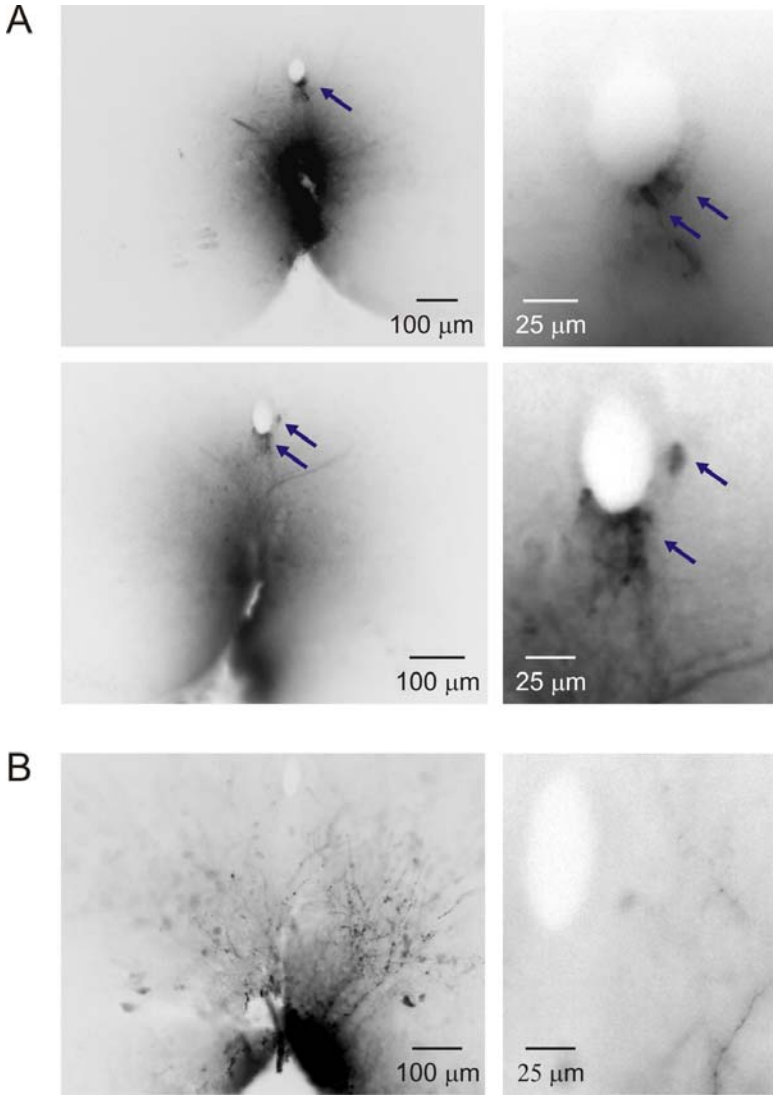

Figure 3.7: Central canal cells project to the ventral funiculus

DiI crystals were placed on fixed lumbar spinal cords at the medial ventral funiculus or just lateral to it and allowed to diffuse to identify grey matter projections sites. **A.** When dye placement contacts the midline tract, neurons associated with the central canal were retrogradely labeled. P14 rat 1 week after with a midthoracic spinalization. **B.** Neurons in the central canal were not labeled. Sections are 70 μm in A and 30 μm in B.

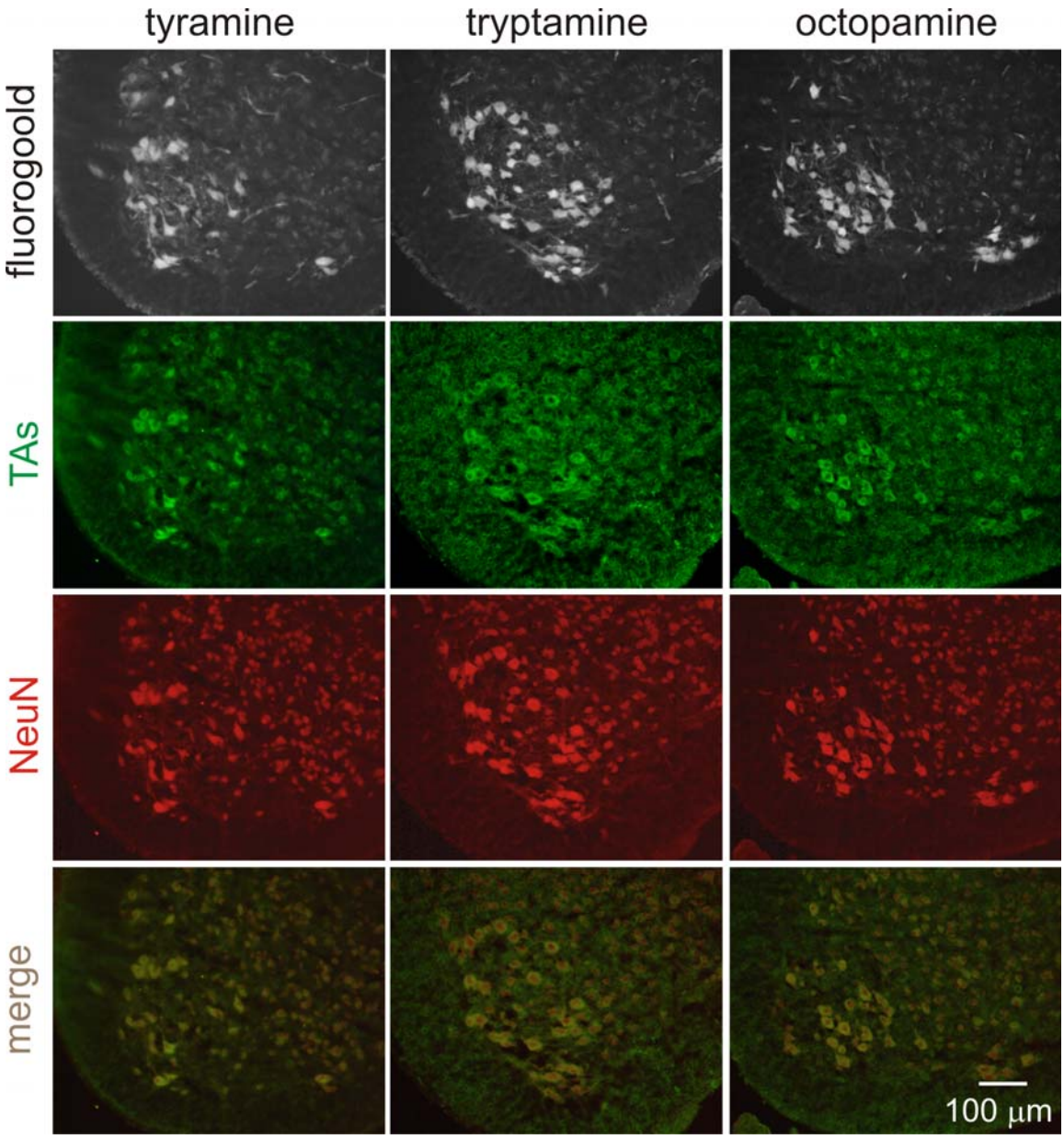

Figure 3.8: Trace amine labeling appears to preferentially label neurons

The isolated spinal cords of P2 rat were pre-incubated in TAs for 2 hours. The day before the treatments, the rat pups were injected with Fluorogold to retrogradely label motoneurons (top row). Row 2 shows immunostaining for tyramine (left), tryptamine (middle), and octopamine (right). Row 3 provides immunostaning for the neuron-specific marker, NeuN. Row 4 is a merge of the trace amine with NeuN to show that the TAs were observed in many neurons, including motoneurons.

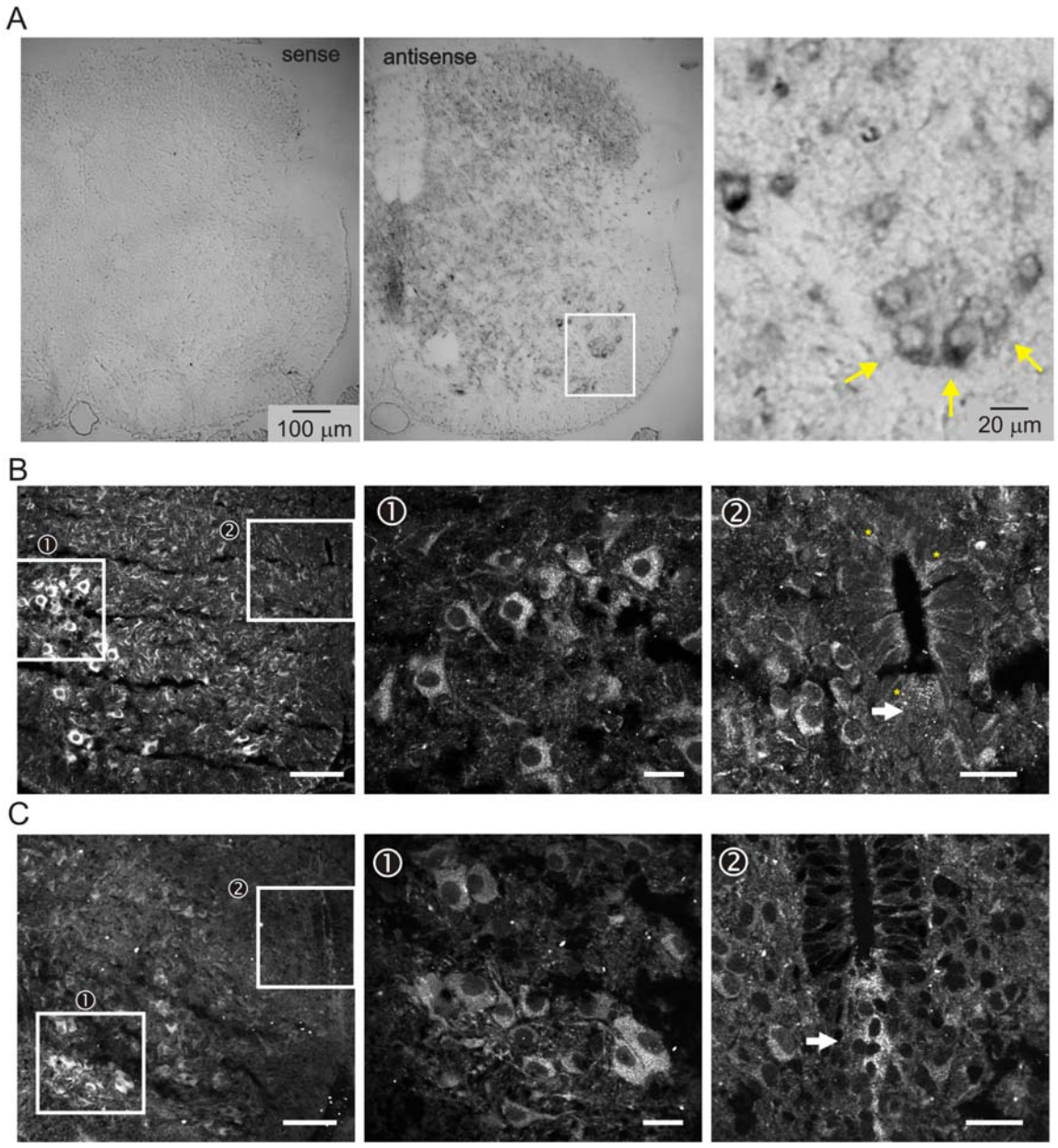

Figure 3.9: TAAR1 and TAAR4 receptors are expressed in the ventral horn

A.TAAR1 mRNA expression in spinal neurons. *In situ* hybridization reveals TAAR1 labeling throughout the P2 rat spinal cord (compare sense to antisense). Particularly notable was the labeling found in the ventral horn, including motoneurons (arrows). **B.** TAAR1 receptor immunolabeling was found in many spinal neurons in the gray matter of the ventral horn, with particularly strong labeling in motoneurons (region expanded in middle panel). There was apparent labeling of cells surrounding the central canal (see asterisks in region expanded in right panel). Note also the punctate labeling that extends as a thread adjacent to epithelial cells with expansion in central canal interior. **C.** TAAR4 receptor immunolabeling was also found in many spinal neurons in the gray matter ventral horn, including strong labeling in motoneurons (region expanded in middle panel). TAAR4 receptors appears to localize in cells ventral to the central canal. Scale bar: 100 µm, left panels; 25 µm, middle and right panels. All images in A and B are confocal images taken at optical section thickness of 1.14 µm, left panels; 0.30 µm, middle and right panels.

selective uptake via passive diffusion. Moreover, this suggests that spinal cord neurons are sensitive to extracellular TA levels.

3.4.5 Trace amine-associated receptors 1 and 4 were widely expressed in the spinal cord

In situ hybridization for TAAR1 in the neonate demonstrated widespread, albeit weak, labeling throughout the spinal cord including motoneurons (**Figure 3.9A**). In P1 neonatal rat spinal cord, immunolabeling for both TAAR1 and TAAR4 was found throughout the spinal cord, especially in motoneurons (**Figure 3.9B and C**). Cells around the central canal were also TAAR1 and TAAR4 immunopositive. Labeling in all neurons appear cytoplasmic. The cytoplasmic location of the ligand and the receptor (e.g. tyramine and TAAR1) supports intracellular activation of signal transduction pathways, as suggested previously. The presence of TAAR1 and TAAR4 provides potential binding sites for the TAs to have independent actions in the spinal cord.

3.5 Discussion

I showed that an anatomical substrate exists for the TAs, a little studied but pervasive class of neuroactive molecules, to be produced and have actions in the spinal cord. The synthesis enzyme, AADC, which is required for production of all of the TAs, was found throughout the spinal cord, but especially around the central canal, ventral funiculus, and in ventral neurons, including motoneurons. Tyramine, tryptamine, and octopamine were also found in similar locations in the spinal cord. It seems highly likely that tyramine, tryptamine, and PEA (which was not examined due to a lack of a specific antibody) are produced in the spinal cord by AADC. AADC expression was still present after

spinalization, but TH labeling was absent, suggesting that after spinalization tyramine, tryptamine, and PEA can be intrinsically produced in the spinal cord.

Octopamine further requires DBH, which is located only in descending neurons, implying that octopamine is only produced in descending noradrenergic neurons. However as stated above, another enzyme, Moxd1 has comparable function (Chambers et al. 1998; Xin et al. 2004) and could also theoretically convert tyramine to octopamine. Moxd1is strongly expressed in the mouse spinal cord including in presumed motoneurons and around the central canal (**Appendix A.1**) (Allen_Spinal_Cord_Atlas 2009). Further studies will be required to determine if Moxd1 does in fact produce octopamine, and hence, if octopamine is also intrinsically produced in the spinal cord. Further, as indicated in our studies and in those of others, the lack of both TH and DBH in the spinal cord indicates that the monoamines are not produced in the spinal cord neurons, but are made in terminal of descending neurons. **Figure 3.10** shows the possible locations of production of the TAs and monoamines.

Since the TAs appear to be transported into neurons (explored in more detail later), this would explain how the TAs, including octopamine, could be found in neurons besides those where it could be produced. The presence of the TAs into neurons is a mechanism by which they can be having actions on the intracellularly located TAARs. TAAR1 and TAAR4 also have widespread expression in the spinal cord and are activated by the TAs: TAAR1 by tyramine and PEA, and TAAR 4 by tryptamine and PEA (Borowsky et al. 2001). That the TAs have a family of receptor to which they preferentially bind, suggests that they can produce effects on their own independent of

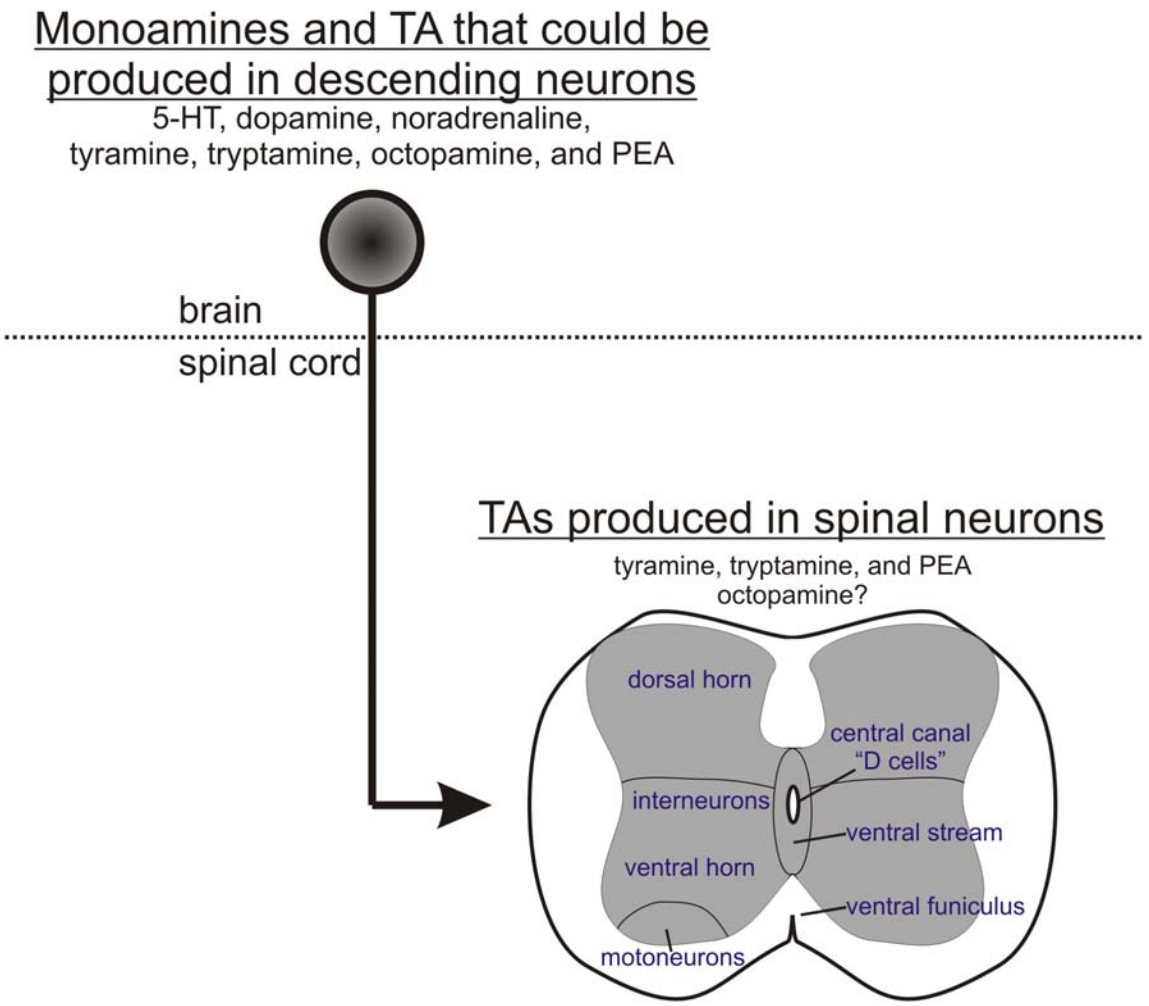

Figure 3.10: Summary of the locations that the trace amine and monoamines can be produced
The TAs, tyramine, tryptamine, and PEA, are likely produced in the spinal cord. AADC, the only enzyme required for their production, was found with widespread labeling throughout the spinal cord in neurons and axons that are labeled above in the spinal cord cross section. Labeling of tyramine and tryptamine supports widespread production of the TAs. DBH one of the required enzymes for octopamine is not found in the spinal cord; however, another enzyme Moxd1 that may also produce octopamine is found in the spinal cord. Therefore, it is currently unknown whether octopamine can be produced in the spinal cord. The enzymes necessary for production of both the monoamines and TAs are found in descending neurons.

the monoamines and their receptors, thus giving an anatomical substrate for the TAs to have independent intrinsic biological actions.

3.5.1 AADC and trace amines are widely expressed in the spinal cord

Previously, many neuronal populations were observed to be $AADC^+$ that did not express 5-HT or TH (cannot synthesize DA, NA, or adrenaline). They were called D cells (Jaeger et al. 1984a; Jaeger et al. 1983). One of the 14 identified groups was located in the spinal cord and called D1 cells (Jaeger et al. 1984a). D1 cells project at least one of their processes into the lumen of the central canal, which makes them part of a group of cerebrospinal fluid (CSF)-contacting neurons (Jaeger et al. 1983; Vigh et al. 2004). No neurotransmitter was identified for these neurons, and nothing is known about their function. It has been speculated that D cells could produce the TAs (Berry 2004; Jaeger et al. 1983).

Here, AADC was expressed in spinal cord of adult and neonatal rat. Labeling was widespread but relatively weak in most cells. However, the cells surrounding the central canal, a ventral stream from this region, and blood vessels appeared strongly $AADC^+$. Labeling of cells around the central canal is consistent with the previous identification of D1 cells (Jaeger et al. 1983). The additional $AADC^+$ neurons that were identified confirms that there are other spinal neurons that have yet to be examined and classified in the vicinity (Nagatsu et al. 1988). Motoneurons and other cells were more weakly labeled. Neither the *in situ* hybridization nor immunodetection of AADC have been previously reported in rat spinal motoneurons. However, the *in situ* hybridization performed by the Allen Mouse Spinal Cord Database confirms widespread neuronal

labeling in both adult and neonatal mouse spinal cord. Labeling around the central canal and in motoneurons was notable (Appendix **Figure A.1**).

The AADC$^+$ D cells found near the central canal, as mentioned above, were CSF-contacting neurons. While the function of these D cells is still unknown, there are CSF-contacting neurons positioned very similarly in the zerbrafish larva. These neurons can initiate slow swimming by optogenetic stimulation, their genetic silencing reduces the frequency of spontaneous locomotion, and they provide the necessary tone for spontaneous forward swimming (Wyart et al. 2009). Based on the similarity in location, it seems plausible that the D cells may be contributing to the frequency and tone of locomotion in the rat, but further studies will be needed to determine this conclusively.

It is noteworthy that while the *in situ* hybridization labeling shows widespread AADC expression in a normal animal, AADC immunolabeling post-spinalization was much more restricted after the degradation of descending monoaminergic terminals. The most obvious explanation for these differences is that intrinsic AADC expression was altered by events related to spinalization. AADC can be regulated at both the level of protein activity and gene expression with long term regulation being due to changes in gene expression. It has been shown that there is an excellent correlation between mRNA level and AADC activity (Coge et al. 1990).

The most consistent observation common among all TAs tested was the central cell, midline, and motoneuronal labeling. These locations were consistent with the locations of AADC. The apparent specificity of tyramine to a population of ventral horn interneurons was seen in only a small number of animals tested. I presume the variability reflects the exquisite sensitivity of the TAs to shifts in network activity, substrate

availability, transport efficacy, synthesis, and degradation. Also, there were differences in motoneuron expression of the TAs compared with the adult, suggesting that there were developmental differences. This is not surprising given that AADC expression changes from embryonic to adult rats (Teitelman et al. 1983). For example, somatic motoneuronal tyramine immunolabeling is found in neonate but appears absent in the adult and replaced instead by apparent pericellular labeling. The explanation for this difference in expression pattern is currently unknown.

TA incubation studies showed that tyramine, tryptamine, and octopamine were transported specifically into neurons and not glia, arguing against global uptake via passive diffusion. Many transporters are bi-directional, meaning that the TAs are likely both taken up and released from neurons via transporters. Once in neurons, the TAs would be available to activate TAARs, which I showed to be intracellular. Also, since the TAs are produced in neurons, like motoneurons, they could be acting in the neurons in which they were made, in addition to being transported out of these neuron and acting at nearby neurons. A more detailed discussion of transport mechanisms is provided in Chapter 4.

3.5.2 Trace amine-associated receptors 1 and 4 were widely expressed in the spinal cord

TAAR1 and TAAR4 both had widespread expression in the spinal cord. Of particular relevance was that TAAR expression overlapped with the expression of AADC and TAs. Thus, mechanisms for TA synthesis and actions are anatomically coincident, providing a substrate by which TAs can produce effects on their own. As has been observed before for TAAR1 in HEK cells (Bunzow et al. 2001; Miller et al. 2005), I observed cytoplasmic

labeling for TAAR1 and TAAR4, both of which are activated by the TAs. Tyramine and PEA activate TAAR1, while PEA and tryptamine activate TAAR4 (Borowsky et al. 2001). The cytoplasmic location of the ligand and the receptor (e.g. tyramine and TAAR1) supports intracellular activation of signal transduction pathways, as suggested previously (Miller et al. 2005). Such a co-localization would not require release from vesicles and could explain why the TAs do not appear to be found in vesicles. It could also explain the relatively trace amounts of TAs found in the spinal cord. If the production of the TAs was typically by or adjacent to the cells that had cytoplasmic TAARs, there would be no need to keep the concentration high.

Since access to the TAARs is limited by the presence of transporters (Xie et al. 2007), this would explain the observed low potency of the TAs for TAAR4 expressed in HEK cells (Borowsky et al. 2001). Further, the intracellular location may also explain why studies to identify the ligands for other TAARs have failed despite the prediction that the ligands must be small molecular weight compounds that are structurally similar to the TAs (Lindemann et al. 2005; Lindemann and Hoener 2005). The assays assume cell surface expression of the receptor.

Previously, *In situ* hybridization, RT-PCR and LacZ reporter expression studies all observed labeled TAAR1 in the brain (Borowsky et al. 2001; Bunzow et al. 2001; Lindemann et al. 2008) with one report also examining spinal cord (Borowsky et al. 2001). Additionally, immunohistochemistry for TAAR1 in rhesus monkey observed labeling in substantia nigra, thalamus, and cerebellum, as well as in mouse brain (Torres et al. 2003). On the other hand, Liberles and Buck (2006) could not detect any TAARs in mouse brain with RT-PCR (Liberles and Buck 2006) and the Allen Mouse Spinal Cord

Database *in situ* hybridization expression studies report the TAARs to be absent in both P4 and P56 with similarly little to no expression in brain (**Appendix Figure A.3**) (Allen_Spinal_Cord_Atlas 2009). Here, I present evidence that TAAR1 and TAAR4 are expressed in the neonatal rat spinal cord (**Figure 3.9**), most notably with labeling in motoneurons. Furthermore, close inspection of the Allen Mouse Spinal Cord Database shows clear weak diffuse labeling for both TAAR1 and TAAR4 in both P4 and P45, including in putative motoneurons (**Appendix Figure A.4A**). I assume their designation 'absent' is based on conservative threshold detection.

Wolinsky et al (2007) provided the first conclusive evidence for a role of TAAR1 in CNS function by demonstrating behavioral changes in TAAR1 knockout mice. There, the TAAR1 receptor agonist amphetamine, in the absence of its action on TAAR1, showed an enhanced psychomotor-stimulating effect, temporally correlated with larger increases in striatal DA and NA release, and 2.6 fold increase in the proportion of striatal high-affinity D2 receptors. TAAR1 therefore appears to play a depressant modulatory role in brain catecholaminergic function (Wolinsky et al. 2007). Lindemann et al (2008) also studied TAAR1 knockout mice and corroborated several of the above findings. They also found that these mice display enhanced amphetamine-triggered increases in locomotor activity and an elevated spontaneous firing rate of dopaminergic neurons in the ventral tegmental area. Tyramine specifically decreased the spike frequency of these neurons in wild-type but not in TAAR1 knockout mice (Lindemann et al. 2008). It is important to note that the locomotor-enhancing actions of TAAR1knockout mice is associated with modulation of basal ganglia dopaminergic neurons. Here, in the isolated spinal cord it is possible that the effects would be different, and based on our

electrophysiologiy studies described below, I would predict that TAAR1 knockout would depress spinal locomotion. Xie and Miller (2008) also examined TAAR1 knockout mice. PEA significantly inhibited uptake and induced efflux of DA, NA, and 5-HT in transfected cells and brain synaptosome of rhesus monkeys and wild-type mice, but not in synaptosomes of TAAR1 knockout mice. The PEA effect was blocked by transporter inhibitors demonstrating that TAAR1 signaling is required for PEA to alter monoamine transporter function. They also showed these effects to be independent of monoamine autoreceptors (Xie and Miller 2008). As TAAR1 receptor knockout mice have shown clear modifications in CNS behavior, these receptors seem to play a functional role in the nervous system (Sotnikova et al. 2009; Wolinsky et al. 2007).

3.5.3 Summary

This chapter establishes an anatomical substrate onto which the TAs could be having intrinsic spinal cord biological actions. AADC, the TAs, and TAARs were found to be widely present in the spinal cord and in neurons with similar locations. Since the TAARs are intracellular there are two likely mechanisms by which the TAs could have effects in spinal neurons. The first would be paracrine signaling of nearby neurons with the TAs using transporters to enter the cell, and the second would be that the TAs, with the possible exception of octopamine, were acting in autocrine fashion in the neurons in which they are produced.

CHAPTER 4

NEUROMODULATORY ACTIONS OF TRYPTAMINE, TYRAMINE, OCTOPAMINE, AND B-PHENYLETHYLAMINE ON MOTOR AND LOCOMOTOR ACTIVITY IN THE MAMMALIAN SPINAL CORD

4.1 Abstract

An anatomical substrate for trace amine (TA) effects in the spinal cord was demonstrated. Here, I tested whether the TAs were able to have effects on motor activity by comparing the actions of the TAs to those of the classical monoamine transmitters in the *in vitro* isolated neonatal rat spinal cord. Tyramine and tryptamine most consistently increased motor activity, including by direct actions on motoneurons. When applied alone tyramine, tryptamine, and octopamine could also activate spinal pattern-generating circuits. In the presence of NMDA, all TAs produced a locomotor-like activity indistinguishable from that observed with serotonin (5-HT), supporting TA-induced actions on conventional locomotor central pattern generating interneurons. Intriguingly, the TAs also produced unique rhythms characterized by episodic bouts of rhythmic bursting. *In vivo* injections of the TAs into previously spinalized animals also activated hindlimb locomotor patterns consistent with bath application thereby supporting a role of the TAs independent of descending monoaminergic systems.

TA and DA evoked actions were observed to be initiated at much longer latency than 5-HT or NA, supporting a requirement for intracellular transport. To test whether TA and DA transport into neurons was required for their effects, I blocked Na^+-dependent uptake with monoamine transport inhibiters. TA and dopamine-induced bursting events

were attenuated but not blocked while 5-HT and noradrenaline actions were unaffected. Evidence of a further contribution from Na^+-independent transporters was shown with increased neuronal labeling after incubation of the spinal cord with TAs in a nominally Na^+-free solution. As multiple Na^+-independent transporters that transport the TAs are shown to be widely expressed in the spinal cord, an intrinsic spinal neuronal transport system likely exists that is independent of transport-mediated action at descending monoaminergic.

These results demonstrate that the TAs are capable of producing complex and differentiable modulatory actions on motor function. It is likely that distinct actions of the TAs are complex and based on interactions with transporters, synthesis and metabolic enzymes, and receptors found heterogeneously in the spinal cord. Overall, I assert that the TAs represent an intrinsic modulatory control system that works with the conventional monoamines to control spinal motor behavior.

4.2 Introduction

The classical monoamine neurotransmitters, dopamine (DA), noradrenaline (NA), and serotonin (5-HT), modulate spinal cord sensory and motor function (Clarac et al. 2004; Hochman et al. 2001; Jacobs and Fornal 1993; Millan 2002; Rekling et al. 2000; Schmidt and Jordan 2000). They have structural, metabolic, physiologic, and pharmacologic similarities to another group of endogenous amines called trace amines (TAs) (Saavedra 1989), whose function is unknown. The TAs, tryptamine, tyramine, octopamine, and β-phenylethylamine (PEA), are synthesized from the same precursor amino acids by the enzyme aromatic-L-amino acid decarboxylase (AADC) with octopamine (like NA) further requiring dopamine β-hydroxylase (DBH). Conversion from the TAs to the

monoamines does not appear to occur (Berry 2007). Degradation is primarily via monoamine oxidases (Shimazu and Miklya 2004). TA synthesis rates are comparable to the classical monoamines, but their exceedingly rapid turnover rates (half-life ~ 30 s) and limited vesicular storage results in low endogenous levels (Boulton et al. 1977; Durden and Philips 1980; Dyck 1989; Juorio et al. 1988; Wu and Boulton 1973; 1974; 1975).

The discovery of the trace amine-associated receptors (TAARs) establishes a mechanism by which the TAs can produce effects on their own (Borowsky et al. 2001; Bunzow et al. 2001; Hauger et al. 1982; Kellar and Cascio 1982; Lindemann and Hoener 2005; Nguyen and Juorio 1989; Vaccari 1986; van Nguyen et al. 1989) rather than function as metabolic-by-products or false transmitters as the TAs have traditionally been viewed (Berry 2004; Boulton 1976; Grandy 2007; Kopin et al. 1965).

In the previous chapter, I established that AADC, the TAs, TAAR1, and TAAR4 were found in the spinal cord, and hence provide and anatomical substrate by which the TAs can have their own actions. I also found that the TAs were able to be transported into neurons, and that the TAARs were found intracellularly. Consistent with the TAARs being intracellular, activation of TAAR1 by the TAs was shown to be greatly enhanced after co-expression of monoamine transporters in HEK cells, an enhancement that is subsequently reduced by monoamine transport inhibitors (Xie et al. 2007).

I hypothesized that TA affect spinal motor networks and can produce rhythmic motor behaviors. To test this hypothesis, I used the isolated intact neonatal rat spinal cord maintained *in vitro*. The spinal cord contains neural circuitry capable of generating locomotion, and its properties have been thoroughly studied in this model system in rodents. Fundamental insights into the neuromodulatory role of the classical

monoamines have been gained through the use of this model system, which makes in appropriate for studying the TAs (Hochman et al. 2001; Schmidt and Jordan 2000; Wallis 1994; Whelan 2003). I show that the TAs can produce rhythmic motor behaviors and have distinct actions. Some of these results have been presented in abstract form (Gieseker et al. 2004; Gozal et al. 2007a; Gozal et al. 2010; Gozal et al. 2006).

4.3 Methods

All experimental procedures complied with the NIH guidelines for animal care and the Emory Institutional Animal Care and Use Committee.

4.3.1 Electrophysiology

4.3.1.1 General setup

Sprague-Dawley rats postnatal (P) day 0-5 were decapitated, eviscerated, and placed in a bath containing oxygenated (95% O_2, 5% CO_2) artificial cerebral spinal fluid (aCSF) containing the following (in mM): 128 NaCl, 1.9 KCl, 1.2 KH_2P0_4, 26 $NaHCO_3$, 2.4 $CaCl_2$, 1.3 $MgSO_4$, and 10 glucose (pH of 7.4). The spinal cord was exposed by a ventral vertebrectomy and carefully dissected out of the body cavity leaving the dorsal and ventral roots attached. The spinal cord was secured with insect pins to a chamber with Sylgard (Dow) on the bottom. Glass suction electrodes were applied to dorsal and/or ventral roots, after which the preparation was allowed to recover for at least 1 hour before experimentation at room temperature. The ventral root electroneurographic activity was amplified (10,000x), band-pass filtered at 10-3,000 Hz and digitized at 5 kHz (Digidata 1321A, 16-bit; Axon Instruments). Band-pass filter frequency settings were selected with consideration to observed frequency components with the low-pass filter set ant mush

greater than the Nyqist frequency. Data was captured on a computer with the pCLAMP acquisition software (v8-9, Molecular Devices; Union City, CA). Electrophysiological data analysis was performed using pCLAMP analysis software (Clampfit) or software written in-house using MATLAB. Statistical comparisons were made in MATLAB using ANOVA or Student's t-test. The means is reported as mean ± SD.

4.3.1.2 Motor activity and motor patterning experiments

Motor activity was monitored using glass suction electrodes attached to ventral lumbar roots, typically bilaterally to L2 and L5. L2 ventral root activity primarily indicates activity in flexors, while L5 ventral root activity primarily indicates activity in extensors (Kiehn and Kjaerulff 1998).

4.3.1.2.1 General motor activity

General motor activity was examined by first applying a RC high-pass filter at 1Hz to reduce drift and then calculating the root mean square (RMS) of a representative ventral root signal and comparing 100 second periods before application of these TAs and monoamines and during their period of maximal response. Changes were expressed as a percent increase over baseline.

4.3.1.2.2 Locomotor-like activity

Locomotor-like activity was analyzed using the in-house MATLAB software, SpinalMOD, which calculated the frequency, peak amplitude, and phase, which was calculated using the middle of the burst (Matsushima and Grillner 1992). I wrote SpinalMOD, and it is discussed in chapter 2.

4.3.1.2.3 Neurochemicals

Neurochemicals, which were stored in 10mM or 100mM stock solutions at -20°C, were added to the bath (typically 30mL) to achieve the final concentration in the chamber. Neurochemicals were obtained from Sigma-Aldrich (St. Louis, MO), including N-methyl-D-aspartate (NMDA) (3-5 μM) and methysergide (5-HT_1, 5-HT_2, 5-HT_7 and tryptamine binding site antagonist; 1-10 μM).

The following monoamines were used: 5-HT (50 μM), noradrenaline (50 μM), and dopamine (50 μM). Drug concentrations were chosen at values comparable to those previously used. The following TAs were used: tryptamine (50 μM), tyramine (1-100 μM), octopamine (50-100 μM), PEA (50-100 μM). TA doses were chosen to match those of the monoamines under the assumption that they have equivalent transporter uptake and degradation following exogenous application. Due to the efficiency of the monoamine transporters, it has been estimated that the actual dose is $1/30^{th}$ that applied (David Bennett, personal communication).

4.3.1.3 Reflex experiments

To evoke the reflexes, constant current stimuli were applied to the dorsal roots while motor activity was recorded from ventral lumbar roots, typically L5. Stimulus intensities were 500 μA and durations ranging from 100 to 500 μs. For experiments examining motoneuron activity in the absence of synaptic transmission, reflexes were abolished after switching from regular aCSF to with high Mg^{2+}, low Ca^{2+} aCSF or zero Ca^{2+} aCSF.

4.3.1.4 Transport inhibitors

First, either the TAs or monoamines were applied with NMDA to induce motor rhythmicity. The bath was then washed, and a cocktail of transport inhibitors was applied. Finally, the TAs and monoamines were re-applied with NMDA in the transport

inhibitors and compared. The following monoamine uptake transport blockers were used: citalopram (SERT inhibitor, 1 μM), bupropion (DAT inhibitor, 1 μM), and clomipramine (SERT and NET inhibitor, 5 μM).

4.3.2 Behavioral studies in transected neonates

Rodents were anesthetized with isoflurane via inhalation. Following dorsal laminectomy to expose lower-thoracic segments of the cord, one section of the cord between T8-T12 was removed using surgical microdissection scissors. Gel foam was placed in the site of transection to maintain the gap between rostral and caudal cord before being closed up.

Rats were spinalized at P2-3 and behavioral experiments were conducted at either P4-5 to complement the *in vitro* work or at P11-12 to allow a longer time from transection to allow sufficient time for degeneration of descending monoaminergic axonal terminals (Commissiong and Toffano 1989). The spinal cord-transected rats were intraperitoneal (i.p.) injected with TAs at 50 mg/kg (or saline control) and videotaped. . The rhythmic locomotor hindlimb movements were observed with the rats either held in a harness to monitor air-stepping or in a half-body chamber with hindlimbs resting on a moving treadmill belt. The half-body chamber was an adjustable horizontal apparatus where the neonatal rats voluntarily crawl into a size-limited and depth-adjustable tube with their hindlimbs suspended, similar to the harness, except that the rats were more supported, less stressed, and in a more physiologic relevant postural position. The results for the harness and half-body chamber were considered equivalent regarding the ability to monitor leg movements and were grouped together. A rough frequency of leg

movements was calculated by counting leg movements over a period of time and dividing the leg movements by the time.

4.3.3 Immunohistochemistry

The spinal cord was isolated from rats who had been i.p. injected with Fluorogold 24 hours prior to sacrifice to retrogradely label most spinal motoneurons (Ambalavanar and Morris 1989) (Merchenthaler 1991). A set of incubation experiments was done to examine whether exogenously applied TAs could increase cytoplasmic expression levels in spinal neurons, and whether uptake was NA^+-dependent. Isolated cords were incubated in one of three different treatments for 2 hours: (1) control regular aCSF, (2) the TAs, octopamine, tyramine, and tryptamine (all at 100 μM), in regular aCSF, and (3) the TAs (all at 100 μM) in zero Na^+ (replaced with equimolar choline).

The spinal cords were then post-fixed for 1 hour in Lana's fixative than cryoprotected in 10% sucrose, 0.1M PO_3 until sectioned into 10 um thick sections on a cryostat and processed for immunohistochemistry. All incubations and washes were performed in 0.1M PO_3-buffered saline containing 0.3% triton X-100 (PBS-T). Tissue was washed overnight in PBS-T at 4°C followed by incubation in primary antibody for 48-72 hours. Slides were then washed three times for 30 minutes and incubated in secondary antibody. The following antibodies combinations were used:

Table 4.1: Antibodies used for immunohistochemistry expression

Primary Antibody	Secondary Antibody
Rabbit anti-tyramine 1:100 or 1:1000 (Chemicon)	cy3 anti-rabbit (Jackson Immunoresearch)
Rabbit anti-tryptamine 1:50 (Chemicon)	
Rabbit anti-octopamine 1:500 (Chemicon)	

In all experiments, omission controls for the primary antibodies were used. Others have provided pre-absorption controls abolishing staining for tryptamine (Dabadie et al. 1990) and octopamine (Karhunen et al. 1993), but there is only evidence for displaced tyramine binding (Geffard et al. 1984). Thus, tyramine pre-absorption controls were performed. Appropriate antibody concentrations (1:100 and 1:1000) were absorbed with 1 mg of antigen (Cell Sciences) for 1 hour prior to incubation. This abolished staining for tyramine in 6 of 7 animals. Images were photographed with a Nikon (Tokyo, Japan) digital camera through a Nikon E800 microscope or using an Olympus FV1000 inverted confocal microscope. Images were processed using Corel Draw (Corel, Ottawa, Ontario, Canada).

4.4 Results

4.4.1 Trace amines can induce motor activity and rhythmic locomotor bursting patterns

To examine whether the TAs have actions on the recruitment of motoneurons while synaptic transmission was intact, the motor output before and after application of the TAs and monoamines were compared). As shown in **Figure 4.1A and C,** the increases in activity were overt and significant for 5-HT and NA, where the increases in activity were 279% (n=11) and 253% (n=14), respectively. DA, tyramine, and tryptamine, also significantly increased activity by 50% (n=14), 67% (n=10), and 80% (n=18), respectively. Octopamine and PEA did not have significant actions on motor activity (26±50% [n=9] and -3±5% [n=7], respectively).

In a subset of experiments, monoamine application initiated rhythmic motor activity. 5-HT (n=4), NA (n=8), and DA (n=1) produce rhythmic motor activity similar to that

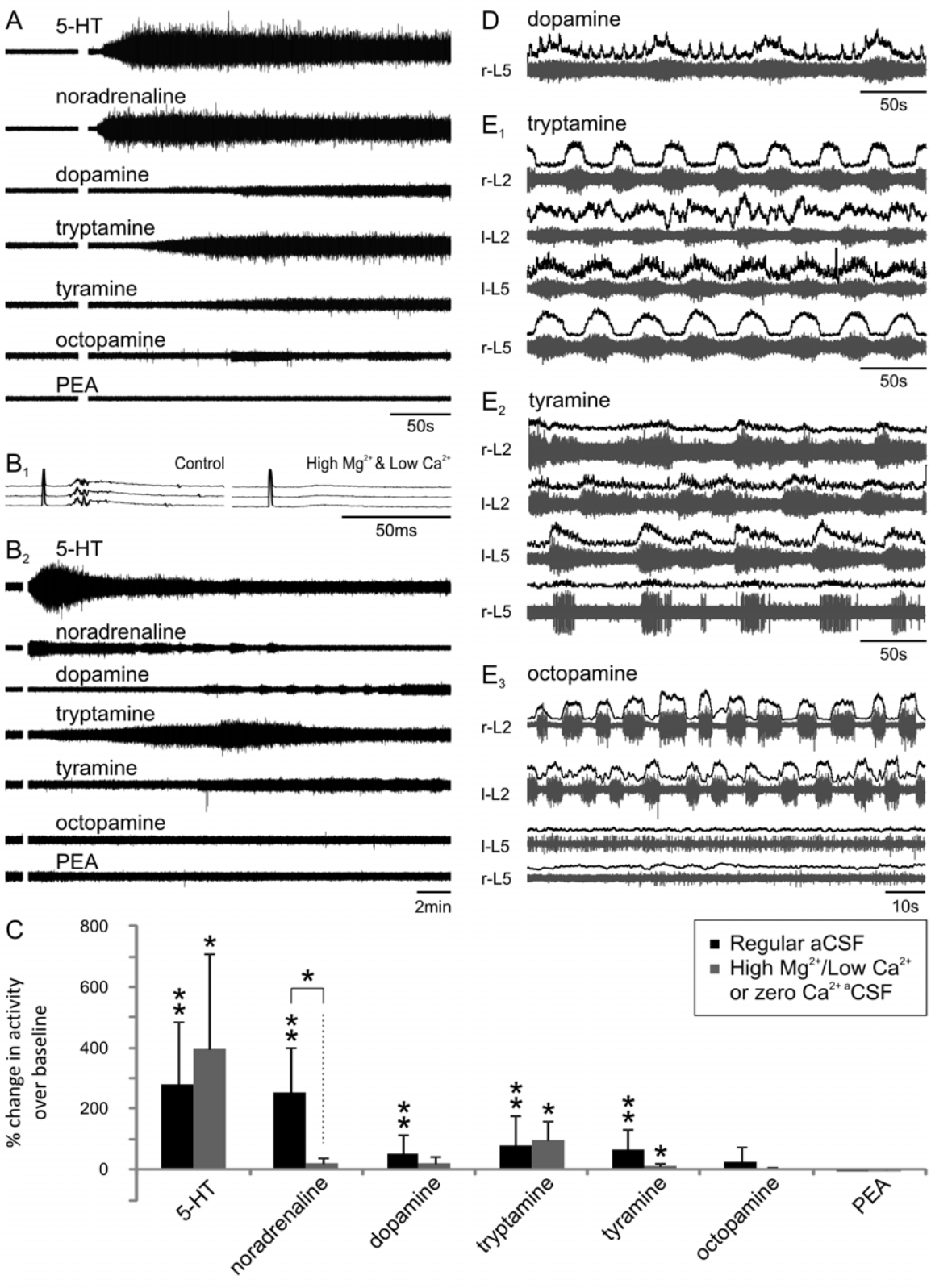

Figure 4.1: Trace amines increased motor activity and induced rhythmic motor bursting patterns

Figure 4.1: continued

A. All TAs and monoamines were able to significantly increase lumbar ventral root activity, except octopamine and PEA. **B1.** Dorsal root stimulation evoked reflexes (left) were abolished when synaptic transmission was blocked in low Ca^{2+}, high Mg^{2+} ACSF (right). **B2.** Under these conditions, direct excitatory actions were still observed. **C.** The changes in ventral root activity were quantified with and without synaptic transmission for the TAs and monoamines. (* indicates $p<0.05$ and ** indicates $p<0.01$). **D.** Dopamine produced high frequency bursting overlaid by a slow frequency bursting, typically on only one ventral root. **E.** Tryptamine, tyramine, and octopamine occasionally induced rhythmic motor bursting patterns. Here, tryptamine and tyramine produced synchronous bursts on the L5 ventral roots and alternating bursts on the L2 ventral roots. Octopamine generated alternating bursts on the L2 ventral roots. Drug concentrations in the figure were 50 μM for tyramine, octopamine, tryptamine, dopamine, and noradrenaline, and 100 μM for PEA, except in E2 were the concentration was 10 μM for tyramine. The ventral roots are denoted as right lumbar segment 2 (r-L2), left lumbar segment 2 (l-L2), left lumbar segment 5 (l-L5), and right lumbar segment 5 (r-L5). In D, E, and subsequent figures, the upper traces in black at each lumbar root have been rectified and low-pass filtered.

previously reported (Barriere et al. 2004; Cazalets et al. 1990; Cowley and Schmidt 1994a; Kiehn and Kjaerulff 1996; Kiehn et al. 1999; Sqalli-Houssaini and Cazalets 2000); however, in my experiments DA (n=2) also produced higher frequency bursting, on only the right L5 ventral root (0.125 ± 0.002 Hz, n=2) that was overlaid by a second slower bursting frequency (0.012 ± 0.001 Hz, n=2; **Figure 4.1D**).

A subset of tryptamine, tyramine, and octopamine experiments also produced rhythmic motor activity that was different than the rhythmic patterns produced by the monoamines. Tryptamine (n=3) induced rhythms varied with a mean frequency of 0.026 ± 0.001 Hz (n=3). The patterns observed were: alternation between L3 roots, rhythmicity in an individual root, and synchronous bursts on the L5 ventral roots with alternating bursts on L2 (**Figure 4.1E$_1$**). Tyramine (n=3) produced two different bursting patterns. First, tyramine produced synchronous bursts on the L5 ventral roots and alternating bursts on the L2 ventral roots (0.018 ± 0.003 Hz, n = 2). The pattern was relatively stable and lasted until tyramine was removed (40 minutes or 3 hours) (**Figure 4.1E$_2$**). The second pattern was restricted to the right L5 ventral root with a very slow frequency (0.008 Hz; not shown). Octopamine (n=1) produced a faster motor rhythm (0.12 Hz) with alternating bursts recorded from L2 ventral roots and tonic activity from L5 ventral roots (**Figure 4.1E$_3$**). These results demonstrate that tyramine, octopamine, and tryptamine can act on pattern-generating circuits.

To test whether the TAs were acting directly on motoneurons, chemical synaptic transmission was minimized by replacing the regular aCSF with either a high Mg^{2+}, low Ca^{2+} aCSF or zero Ca^{2+} aCSF. Under these conditions, recorded reflexes were completely abolished (**Figure 4.1B$_1$**). Subsequently, the motor output before and after application of

the TAs and monoamines were compared (**Figure 4.1B$_2$ and C**). 5-HT, tryptamine, and tyramine significantly increased activity (397±312% [n=4], 98±61% [n=4], and 13% ± 10% [n=4], respectively); however, these changes in activity were not statistically different than observed with intact synaptic transmission. Octopamine (n=4), PEA (n=4), NA (n=3), and DA (n=3) did not have significant effects on motor activity, although there were visual increases in activity in some experiments. Only the changes in activity for NA were significantly different (reduced) following synaptic isolation, supporting activity derived more prominently from premotoneuronal actions. Despite not having synaptic transmission, 5-HT, NA, DA, and tyramine had rhythmic bursting (**Figure 4.1B$_2$**), which was likely due to synchronization of motor neuron oscillations across gap junctions (Tresch and Kiehn 2000).

4.4.2 Trace amines produce both regular locomotor-like rhythms and episodic rhythms

NMDA is commonly co-applied with 5-HT to produce stable, regular, and physiologically comparable locomotor-like rhythm (Kiehn et al. 1999; Kjaerulff and Kiehn 1996; Sqalli-Houssaini et al. 1993). The locomotor-like pattern seen with 5-HT and NMDA is seen as left/right and ipsilateral alternation of bursts between flexors (L2) and extensors (L5) (Kiehn and Kjaerulff 1998; Sqalli-Houssaini et al. 1993). Here, NMDA was applied at concentrations that never produced rhythmic motor locomotor-like activity on its own (3-5 µM; n=15). In the presence of NMDA, tryptamine (n=14/19), tyramine (n=24/26), octopamine (n=11/13), PEA (n=10/10), NA (n=14/14), DA (n=14/14), and 5-HT (n=24/24) almost always recruited rhythmic motor activity.

4.4.2.1 Trace amines can produce locomotor-like rhythms comparable to 5-HT

In several instances, all monoamines and TAs could recruit a locomotor-like pattern broadly comparable to 5-HT (n=22). Incidences are: tryptamine (13/14), tyramine (12/24), octopamine (3/11), PEA (3/10), DA (5/14), and NA (2/14). Examples are shown in **Figure 4.2A**. All amines burst at frequencies statistically indistinguishable from 5-HT (**Figure 4.3)**. Tyramine also produced a second rhythm that was significantly slower, but with comparable coordination ($p<0.0001$; n=5/24) (**Figure 4.2B** and **Figure 4.3**). Since the bursting patterns produced by the TAs and NMDA are often similar to 5-HT and NMDA, the TAs can recruit either the same pattern-generating circuits or different circuits that lead to the same output.

4.4.2.2 Trace amines also produce episodic rhythmic motor behaviors

Additionally, tryptamine (n=1/14), tyramine (n=8/24), octopamine (n=9/11), and PEA (n=7/10) were able to generate a bursting pattern never seen with 5-HT in this preparation. The pattern was more complex with episodic bouts of bursting where the frequencies of the bursts within the bouts were statistically indistinguishable compared to that observed during stable 5-HT locomotion. The episodic bouts of bursting alternated with comparatively quiescent periods. The average frequencies of these episodic bouts were very slow (**Figure 4.4A-D** and **Figure 4.3**). Notably, this was the dominant temporal pattern of bursting for octopamine and PEA. Overall, the profile of the episodic bursting was highly variable, containing 1-47 bursts within a bout and with quiescent periods varying from 5 to 230 seconds. Typically, there were progressive increases and decreases in the locomotor burst amplitude within each episode, which are noticeable in at least some bouts for all TAs tested (**Figure 4.4**). Similarly, locomotor frequency

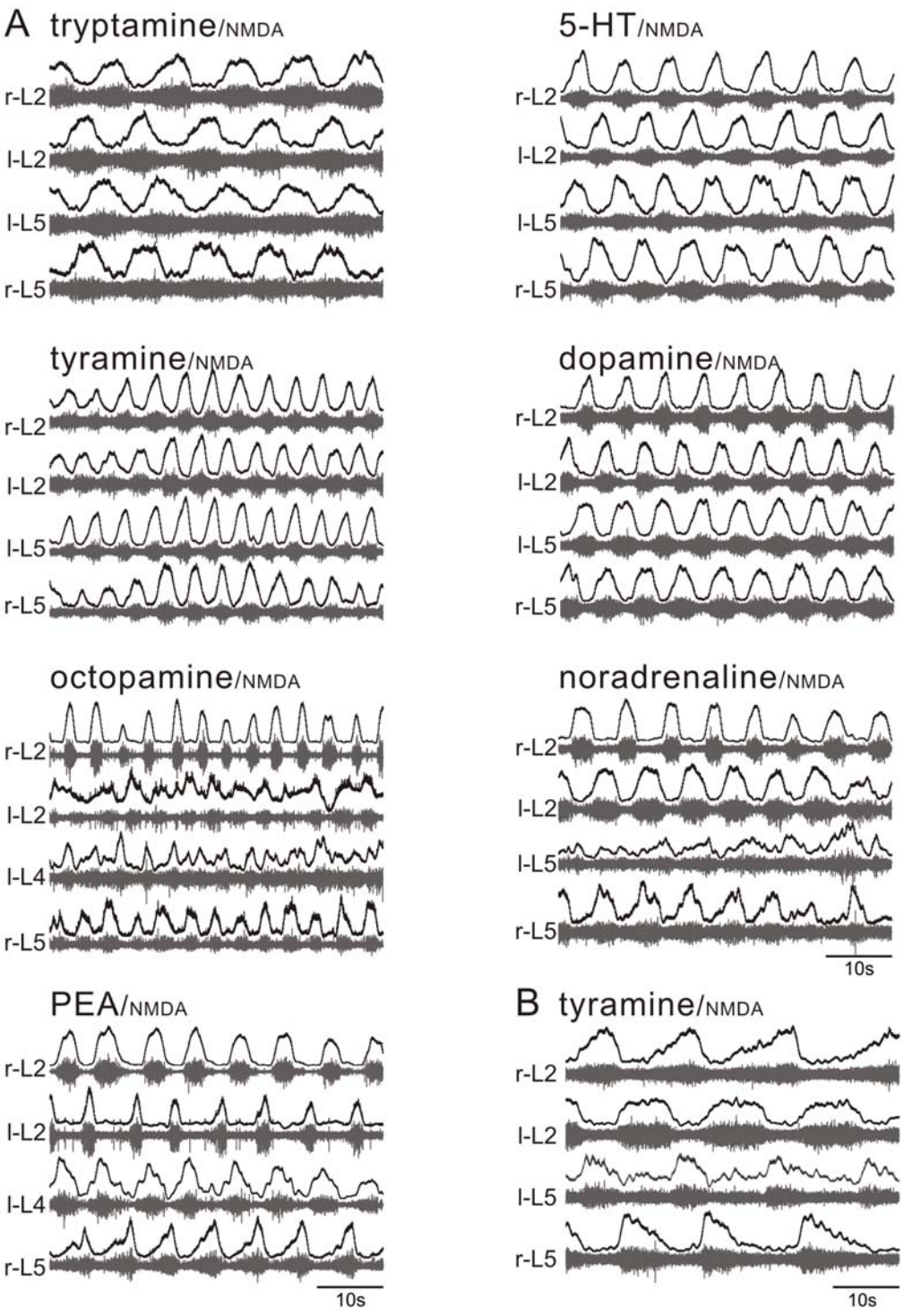

Figure 4.2: The trace amines and monoamines can all produce a continuous locomotor-like activity pattern

A. Tryptamine, tyramine, octopamine, PEA, 5-HT, dopamine, and noradrenaline can all produced a continuous locomotor-like activity pattern in the presence of NMDA. The patterns were similar. **B.** Tyramine also produces a slower locomotor-like activity pattern that was statistically slower.

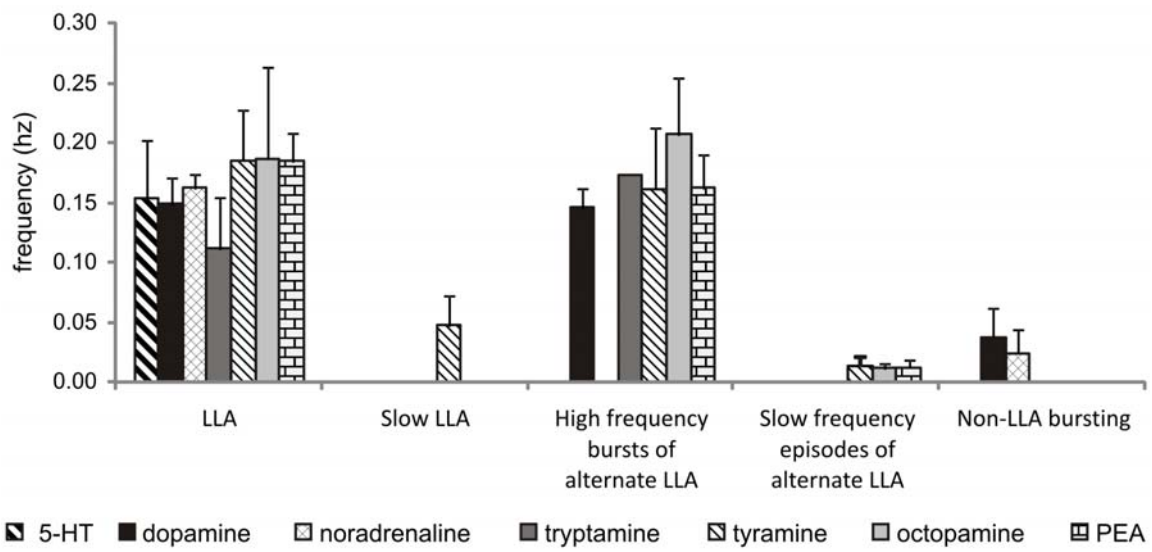

Figure 4.3: Differences in frequency between the different types of bursting activity for the monoamines and trace amines

The monoamines and TAs were able to produce different types of bursting activity in the presence of NMDA. The frequencies varied depending on the type of bursting. Note that not every monoamine or TA produced each type of activity. The DA pattern and the episodic pattern that the TAs produced were grouped together and classified as alternate LLA. Since the episodic TA LLA has both a fast and a slow component in the same traces, both were represented in the graph under high frequency bursts of alternate LLA and slow frequency episodes of alternate LLA. The frequencies of the slow LLA, slow frequency episodes of alternate LLA, and non-LLA bursting were statistically slower than the frequencies for LLA and high frequency burst of alternate LLA. LLA stands for locomotor-like activity.

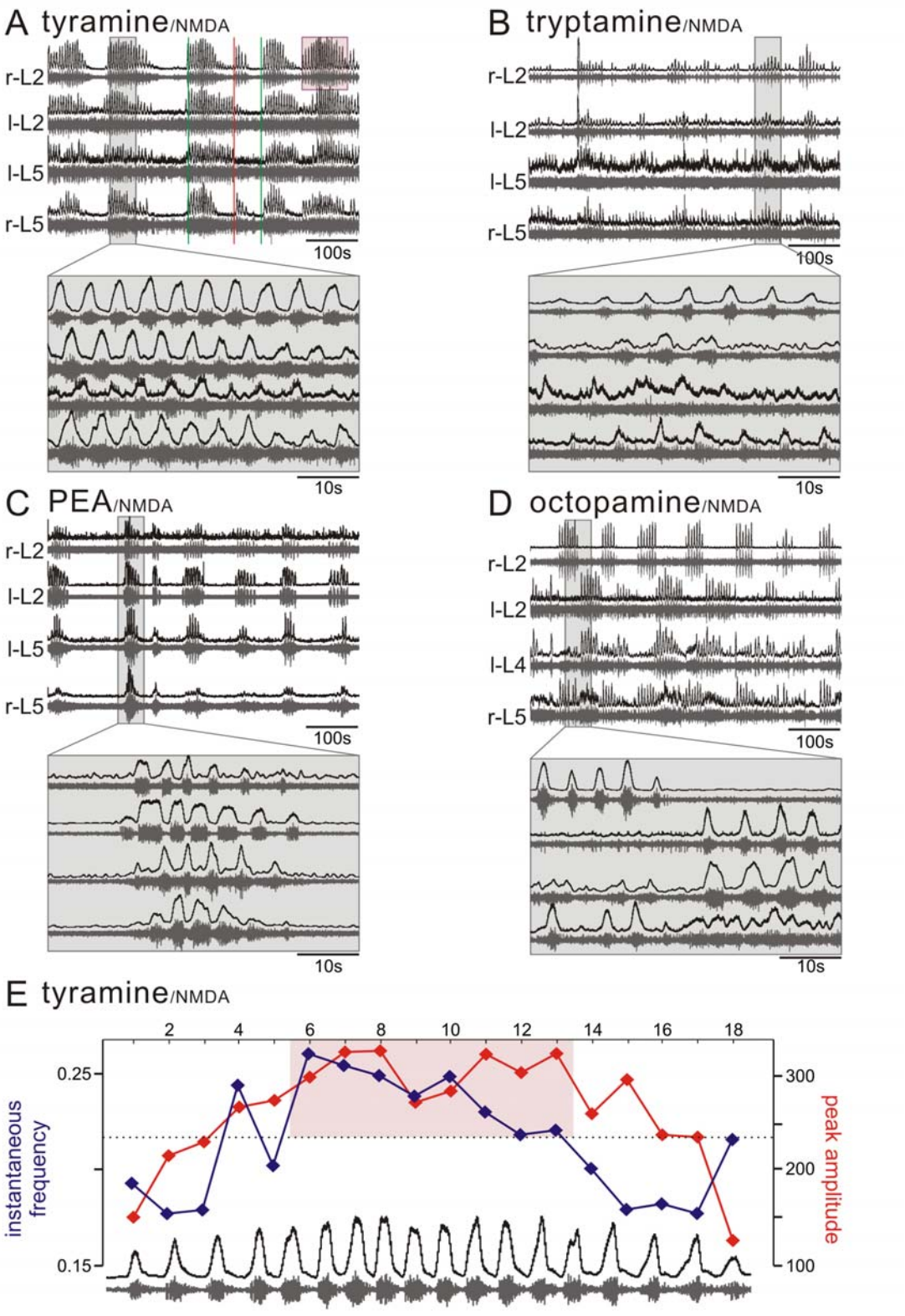

Figure 4.4: The trace amines produce episodic bursting patterns that are different than the regular pattern seen with 5-HT in the presence of NMDA

Figure 4.4: continued

A-D. Tyramine, tryptamine, PEA, and octopamine can produce episodic rhythmic motor bursting patterns where bouts of locomotor-like bursting were interrupted by relative quiescent periods. Typically, the bouts were concurrently bursting on all of the ventral roots. However, occasionally, as shown for octopamine (D) and also seen with PEA, the bouts of bursting alternated on the right and left side. **E.** Episodic bouts of locomotor activity were commonly associated with wax and wane changes in amplitude and frequency. Shown is the bout highlighted in the tyramine example for r-L2 in panel A. The burst numbers in the bout are on the x-axis, and overlaid plots of instantaneous locomotor frequency and peak amplitude of the rectified filtered response are on the y-axis. Note the trend for both amplitude and frequency to increase then decrease over the episodic bout of locomotion. Events within the shaded box emphasize the higher frequency, higher amplitude values observed in the middle of the bout.

appeared to wax and wane during these bouts, supporting a sinusoidal drive to the CPG. This is highlighted in **Figure 4.4A** in a bout of r-L2 rhythmical activity, whose amplitude and frequency are further quantified in **Figure 4.4E**. As shown, there are large progressive increases then decreases in burst amplitude, and locomotor frequency increased then decreased with the middle bursts clearly being of the highest frequency.

Coordination between roots was also variable for this pattern. The predominant pattern for all the TAs was that the bouts would be concurrent on all of the ventral roots, while the bursts within these bouts were locomotor-like (shown for tyramine, tryptamine, and PEA in **Figure 4.4A-C**) (cf. (Beato and Nistri 1999; Whelan et al. 2000)). The number of bursts within these bouts varied between roots, but typically with clear transitions between bouts of bursting and quiescent periods. Besides this pattern, octopamine (n=2/9) and PEA (n=1/7) produced bouts of bursting that alternated between the right and left side of the spinal cord (shown for octopamine in **Figure 4.4D**). Sometimes there was a slight overlap between bouts on the right and the left, and during these times coordination was consistent with locomotor-like pattern (not shown). For tyramine and octopamine, there was an experiment for each where the bursting pattern changed from the continuous locomotion shown in **Figure 4.2** to the more complex pattern with quiescent periods shown in **Figure 4.4**.

4.4.3 Dopamine can produce fast and slow locomotor-like rhythms simultaneously

DA (n=3/14) in the presence of NMDA produced its own unique alternate pattern where the typical locomotor-like frequency occurs in conjunction with a much slower locomotor-like rhythm (**Figure 4.3** and **Figure 4.5A**). In essence there appear to be two distinct locomotor CPGs operating simultaneously. These longer bursts interact with the

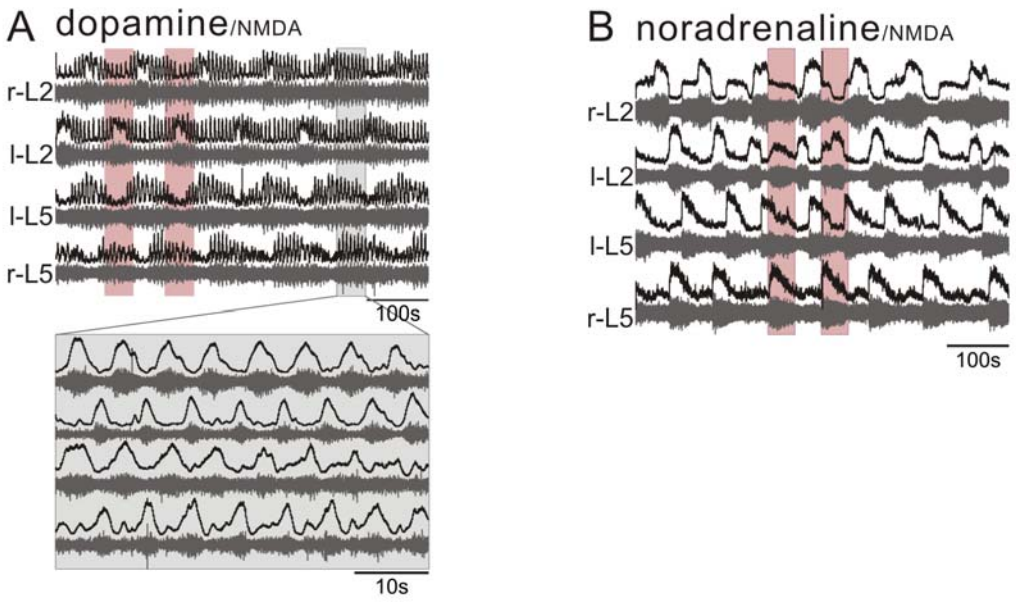

Figure 4.5: Dopamine and noradrenaline produce bursting patterns that are different than the regular pattern seen with 5-HT in the presence of NMDA

A. DA produced a pattern where the typical locomotor-like pattern occurs and then a long burst appears to overlay the bursting, increasing the amplitude at the time, but not disrupting the frequency of bursts. Grey boxes provide expanded timescale to highlight burst structure within bouts of bursting. **B.** NA and DA (not shown) often produced continuous bursting that was slow and not locomotor-like. Pink highlighted areas identify duration of the slow rhythms in A and B.

higher frequency locomotor bursts to increase their amplitudes. However, they do not alter locomotor frequency of the bursts or produce quiescent periods.

In many experiments also in the presence of NMDA, DA (0.04 ± 0.02 Hz; n=6/14) and NA (0.02 ± 0.02; n=12/14) also produced continuous bursting that was not locomotor-like (**Figure 4.5B** shows NA), and was significantly slower than 5-HT and its own locomotor-like rhythm (**Figure 4.3**).

4.4.4 Trace amines can cause locomotor-like behavior in transected neonates

To investigate behavioral relevance of TAs on spinal motor behavior, the TAs were i.p. injected into spinalized neonatal rats *in vivo* at P4-5 or P11-12 to determine whether actions on spinal motor circuits. A great advantage of the spinal cord is that a chronic transection can lead to near-complete to complete loss of descending monoaminergic transmission (Commissiong and Toffano 1989). Therefore, especially in the P11-12 rats, any change in motor behavior would be due to the effect of the TAs independent of descending monoaminergic axonal terminals.

Octopamine, PEA, and tryptamine were tested in different animals, but not tyramine, since it is not thought to be transported across the blood-brain barrier (Oldendorf 1971). Aside from a transient injection-induced pain reflex response, saline control injections in all of these animals were without effect.

In 9/9 transects, the TA injected produced hindlimb motor activity. Octopamine abruptly activated hindlimb locomotion that lasted several minutes with frequencies ranging between 1.2-1.8 Hz (n=4/4). As observed *in vitro*, octopamine induced locomotion was episodic, occurring in bouts with intervening quiescent periods. PEA activated alternating locomotor-like activity (n=3/3). Also similar to the *in vitro*, the legs

alternated between periods of rhythmic leg movements and quiescent periods. In comparison, tryptamine produced adduction / hyperflexion (n=2/2), which was accompanied by very slow alternating leg movements that were ~0.2 Hz. These studies support TA actions on spinal motor systems independent of descending monoaminergic systems.

4.4.5 Transport inhibitors attenuate trace amine and dopamine induced bursting

The monoamine transporters, SERT, DAT, and NET, are expressed in monoaminergic neurons. In spinal cord, they are located on presynaptic descending aminergic axon terminals, but not in spinal neurons. These transporters are responsible for both monoamine and TA uptake. To examine whether the motor actions of the TAs observed here require transport via monoamine transporters, I examined their actions in the presence of monoamine transport inhibitors: Citalopram, a SERT Inhibitor; Bupropion, a DAT Inhibitor; and Clomipramine, a SERT and NET Inhibitor] **Figure 4.6**).

Application of the transport inhibitors alone did not change motor activity. 5-HT (n=3/3) and NA (n=4/4) produced similar motor patterns both before and after transport inhibitor application. In comparison, the motor patterns for DA and the TAs were attenuated and variably affected (**Figure 4.6**). There was a trend toward decreasing frequency for all the TAs and DA (**Figure 4.7A**), which reached statistical significance when the TAs and DA were pooled ($p<0.05$). Burst amplitude was significantly reduced after application of the transport inhibitors for NA, tryptamine, and tyramine (**Figure 4.7B**; $p<0.05$) with a trend toward reduction for octopamine ($p=0.08$). There was also a trend towards a reduction in total number of induced bursts for the TAs and DA (**Figure4.7C**). The effect was significant for octopamine ($p<0.05$). For all applied

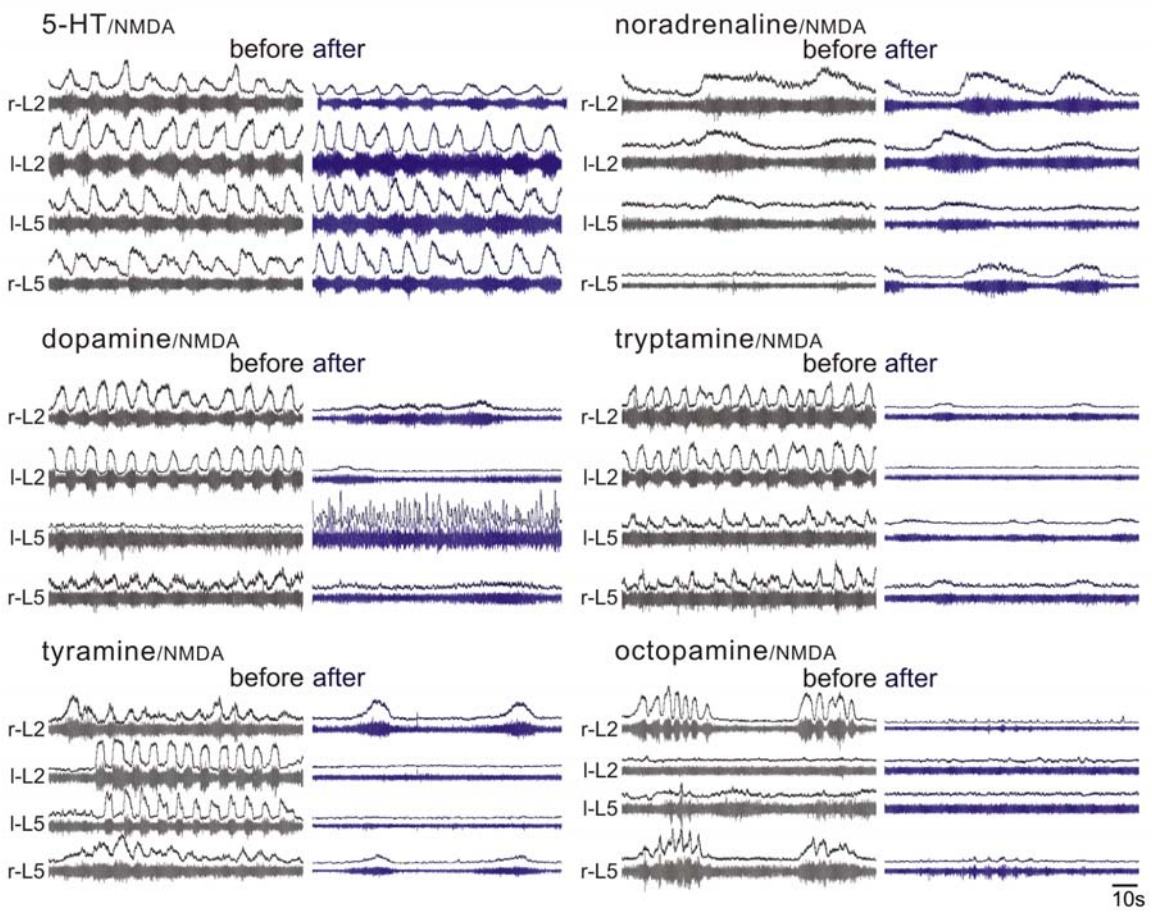

Figure 4.6: The monoamine transport inhibitors preferentially depress trace amines and dopamine modulatory actions.

The monoamines and TAs were applied in the presence of NMDA before and after the transport inhibitors [1 µM Citalopram, a SERT Inhibitor; 1 µM Bupropion, a DAT Inhibitor; 5 µM Clomipramine, a SERT and NET Inhibitor]. The waveforms show before transport inhibitors on the left and after transport inhibitors on the right. The patterning of 5-HT and NA remained similar before and after transport inhibitors, including their frequencies.

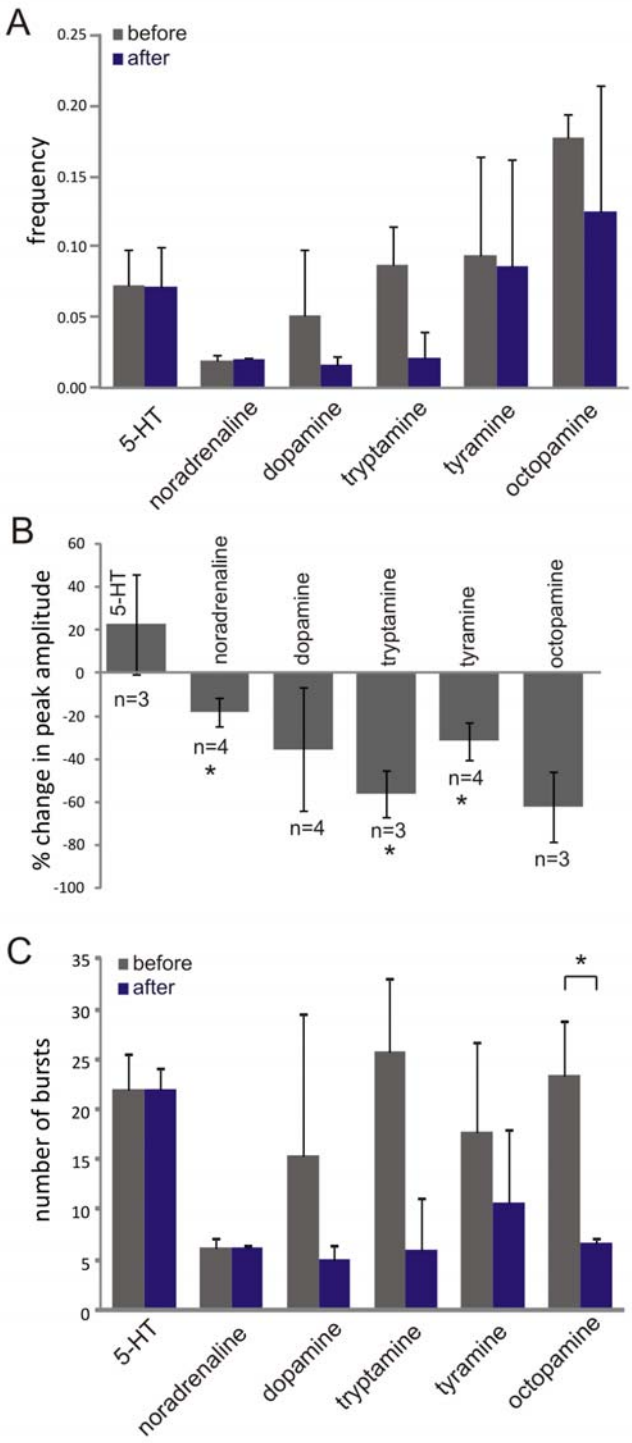

Figure 4.7: Effects of monoamine transport inhibitors on bursting properties

A. There was a trend toward reduced burst frequency in the presence of the TAs and DA, which when pooled attained statistical significance. **B.** There were significant decreases in the peak amplitude for NA, tryptamine, and tyramine. The percent change is in reference to before the addition of the transport inhibitors. **C.** There was a trend toward a reduced number of evoked motor bursts in the presence of the transport inhibitors which reached statistical significance for octopamine. (* indicates $p<0.05$).

neurochemicals, there was a trend for longer times to initiate bursting in the presence of transport inhibitors. Only for NA was this significant ($p=0.02$). However, if all TAs and DA were pooled, significance also emerges ($p<0.05$).

Overall, it appears that unlike 5-HT and NA, the action of TAs and DA were attenuated after block of intracellular transport. This suggests that TA and DA transport into presynaptic terminals contributes to the bath application-induced motor activity, but that the bursting is partially independent since bursting remains following transporter inhibition.

4.4.6 Trace amines can be transported into spinal neurons by a Na^+-independent mechanism

In the previous chapter, I found that spinal neurons increased cytoplasmic expression levels in spinal neurons. To confirm this and determine whether the uptake was NA^+-dependent or Na^+-independent, spinal cords were incubated in control aCSF, control aCSF in the presence of TAs, or TAs in a solution having nominally zero Na^+ aCSF. Qualitative increases in the immunolabeling of the TAs suggested that the TAs were taken up in a Na^+-independent manner (**Figure 4.8**). Importantly, since the monoamine transporters SERT, DAT, and NET are all Na^+-dependent (Torres et al. 2003) and not produced in the spinal cord (Allen_Spinal_Cord_Atlas 2009), uptake in zero Na^+ must be by other mechanisms. Three families of Na^+-independent transporters are known to transport TAs across the plasma membrane in the spinal cord. These are the L-type amino acid transporters (LATs), the organic cation transporters (OCTs), and the plasma membrane monoamine transporter (PMAT) (**Appendix Figures A.6-7)** (Allen_Spinal_Cord_Atlas 2009).

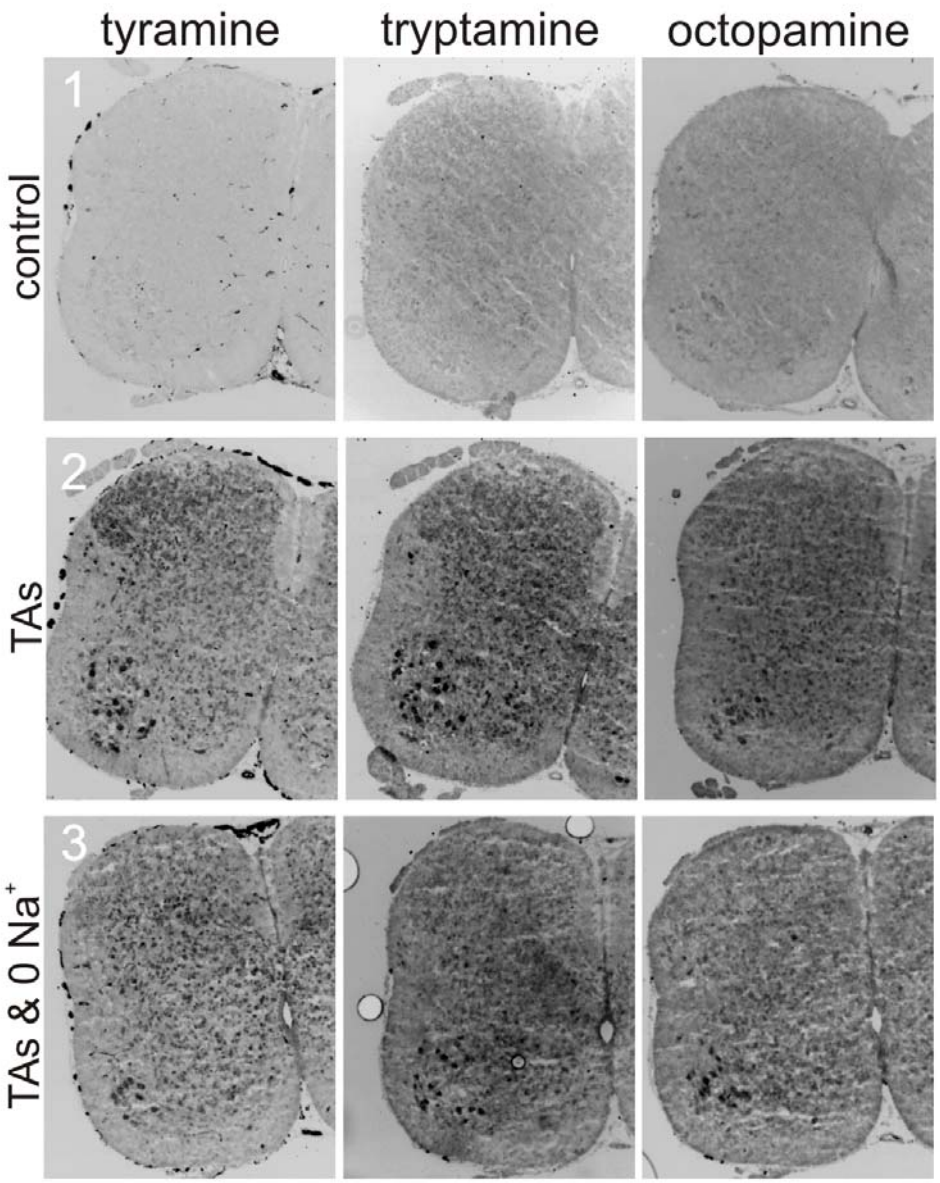

Figure 4.8: The trace amines were transported into spinal neurons by a Na^+-independent mechanism Apparent increase in TA expression following pre-incubation in TAs. Presented are low-power photomicrographs of a 10 µm section showing a complete hemicord. The isolated cords of P2 littermates were incubated in one of 3 different treatments for 2 hours: (1) control aCSF, (2) control aCSF in the presence of TAs, or (3) TAs in a solution having nominally zero Na^+ aCSF. Note that TA transport was Na^+-independent and thus independent of the monoamine transporters.

4.4.7 Trace amines and dopamine actions have much slower kinetics of activation

Given the intracellular location of the TAARs and that the TAs may need to be transported into the neurons to be activated, I compared the time to burst onset after bath application of the TAs and monoamines in the presence of NMDA (**Figure 4.9**). 5-HT (n=12) and NA (n=4) had rapid burst onset with medians of 7.5 and 2.5 seconds respectively. In comparison, it took dramatically longer for DA (480 seconds, n=4), tryptamine (300 seconds, n=5), tyramine (300 seconds, n=7), octopamine (300 seconds, n=5), and PEA (540 seconds, n=3) to initiate bursting (all are $p<0.05$). That the time to initiate bursting was longer for the TAs and DA indicates that they are not acting predominantly at classical plasma membrane monoaminergic receptors. Behaviorally, it would appear that the TAs would not be recruited to initiate descending commands for burst initiation. Rather it seems their recruitment is more consistent with modulation of ongoing activity. The actions of the TAs during ongoing 5-HT-induced locomotion are explored in Chapter 5.

4.4.8 Differences in methysergide sensitivity to activity block

Methysergide is a 5-HT_1, 5-HT_2, 5-HT_7 and tryptamine binding site antagonist. To test whether the TAs and monoamines bound the receptor sites with similar affinities, methysergide was added to TA and monoamine–induced rhythms (with NMDA) at progressively increasing doses. Concentration dependent differences were discovered (**Table 4.1**). Tryptamine was by far the most sensitive with 1 µM of methysergide always stopping locomotion. Methysergide generally blocked 5-HT locomotion at 2 µM. In comparison, tyramine and octopamine were more insensitive to methysergide, while PEA, NA, and DA were methysergide insensitive even at the highest dose tested (10

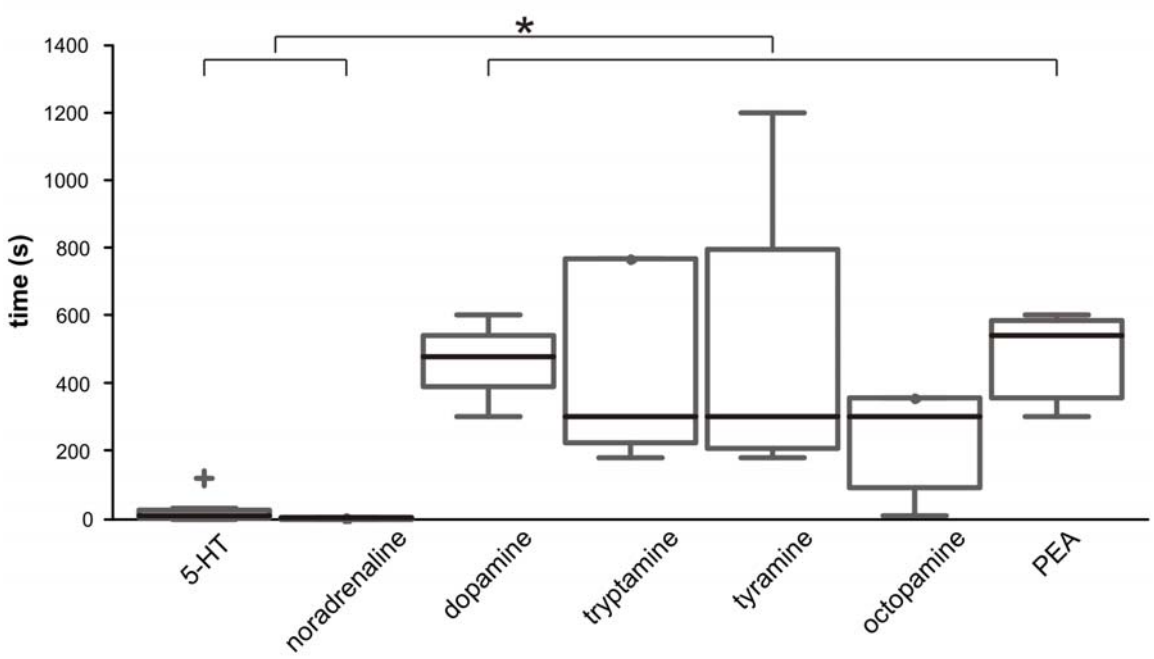

Figure 4.9: Differences in time to burst onset of bursting between the monoamines and trace amines. DA and the TAs take a significantly longer time to initiate bursting than 5-HT and NA. The time was defined as the time it took to initiate bursting after the drugs were added (always in the presence of NMDA). The box-and-whisker plots show the time it takes to initiate bursting. The black line is the median. Outliers were defined as more than 1.5 times the interquartile range. An outlier for tryptamine at 1800 seconds is not shown. (* indicates $p<0.05$).

μM). Based on methysergide sensitivity it appears likely that tyramine, octopamine, and PEA do not act predominantly on the 5-HT receptors implicated in locomotion, while tryptamine may act either at a tryptamine binding site, possibly TAAR4, or at 5-HT receptors.

Table 4.2: Methysergide blocks tryptaminergic bursting.
The number of animals in which locomotion was blocked following methysergide administration in the presence of the listed drug and NMDA.

	Total Methysergide			
Drug	**1μM**	**2μM**	**5μM**	**10μM**
5-HT	2 / 8	5 / 5		
tryptamine	6 / 6			
tyramine	1 / 6	0 / 5	2 / 5	0 / 3
octopamine	0 / 3	0 / 3	1 / 3	0 / 2
PEA	0 / 3	0 / 3	0 / 3	0 / 3
noradrenaline	0 / 4	0 / 4	0 / 4	0 / 4
dopamine	0 / 6	0 / 4	0 / 4	0 /4

4.5 Discussion

I have shown that the TAs have distinct actions on the spinal motor circuitry. Tryptamine, tyramine, octopamine, and PEA all recruited locomotor-like patterns similar to that observed for 5-HT as well as more complex episodic patterning. Both continuous and episodic locomotor phenotypes could be observed within individual animals indicating that the episodic pattern involves activation of additional cellular/network interactions that influence the output of the spinal locomotor central pattern generator.

I have shown evidence that the TAs are likely having actions both at descending terminals of monoaminergic neurons, as well as in spinal cord neurons. The high-affinity Na^+-dependent monoamine transporters NET, DAT and SERT, are found only on descending adrenergic, dopaminergic and serotonergic terminals, respectively. As monoamine transport inhibitors attenuated the action of the TAs on the motor system, TA

uptake into descending terminals contributes to the observed response. However, as responses were not abolished and rhythmicity persisted, additional TA effects must be due to actions at other sites. Further, support of TA actions occurring independent of descending monoaminergic systems was provided with *in vivo* i.p. TA administration experiments in rats having undergone chronic mid-thoracic cord transection. Under these conditions, all TAs tested induced rhythmic hindlimb motor activity, strongly supporting TA-induced actions via intrinsic modulation of spinal neural networks. While it is possible the TA actions on spinal circuits are secondarily due to unknown TA action on peripheral primary afferent receptors, the similarity in patterning seen to that in the *in vitro* isolated spinal cord makes this unlikely.

One possible means of TA actions is via activation of TAAR receptors, as I have shown that TAAR1 and TAAR4 receptors are found intracellularly in spinal neurons (see Chapter 3). To activate the intracellular receptors, the TAs must be transported into the neurons. However, spinal neurons lack the Na^+-dependent monoamine transporters known to transport TAs. To demonstrate that alternate routes of transport exist for spinal neurons, I showed that TAs can be transported into spinal neurons in the absence of external Na^+, supporting the presence of Na^+-independent transport mechanisms in spinal neurons. Indeed, as shown in the Allen Institute *in situ* hybridization studies provided in the Appendix figures, multiple Na^+-independent transporters, including LATs, OCTs and PMAT, have been shown to transport PEA, tyramine, and tryptamine and the are widely expressed in the spinal cord. Based on these data, an intrinsic TA transport system for spinal neurons seems highly likely.

4.5.1 Trace amines can induce motor activity and rhythmic locomotor bursting patterns

When applied alone, the monoamines, tryptamine, and tyramine increased motor activity, including excitatory actions on motoneurons. This agrees with a previous study showing that tyramine can act directly on motoneurons (Kitazawa et al. 1985). NA action of motor activity was significantly reduced following synaptic isolation, supporting a more prominent NA-induced activity derived from premotoneuronal actions. PEA is an agonist at both TAAR1 and TAAR4 (Borowsky et al. 2001; Bunzow et al. 2001), and therefore, the prediction would be that it would facilitate motor activity. However, no overt actions were observed. One possibility is that PEA is also acting on one of the other TAARs whose ligand has yet to be determined and which are G_i-coupled. Another possibility is that PEA has currently unstudied actions on classical monoamine receptors which are G_i-coupled (e.g. α_2-adrenergic) and compete with TAAR1 and TAAR4 G_s-coupled activity.

Application of 5-HT, NA, and DA are each known to produce signature patterns of rhythmic motor activity when applied alone. I found them to produce rhythmic motor activity similar to those previously reported (Barriere et al. 2004; Cazalets et al. 1990; Cowley and Schmidt 1994a; Kiehn and Kjaerulff 1996; Kiehn et al. 1999; Sqalli-Houssaini and Cazalets 2000). However, I also saw a pattern not reported for DA where the high frequency bursting was overlaid by a second slower bursting frequency (**Figure 4.1D**). Both DA frequencies observed were within the range found previously (Kiehn and Kjaerulff 1996). Like the monoamines, the TAs, tyramine, octopamine, and tryptamine, but not PEA, can recruit rhythmic motor patterns and with distinctive

appearance. Tyramine and tryptamine produced patterns of long and variable cycle periods whereas octopamine had a much shorter cycle period.

4.5.2 Trace amines produce continuous and episodic rhythmic bursting patterns when added with NMDA

NMDA is commonly used to stabilize 5-HT induced locomotor–like activity (Kiehn et al. 1999; Kjaerulff and Kiehn 1996; Sqalli-Houssaini et al. 1993). The use of NMDA as an adjunct neuroexcitant to facilitate TA and monoamine-induced rhythmical motor behavior in complicated by the fact that it can activate locomotion by itself at higher doses, and even at the doses I used as reported by other labs (e.g. (Beato and Nistri 1998; Bracci et al. 1998)). Regardless, there is well documented inter-strain and intra-strain differences in spinal cord function (Shay et al. 2005), and I emphasize that the doses I used here never activated locomotion in my studies. Moreover, it is clear that there is a complex interplay between 5-HT and NMDA receptors in motor rhythm generation (MacLean et al. 1998).

Here, in the presence of sub-locomotor concentrations of NMDA, the TAs produced locomotor-like activity similar to 5-HT suggesting that the TAs can recruit either the same pattern-generating circuits or different circuits that lead to the same output. Based on flexor/extensor and left/right coordination during the continuous locomotor-like pattern, it is very likely that the TAs are acting on neurons at the level of the central pattern generator (CPG) to produce the patterning. The TAs also produced episodic burst patterns. Whelan et al (2000) reported spontaneous episodes of rhythmic ventral root activity in the mouse very similar to those seen here (Whelan et al. 2000). This supports the observed TA-induced episodic pattern of motor activity as a physiologic pattern also

recruited by endogenous mechanisms. Speculation on behavioral relevance is in the discussion of Chapter 5.

Both continuous and episodic locomotor phenotypes could be observed within individual animals indicating that the episodic pattern involves activation of additional cellular/network interactions that influence the output of the spinal locomotor central pattern generator. Assuming that these different modulatory actions were due to actions at distinct spinal cord sites, there are three possible levels of network neurons that could be affected by tyramine, octopamine, and PEA to give the unique episodic patterns (**Figure 4.10**). In all cases in Figure 4.10, it was assumed that the regular locomotor-like activity pattern was produced at the CPG level by the TAs (**Figure 10A**). The first level where the modulation could be occurring would be in neurons that project onto the CPG (**Figure 10B**, left circuit). These neurons could produce an alternation of excitatory and inhibitory drive to the CPG, thus causing the waxing and waning of activity. The second level where the modulation could be occurring would be in neurons within the CPG where both slow episodic events and locomotor rhythmicity co-exist (**Figure 10B**, middle circuit). For example, rhythms at locomotor frequency could be associated with synaptic network interactions while a second slower modulation of neural excitability could be via non-synaptic somatic biochemical pathways (Katz and Clemens 2001). As the slow rhythm changes, locomotor frequency changes (e.g. **Figure 4.4E**). The third level where the modulation could be occurring would be in neurons downstream of the CPG (**Figure 10B**, right circuit). For example, motoneurons can enter high conductance states (Heckman et al. 2003; Kiehn 1991). Conceivably, a high conductance state may be sufficient to prevent synaptic input from the CPG to reach threshold effectively silencing

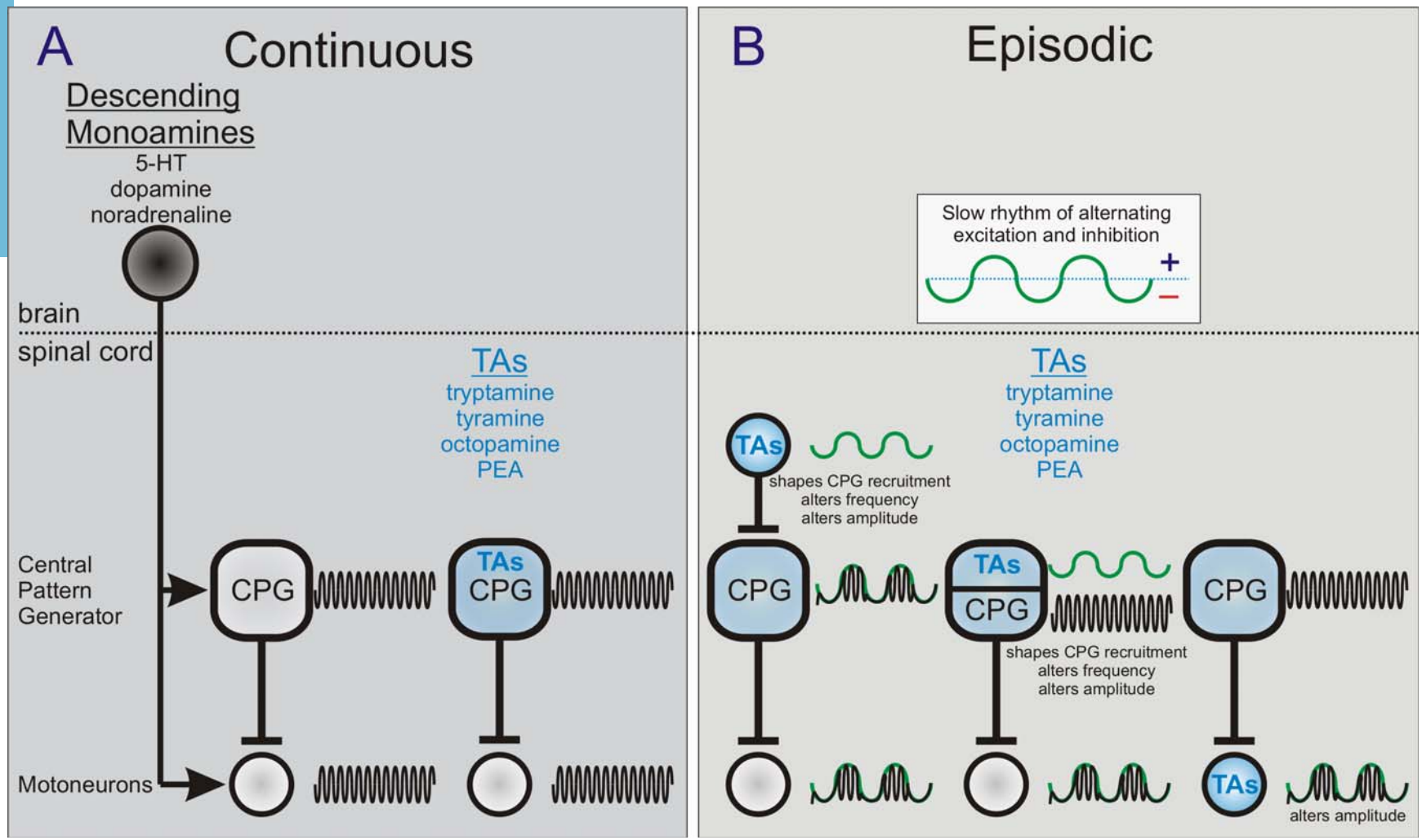

Figure 4.10: Summary diagram for possible locations of action to produce continuous and episodic bursting

Figure 4.10: continued

Both the monoamines and the TAs were able to produce continuous bursting, while tryptamine, tyramine, octopamine, and PEA additionally produced episodic bursting. Continuous locomotion is depicted in panel **A**, and putative episodic locomotor circuits are shown in panel **B**. There are three possible levels of TA actions that could result in unique episodic patterns. The first level where the modulation could be occurring would be neurons that project onto the CPG. The second level where the modulation could be occurring would be in neurons within the CPG where two events co-exist with distinct time courses. The third level where the modulation could be occurring would be in neurons downstream of the CPG (e.g. motoneurons).

motor output (Alaburda et al. 2005; Prescott et al. 2006; Raastad et al. 1996). In this case, locomotor frequency would be unchanged, but amplitude would alternate between more excitable and less excitable states. I think that since there appears to be coordination between the bouts of bursting across flexors/extensors and left/right alternation, that the first two possibilities are more likely.

Reith and Sillar (1998) observed episodic locomotor rhythms in *Xenopus* larvae with 5-HT and NMDA which was associated with a 'slow' NMDA receptor dependent motoneuron membrane bistability. However, their 'slower' modulatory rhythm (~0.5 Hz) is comparable to frequencies of NMDA receptor-induced oscillations in neonatal rat motoneurons (Hochman et al. 1994b; MacLean et al. 1997) and orders of magnitude faster than the slow rhythms here (~0.013 Hz). NMDA receptor dependent oscillations of comparable frequency to those observed here are more consistent with that seen in medial intermediate grey interneurons (Hochman et al. 1994a) and so would be more consistent with actions occurring in spinal interneurons.

4.5.3 Dopamine can produce fast and slow rhythms simultaneously

The observation that both a slow rhythm and normal locomotor rhythm can be observed simultaneously has important implications regarding the organization of the CPG. First, it suggests that, as with larval zebrafish (McLean et al. 2007), there are two distinct CPGs with network connectivity consistent with a coordination required for locomotion. Thus, the presumption that V1 interneurons are responsible for locomotor speed but not locomotor CPG operation may be incorrect (Gosgnach et al. 2006). Instead, it is seems possible that genetic deletion of the V1 interneurons was actually essential for the fast locomotor CPG, and in its absence, the slow rhythm with locomotor-

like coordination becomes predominant. Here, it is possible that DA activated two distinct neuronal interneuron populations; both a fast CPG and a slow rhythm generator whose outputs were integrated. That both rhythms have not been previously reported to be observed concurrently suggests that under normal conditions these circuits may be mutually inhibitory. In this regard, they may related to the observed short- and long-latency flexor reflex afferent (FRA) pathways observed with administrations of L-dopa in the cat (Jankowska et al. 1967; Lundberg 1969). FRA pathways represent higher threshold afferent-evoked reflex pathways. Afferents involved include group II-IV muscle, joint, and high threshold cutaneous afferents. It has been shown that following intravenous administration of L-dopa in the acute spinal cat, short-latency FRA pathways that normally produce the flexion reflex are depressed and longer-latency pathways emerge that are associated with the production of spinal stepping (Jankowska et al. 1967). While it has been assumed that L-dopa acted by increasing NA, the present observations with DA support a contribution of dopaminergic mechanisms in this switch as well, given that both tend to recruit the slower rhythms. It is also possible that the slow rhythms occasionally seen with the TAs in the absence of NMDA activate the same slow CPG recruited by NA and DA in the presence of NMDA. Additional studies are required to examine these interactions at the synaptic, cellular and network level.

4.5.4 Trace amines can cause locomotor-like behavior in transected neonates

Assuming that the duration of spinalization was sufficient to permit complete or near-complete degeneration of descending monoaminergic terminals, the observation that i.p. administered TAs were capable of evoking robust and consistent rhythmical hindlimb

movements in previously spinalized animals provides the strongest evidence that TAs can modulate function independently of monoamine transmitter release.

It could be argued that the injected doses of TAs (50 mg/kg) were not within therapeutic dose range. It is difficult to estimate the circulating levels of injected drugs without formal quantitative study. This could be done using high performance liquid chromatography and mass spectrometry or negative chemical ion gas chromatography and mass spectrometry (Durden and Davis 1993). Based on similarity of actions to the *in vitro* studies it is not unreasonable to assume that these injected doses lead to concentration in the high μM range (e.g. 50-250 μM). The injected doses chosen were based on those used by others (Borison et al. 1977) and so may have clinical relevance for subsequent pharmacological approaches aimed at modulating motor function after spinal cord injury.

4.5.5 Transport inhibitors attenuate trace amine and dopamine induced bursting

Two differences common to all TAs were their slow onset time and sensitivity to the transport inhibitors. Both differences support a requirement for the TAs to be transported across the membrane prior to having biological action. The 5-HT, DA, and NA transporters SERT, DAT, and NET respectively, are symporters that move these monoamines across the cell membrane by coupling their movement to the movement of Na^+ down its concentration gradient. In the spinal cord, only descending monoaminergic axons contain the monoamine transporters. Indeed, the Allen Institute *in situ* hybridization studies in spinal cord show an absence of SERT, DAT, and NET expression in either neonatal or adult spinal cord neurons (**Appendix Figure A.4A**); therefore, in my experiments, the attenuation of motor activity in the isolated spinal cord

caused by the transport blockers is likely via prevention of TA entry into descending monoaminergic nerve terminals. However, we cannot exclude the possibility that the transport blockers had actions at other sites, including at Na^+-independent transporters on spinal neurons (discussed below) (Haenisch and Bonisch 2010).

Xie et al. (2007) found that TA uptake is facilitated by monoamine transporters, and that such uptake greatly enhances activation of TAAR1 in HEK cells (Xie et al. 2007). Further, when TAAR1 was co-expressed with monoamine transporters in HEK cells, PEA activation of TAAR1 inhibited uptake and increased efflux of the monoamines, which did not occur when only the monoamine transporters were expressed (Xie and Miller 2008). Since it has been reported that TAs can facilitate monoamine transmitter efflux in the CNS via reverse transport (Paterson et al. 1990; Sulzer et al. 2005), it seems likely that TAARs play an important modulatory role in regulating monoamine transporter function. Therefore, one very plausible site for TAs actions is on presynaptic descending terminals by causing monoamine efflux (**Figure 4.11**). Based on the above, such release may depend on the descending systems expressing TAARs. Once the monoamines have been released they can act at receptors on the postsynaptic neuron. For tryptamine, transport across SERT and activation of TAAR4, for which it is an agonist (Borowsky et al. 2001), could lead to 5-HT release with subsequent 5-HT receptor activation mediated induction of locomotion. Like 5-HT, tryptamine-induced locomotion is blocked by methysergide, so efflux of 5-HT from descending monoaminergic axons may explain the observed actions of tryptamine. It is important to note however that even after complete inhibition of monoamine transport with inhibiters, TA actions including those of tryptamine remained albeit attenuated (**Figure 4.6 and 4.7**). I conclude that

while TA induced monoamine efflux contributes to the observed motor actions, TAs are also binding at sites independent of descending monoamine presynaptic terminals.

There is evidence that at least PEA has actions both at DAT and independent of DAT. Sotnikova et al (2004) looked at the effects of PEA in wildtype, heterozygous, and DAT knockout mice. PEA produced pronounced striatal DA release in wildtype mice, but no DA release was detected in DAT knockout mice. Behaviorally, PEA produced a strong transient increase in locomotion in wildtype and heterozygous mice, whereas in knockout mice, PEA acted as a potent depressant (Sotnikova et al. 2004).

It was unexpected that DA had actions resembling those of the TAs. Both the slow onset time and sensitivity to transport inhibitors of DA support a requirement for the DA to be transported across the membrane prior to having biological actions. Since dopamine receptors are thought to exert their actions on the plasma membrane, and there is no evidence that they are active once internalized, DA actions could be via activation of TAARs. Indeed, it is known that the monoamines also bind to TAAR1, although at lower affinity (Borowsky et al. 2001; Bunzow et al. 2001). In HEK cells co-expressing DAT and TAAR1, at high enough concentrations, DA halts DA uptake and initiate DA efflux starting around 3 minutes of incubation. This was not seen in cells expressing only DAT (Xie and Miller 2007). This suggests that DA transport across DAT and subsequent activation of TAARs similarly to the TAs, and would be consistent with the common actions observed between DA and the TAs in the Results section above.

4.5.6 Possible trace amines actions on monoamine receptors

In addition to the presynaptic actions just described, the TAs are likely acting on postsynaptic neurons as well. Since the TAs are structurally similar to the classical

monoamines and the TAARs have a high homology to the monoamine receptors (Borowsky et al. 2001), the possibility must be considered that the TAs are acting as agonists to the monoamine receptors in the spinal cord. Tryptamine directly activates $5HT_2$ and $5HT_7$ receptors (Boess and Martin 1994), on which 5-HT induced locomotion is dependent (Liu et al. 2009; Liu and Jordan 2005; Madriaga et al. 2004). This would explain why tryptamine and 5HT locomotion appear the same. It is also consistent with its sensitivity to block with methysergide. In comparison to tryptamine, all other TAs appear to have very low affinity to the monoamine receptors tested, such that the applied doses would not be expected to have direct actions on monoamine receptors (Peddi et al. 2003; Shen et al. 1993; U'Prichard et al. 1977).

4.5.7 Evidence supporting trace amine uptake by Na^+-independent transporters

While inhibition by monoamine transport inhibitors attenuated TA bursting, it did not abolish it, suggesting that the TAs are acting at sites in the spinal cord other than just the presynaptic descending monoaminergic terminals. Incubation of spinal cords in TAs and zero Na^+ aCSF showed that the TAs can be transported into spinal neurons via a Na^+-independent mechanism, strongly supporting the presence of Na^+-independent TA transporters. Three families of Na^+-independent plasma membrane transporters, LATs, OCTs, and the PMAT, have been shown to transport TAs across the plasma membrane. They are widely expressed in the spinal cord according to the *in situ* performed by the Allen Institute for Brain Science (**Appendix Figure A.5 and Figure A.6**) (Allen_Spinal_Cord_Atlas 2009), providing an intrinsic substrate for independent actions within spinal neurons (**Figure 4.11**). Importantly, as transport across these transporters is bidirectional, the TAs can be synthesized and effluxed. In this scenario, observed TA

selectivity and expression patterns would depend on levels of available substrate (aromatic amino acids), circulating TAs, synthesis enzymes (AADC and DBH), degradative/metabolic enzymes (MAOA, MAOB, and PNMT), and presence of membrane transporters.

There are three OCTS (OCT1-3), all of which can transport tyramine (Schomig et al. 2006). There are four LATs (LAT1-4). The LATs preferentially transport amino acids, including the aromatic amino acid precursors (Babu et al. 2003; Bodoy et al. 2005). Additionally, LAT3 transports PEA (Babu et al. 2003). Moreover, both tyramine and tryptamine are transported by PMAT. PMAT transport affinity order for the TAs and monoamines were: tryptamine > serotonin > dopamine > tyramine with NA and adrenaline having very low (millimolar) affinity binding to PMAT (Engel and Wang 2005). Importantly, it is possible that the transport inhibitors used in this study are having actions at PMAT, as PMAT is inhibited by commonly used monoamine transport inhibitors, albeit at lower affinity (Haenisch and Bonisch 2010). In particular, I used citalopram, whicht has a much higher affinity to SERT than sertraline, currently the most potent PMAT transport blocker of the antidepressants tested (K_i = 5 μM) (Haenisch and Bonisch 2010). Future studies on the effects of known inhibitors of PMAT on TA modulation should be undertaken to support their role in observed TA and monoamine neuromodulatory actions (e.g. cimetidine).

4.5.8 Possible mechanism for trace amine actions in the spinal cord

It seems highly-likely that the TAs are exerting at least part of their observed actions through the TAARs, since the TAs and the TAARs are found in similar locations in the spinal cord and both TAAR1 and TAAR4 are activated by TAs. The TAs must be found

intracellularly to act on the intracellular TAARs. I showed in Chapter 3 that TAAR1 and TAAR4 are present in spinal neurons. There are two ways that the TAs could be found in spinal neurons (**Figure 4.11**). First, they could be made in AADC-expressing spinal neurons from aromatic amino acids precursors. Second, they could be transported there. Transport presynaptically into descending monoaminergic neurons is likely via the monoamine transporters, which when blocked here, attenuated rhythmic bursting. Postsynaptically, transport is likely via the LATs, OCTs, and PMAT, which are all Na^+-independent. This is consistent with the incubation experiments here where I showed that uptake into neurons was Na^+-independent, as well as with electrophysiological studies where I showed a slow activation time. While transporter mechanisms dominate when TAs are bath applied to the spinal cord, endogenous actions are more likely dominated by autocrine TA synthesis in neurons having AADC and TAARs as well as paracrine actions in local neurons having TAARs via transport across bidirectional Na^+-independent transporters.

Presynaptically, once the TAs are in the neurons, they likely act on TAARs, which lead to modulation of neuronal function that induces an efflux of monoamines into the synapse. This efflux is consistent with reports that the TAs can facilitate monoamine transmitter efflux in the CNS via reverse transport (Paterson et al. 1990; Sulzer et al. 2005). Extracellular TAs could also have action on presynaptic monoamine receptors. Postsynaptically, there are two major routes by which the TAs actions could lead to modulation of neuronal function. First, they could act on TAARs and modulate neuronal function by altering signal transduction pathways. Second, extracellular TAs could act on postsynaptic monoaminergic receptors.

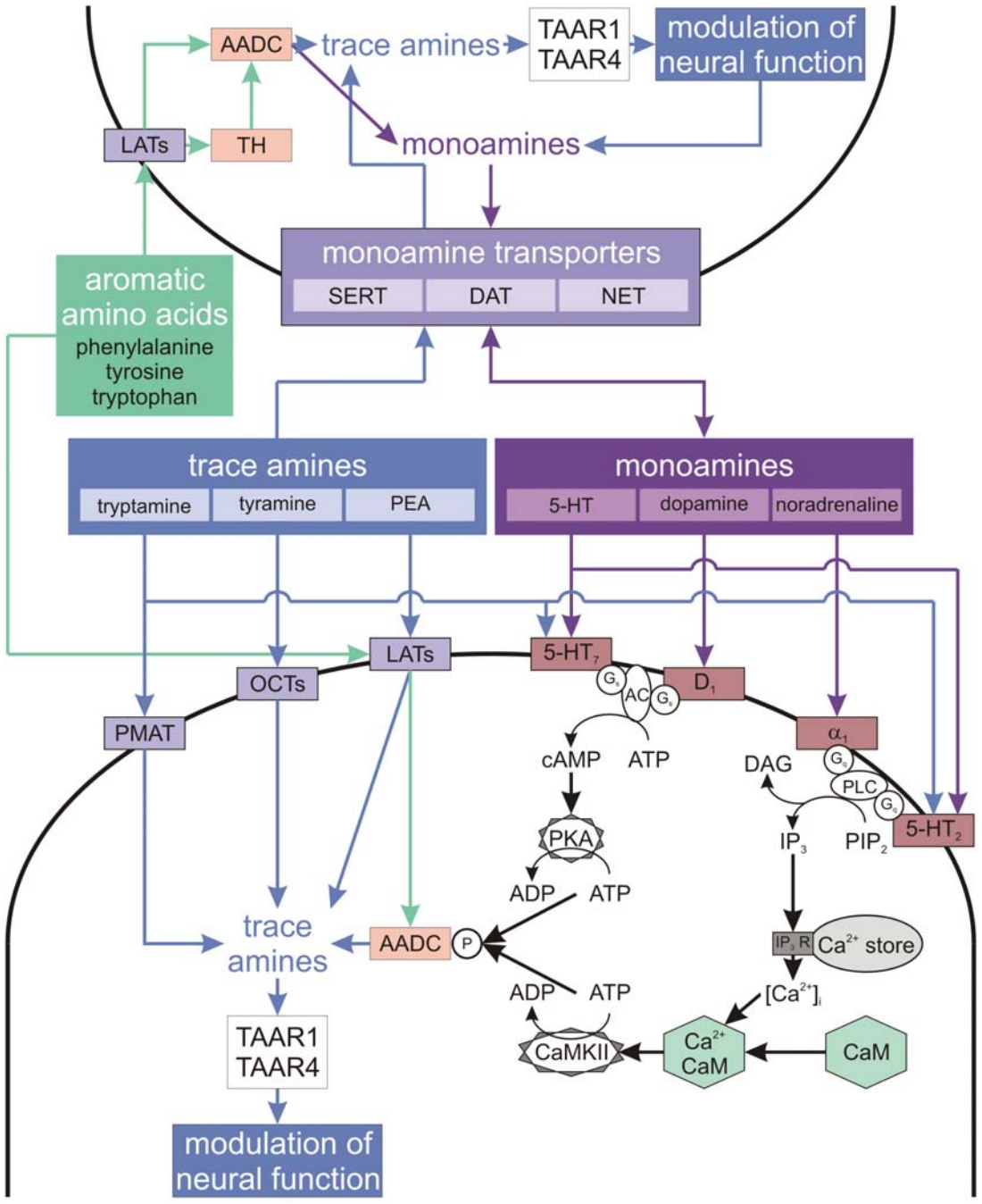

Figure 4.11: Possible intracellular mechanism for trace amine actions

The TAs could be having actions both pre and post-synaptically. It is possible for them to be both made and transported into both neurons. Once inside, the TAs could be having effects on intracellular TAARs. Presynaptically, it appears that the activation of the TAARs could lead to an efflux of monoamines. This efflux of monoamines can lead to activation of monoamine receptors on the postsynaptic neuron, which can lead to intracellular signaling that can activate the phosphorylation of AADC and an increase in TA production. This increase in TAs could activate TAARs, hence leading to modulation of neuronal function. What exactly this modulation is has yet to be determined, but one likely possibility is that it is refining or reinforcing the output of the neuron. Currently, octopamine transport has not been tested at these transporters.

It also seems likely that activation of conventional monoamine receptors interacts with TA signaling. For example, previous reports suggest that the monoamine receptors, 5-HT_2, 5-HT_7, D_1, and α_1, are involved in mediating actions on the locomotor network (Gabbay and Lev-Tov 2004; Liu et al. 2009; Liu and Jordan 2005; Madriaga et al. 2004). Activation of these receptors leads to downstream signal transduction pathways capable of phosphorylating AADC, which increases the activity of AADC and increases the levels of the TAs (Berry 2004; Duchemin et al. 2000; Duchemin et al. 2010; Neff and Duchemin 2002). In this manner, monoaminergic action could lead to subsequent modulation of intrinsic spinal function via enhanced activation of the TAARs.

5-HT_7 and D_1 both activate the $G_s\alpha$ subunit, which stimulates adenylate cyclase (AC) to produce cyclic adenosine monophosphate (cAMP) from adenosine triphosphate (ATP). cAMP, a second messenger, activates protein kinase A (PKA, cAMP-dependent protein kinase) (Gervasi et al. 2007), and PKA phosphorylates AADC (Duchemin et al. 2000).

5-HT_2 and α_1 both activate the $G_q\alpha$ subunit, which stimulates phospholipase C (PLC) to hydrolyzes phosphatidylinositol 4,5-biophosphate (PIP_2) into two second messengers, diacyl glycerol (DAG) and inositol triphophate (IP_3). IP_3 causes the release of calcium from intracellular stores, leading to the activation of Calcium/calmodulin-dependent protein kinase type II (CaMKII) (Dash et al. 2007). CaMKII phosphorylates AADC (Neff and Duchemin 2002).

Currently, the role of the TAARs in the spinal cord is unknown. However, it is known that they that they are $G\alpha_s$-coupled proteins able to activate cAMP (Borowsky et al. 2001; Bunzow et al. 2001). There are a number of classes of downstream substrates which cAMP signaling can activate including transcription factors, voltage-gated ion channels,

ion pumps, and neurotransmitter receptors, all of which are candidates after TAAR activation (Greengard 2001).

An interesting and very plausible role for the anatomical substrate created by the widespread expression of AADC, the TAs, and the TAARs would be as an activity amplifier / reinforcement mechanism. Changes in excitability observed could be based on AADC activity and TAAR activation. Such a role would support a tight interdependence of the TAs with the monoamines on spinal motor function. If true, the the TAs may predominantly serve a downstream role of refinement of monoamine-induced locomotor pattern, not necessarily the initiation of it. This implies that the monoamines and the TAs work together, but that each has its own role.

It is important to emphasize that in this scenario, other pathways independent of the monoamine and their receptors that also co-activate the same signal transduction pathways could also recruit the TAARs leading to the unique intrinsic modulatory actions of the TAs.

4.5.8.1 Possible mechanism for contribution by NMDA *in vitro*

As many of my *in vitro* experiments also used NMDA, intracellular cascades following NMDA receptor activation could also potentially increase TA levels. NMDA receptor activation-induces Ca^{2+} entry into the neurons, which activates CaMKII and nitric oxide synthase (NOS) (Matsumura et al. 2010) (**Figure 4.12**). CaMKII phosphorylates AADC (Neff and Duchemin 2002). NOS produces nitric oxide (NO), which can lead to inhibition of MAO activity (Muriel and Perez-Rojas 2003), and thereby reducing TA degradation. Further, NO leads to the activation of cGMP-dependent

protein kinase (PKG) (Schlossmann and Hofmann 2005), which can then phosphorylate AADC (Duchemin et al. 2010) and lead to modulation of neural function.

Another important role for NMDA to consider is that there may be a critical activity level which a neuron needs to reach for the TA modulatory actions to be significantly altered, and adding NMDA to the bath may help the neuron to achieve this level.

4.5.9 Conclusions

Overall, the present work has laid the groundwork for understanding the complex interplay between the well established role of the monoamine transmitters and the existence of a more diffuse, more variable, more modifiable class of structurally related neuromodulatory amines. Based on the distribution of transporters, the synthesis enzyme AADC, and the TAAR receptors, these so called 'trace' amines appear capable of exerting profound neuromodulatory actions.

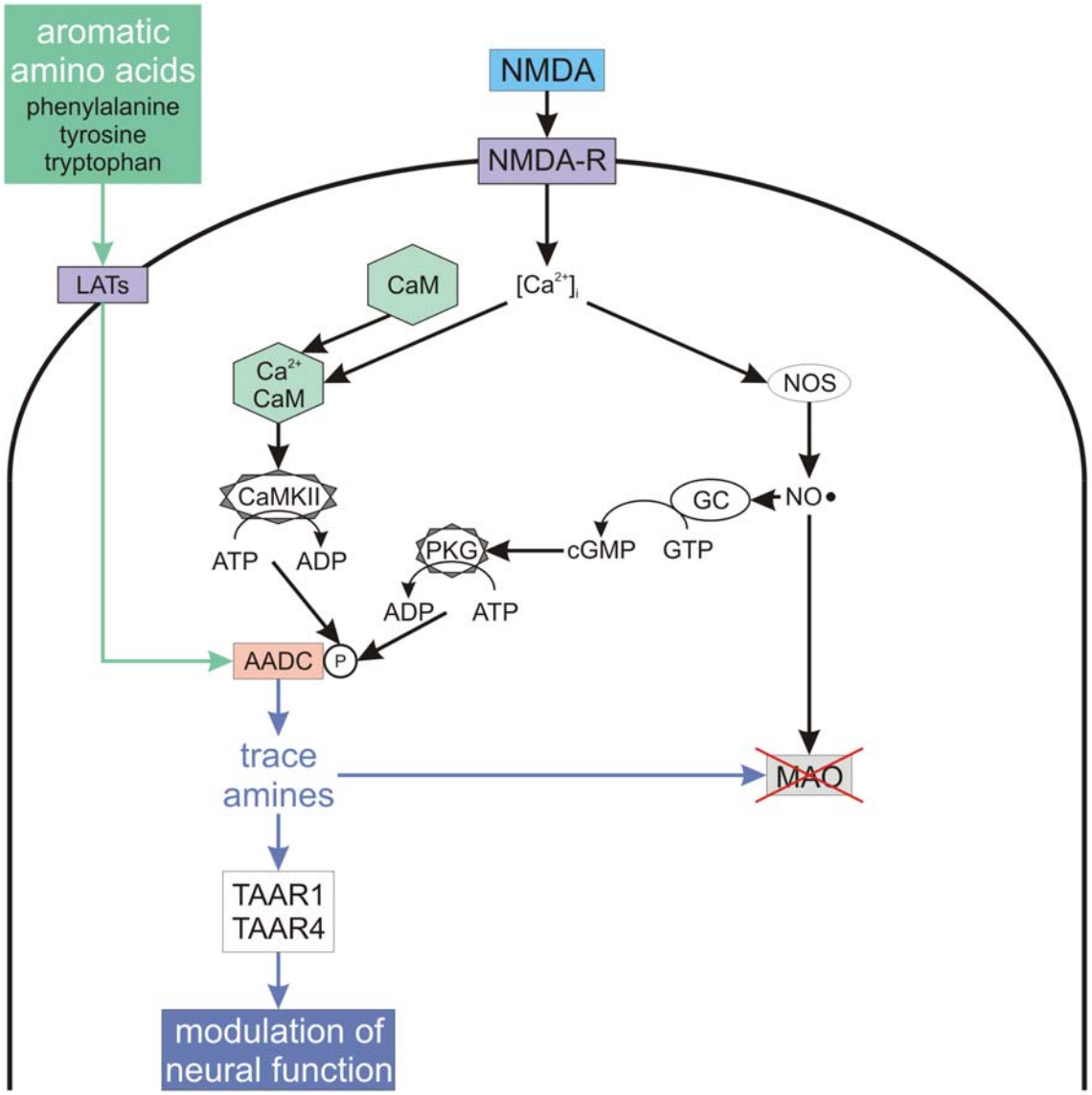

Figure 4.12: Possible mechanism for contribution by NMDA

When NMDA activates the NMDA receptor, it can set off and intracellular cascade that can lead to modulation of the TAs. Increasing intracellular levels of Ca^{2+} leads to activation of CaMKII and PKG, which can phosphorylation of AADC, which increases activity of AADC and hence production of the TAs. The TAs can then activate TAARs, leading to modulation of neural function. Further, NOS produces NO, which can lead to inhibition of MAO activity.

CHAPTER 5

MODULATORY ACTIONS OF THE TRACE AMINES ON HINDLIMB MOTOR COORDINATION

5.1 Abstract

In the previous chapter, I showed that when recording from ventral roots, the trace amines (TAs), in the presence of NMDA, can produce a continuous locomotor pattern as well as a unique episodic rhythm characterized by bouts of rhythmic bursting. Here, using the *in vitro* isolated spinal cord with attached hindlimbs to record electromyographic (EMG) activity from various hindlimb muscles, I found that that the TAs induced the previously observed continuous and episodic locomotor patterns as well as previously undescribed episodic bursting phenotypes. There were differences in phasing as well as amplitude when comparing the TAs to 5-HT.

When the TAs were applied during ongoing 5-HT/NMDA locomotion, all TAs except for tryptamine increased frequency, and all TAs except for PEA increased L2 ventral root amplitude. The most pronounced effects in patterning to 5-HT locomotion were to a subgroup of tyramine and octopamine applications where dramatic changes were observed. By recording from both ventral roots and muscles, the EMG recordings revealed patterns that were undetected by the ventral root alone, demonstrating that ventral root activity can be an inaccurate reflection of the actual motor patterns generated. Overall, I conclude that the TAs increase the strength and frequency of ongoing 5-HT locomotion while also being capable of modifying the rhythmic motor pattern generated.

5.2 Introduction

The spinal cord contains all of the necessary circuitry at birth to produce complex motor outputs including locomotor patterning (Grillner 1981). In the isolated *in vitro* neonatal rodent spinal cord, many different neurochemicals have been used to induce locomotor-like activity (LLA) patterns, which can be characterized as left/right and ipsilateral alternation of bursts between flexors and extensors (Kiehn and Kjaerulff 1998; Sqalli-Houssaini et al. 1993). Locomotor-inducing neurochemicals include serotonin (5-HT), noradrenaline (NA), dopamine (DA), excitatory amino acids, acetylcholine, N-methyl-D-aspartate (NMDA), glutamate, acetylcholine, (Barbeau and Rossignol 1991; Cazalets et al. 1990; Cazalets et al. 1992; Cowley and Schmidt 1994a; Kiehn and Kjaerulff 1996; Kiehn et al. 1999; Kudo and Yamada 1987; Sqalli-Houssaini and Cazalets 2000) and most recently my work with the trace amines (TAs) (see previous chapter).

The TAs, named for their low endogenous concentrations in mammals, are a family of endogenous amines with structural, metabolic, physiological, and pharmacological similarities to classical monoamine neurotransmitters (Saavedra 1989). The TAs include tryptamine, tyramine, octopamine, and β-phenylethylamine (PEA). In chapter 3, I found that an anatomical substrate exists for the TAs to be produced in the spinal cord, and then in chapter 4, I found that the TAs can produced rhythmic bursting patterns. Using ventral root recordings in the isolated spinal cord preparation, the TAs in the presence of NMDA can produce both a continuous LLA pattern similar to 5-HT and NMDA as well as an episodic pattern characterized by bouts of activity with intervening quiescent periods. The episodic bouts of bursting had a locomotor-like pattern and frequency statistically

indistinguishable from the continuous LLA pattern. The quiescent periods had relatively low activity and no bursting.

While ventral root recordings offer a simple way to track the spinal motor output, they do not give the whole story. Ventral root activity may not reflect recruitment of individual muscles (Cowley and Schmidt 1994b). Anatomical studies have shown that ventral roots are actually quite heterogeneous, containing axons projecting to both flexors and extensors (Nicolopoulos-Stournaras and Iles 1983). Important changes in motor coordination between flexor and extensor activity may be lost due to the heterogeneity of these roots. It has been long known that muscle recruitment by spinal cord circuitry is more complicated than just simple flexor/extensor alternation. These distinct complex patterns and sequential activation of muscles have been demonstrated in the *in vitro* spinal cord (Grillner 1981; Kiehn and Kjaerulff 1996). Distinct motor patterns of recruited were observed between different transmitters such as 5-HT and DA as well as pharmacological and electrical stimulation, suggesting that the spinal locomotor network is flexible and that extrinsic modulation can modify the network coordination (Kiehn and Kjaerulff 1996; Klein et al. 2010).

I hypothesized that TA would have distinct differentiable actions on motor pool recruitment during rhythmic motor behaviors and that the TAs can modulate recruited activity with distinct actions. To test this, I used the *in vitro* isolated spinal cord with attached hindlimbs to record muscle activation electromyographically. I found that the TAs can activate distinct motor patterns and modulate ongoing activity in complex fashion. Some of these results have been presented in abstract form (Gozal et al. 2008; Gozal et al. 2007b).

5.3 Methods

All experimental procedures complied with the NIH guidelines for animal care and the Emory Institutional Animal Care and Use Committee.

5.3.1 General setup

Sprague-Dawley neonatal rats postnatal day 0-5 were decapitated, eviscerated, and the skin was removed except that covering the feet. The preparation was placed in a bath containing continuously oxygenated (95% O_2 and 5% CO_2) high magnesium, low calcium artificial cerebral spinal fluid (aCSF) [containing (in mM): 128 NaCl, 1.9 KCl, 1.2 KH_2P0_4, 26 $NaHCO_3$, 0.85 $CaCl_2$, 6.5 $MgSO_4$, and 10 glucose (pH of 7.4)]. The spinal cord was exposed by a ventral vertebractomy and a dorsal laminectomy. The dura mater, connective tissue, and dorsal roots were carefully cut by angled scissors. The ventral roots were cut rostrally down to the thoracic roots 12-13 (T12-13) or lumbar 1 (L1), leaving the spinal cord, lumbar and sacral ventral roots, and innervated hindlimbs intact. To increase superfusion to the spinal cord, the vertebral column surrounding the cervical and most of the thoracic spinal cord was removed. The spinal cord and attached hindlimbs were immobilized ventral side upward with insect pins to a chamber with Sylgard on the bottom. All dorsal roots were cut to remove the influence of afferent feedback on the neurochemically-induced rhythm and simplify the spinal cord output to reflect the changes in spinal cord circuitry due to the TAs. Modulatory actions of the TAs can be inferred to reflect the actions on motor circuits. The perfusing medium was then switched to the standard aCSF solution (same concentrations as above except $MgSO_4$ and $CaCl_2$ adjusted to 1.3 mM and 2.4 mM respectively). A gravity-fed superfusion system that used a Cole Palmer Masterflex L/S compact pump to bring the

perfusate back to the upper beaker to be recirculated was used. Later when the neurochemicals were added, they were added to the upper beaker. Glass suction electrodes were then placed on the left lumbar 2 (l-L2) ventral root and up to 7 muscles of the hindlimb, after which the preparation was allowed to recover for about 1 hour before experimentation at room temperature. Activity was amplified (10,000x), band-pass filtered at 10-3,000 Hz, digitized at 5kHz (Digidata 1321A, 16-bit; Axon Instruments), and captured on a computer with the pCLAMP acquisition software (v8-9, Molecular Devices; Union City, CA).

5.3.2 Recordings from the ventral root and muscles

Recordings were obtained from the following muscles: tibialis anterior (TibA; ankle flexor), medial gastrocnemius (MGas; ankle extensor), semitendinosus (SemT; knee flexor / hip extensor), semimembranosus (SemM; knee flexor / hip extensor), vastus medialis (VasM; knee extensor), rectus femoris (RecF; knee extensor / hip flexor), and adductor magnus (AddM; hip adductor). Some of the muscles (e.g. TibA) were often recorded bilaterally.

The left L2 ventral root was always recorded for two reasons: for comparison of ventral root activity profile in relation to that obtained in the isolated spinal cord preparation reported above, and because its reliable recruitment allowed a common comparison between experiments where muscle EMG recruitment was variable.

In 21 experiments, which always included recordings from the left L2 ventral root and 7 muscle EMGs, 7.1 ± 2.2 recorded channels were active at some point during the experiment (**Table 5.1**). However in a given experiment some muscles were active only in the presence of a specific neurochemical. For the channels that were inactive, I cannot

rule out whether there was damage to them, although I can comment that r-RecF, was the least likely muscle to be active in general.

Table 5.1: Number of experiment where the ventral root/muscles was active

Ventral root or muscle	Number of experiment where the ventral root/muscles was active during at least one drug application	Number of experiment from which the ventral root/muscle was recorded
l-L2	21	21
l-TibA	18	18
r-TibA	18	19
l-MGas	7	7
r-MGas	19	19
l-SemT	6	7
r-SemT	17	17
l-SemM	2	2
r-SemM	17	18
r-VasM	1	2
r-RecF	8	16
r-AddM	2	3

5.3.3 Motor activity and motor patterning experiments

Rhythmic motor activity was evoked by two different types of experiments. First, tryptamine, tyramine, octopamine, PEA, or 5-HT was co-applied with NMDA. In experiments were the TAs and NMDA were co-applied, an application of 5-HT and NMDA was also performed for the sake of later comparison. Second, 5-HT and NMDA were co-applied to obtain ongoing LLA, after 10-20 minutes tryptamine, tyramine, octopamine, and PEA were added. Typically there were 3-7 drug co-applications per experiment, lasting 20-60 minutes each. Within each experiment, the amines were applied in random order. The order did not appear to affect the output. A wash with aCSF was performed between all drug co-applications, lasting a minimum of 30 minutes, long after baseline had been reached. Recorded files were 5 minutes long and continuous

from the start of the 5 minute baseline until right before the drugs were washed out of the bath.

5.3.3.1 Neurochemicals

All neurochemicals were stored in 10mM or 100mM stock solutions at -20°C and were thawed, then diluted into a circulating bath (100mL) to achieve the final desired concentration in the chamber. The following neurochemicals obtained from Sigma-Aldrich (St. Louis, MO) were used: 5-HT (20-50 μM), N-methyl-D-aspartate (NMDA) (3-5 μM), tyramine (40-100 μM), octopamine (40-100 μM), PEA (50-100 μM), and tryptamine (50 μM).

5.3.3.2 Analysis

Electrophysiological data analysis was performed using custom software, SpinalMOD, written in MATLAB (The MathWorks). Frequency, peak amplitude, and phase were calculated. Briefly, to calculate onset and offset of a burst, the data was rectified, then low-pass filtered, creating a burst envelope. A custom algorithm was constructed to detect burst onset and offset.

Typically 10 consecutive representative bursts were selected for analysis. When bouts of episodic bursting contained less than 10 bursts consecutively (see **Figure 5.3**) non-consecutive bursts were selected for analysis. In these cases, the different bouts of bursting were pooled. Calculations made this way allowed for more reliable statistical comparisons between conditions. In the experiments where a TA was added to ongoing 5-HT and NMDA LLA, the 10 cycles were chosen immediately preceding TA application while 10 cycles after the addition of the TAs were chosen once the modified rhythm stabilized

Since the hindlimbs were pinned down and there were no movement to base timing, muscles are classified as flexors and extensors based on terminology from previous studies (Kiehn and Kjaerulff 1996). For the phase diagrams, step cycles are defined in reference to the onset of the left L2 ventral root, which are generally considered to represent primarily flexor activity. Left L2 ventral root was chosen due to its pattern stability and my ability to consistently acquire recordings from it. Muscles on the left side that were in phase with left L2 ventral root are considered active during the flexor phase, while those that are out of phase were active during the extensor phase. The opposite is true of muscles on the right side. Those that were active with left L2 ventral root are considered active during the extensor phase, while those out of phase were active during the flexor phase.

Using MATLAB, a paired Student's t-test was used for statistical comparison of frequency and amplitude, while a Watson-William's test was used for the phase diagrams. A significance level set at $p<0.05$. The data are reported as mean ± SD.

5.4 Results

5.4.1 Trace amines can produce locomotor activity

Previously in the isolated spinal cord preparation, I found that the TAs in the presence of NMDA produced distinct rhythmic motor patterns. I wanted to further investigate how these unique patterns translated to motor coordination patterns in the hindlimb. Here, in the isolated rat spinal cord with attached hindlimbs, NMDA was applied at concentrations that never produced rhythmic motor locomotor-like activity (LLA) on its own (3-5 μM; n=7/7). In the presence of NMDA, 5-HT (n=22/22), tryptamine (n=10/11), tyramine (n=14/14), octopamine (n=10/11), and PEA (n=14/14) almost

always recruited rhythmic motor activity. As expected, the overall patterning was similar to that found in the isolated spinal cord reported in the previous chapter, including the ability to change phenotype over time. The same three main patterns were produced, but with some differences:

5.4.1.1 The trace amines produced three main patterns

5.4.1.1.1 Trace amines produce a continuous locomotor-like activity pattern

First, tryptamine (n=10/10), tyramine (n=10/14), octopamine (n=5/10), and PEA (n=8/14) all produced a continuous LLA pattern (**Figure 5.1A**) with frequency values that were statistically indistinguishable from 5-HT (n=22/22) (**Figure 5.2**), similar to what was found in the isolated spinal cord. The mean frequency values were numerically slightly higher for tryptamine, octopamine, PEA, and 5-HT than in the isolated spinal cord without hindlimbs, but not statistically different.

5.4.1.1.2 Trace amines produce a slow continuous locomotor-like activity pattern

Second, octopamine (n=1/10) and PEA (n=1/14) produced a slow continuous LLA pattern with significantly slower frequency than the faster continuous LLA pattern (**Figure 5.1B** and **Figure 5.2D**, middle left). Although this pattern was only seen in tyramine (n=5/24) in the isolated spinal cord, it was never seen in the hindlimb attached preparation (n=0/14).

5.4.1.1.3 Tyramine, octopamine, and PEA produce episodic activity patterns

Third, tyramine (n=5/14), octopamine (n=5/10), and PEA (n=8/14), but not tryptamine (n=0/14) produced an episodic pattern that was generally characterized by

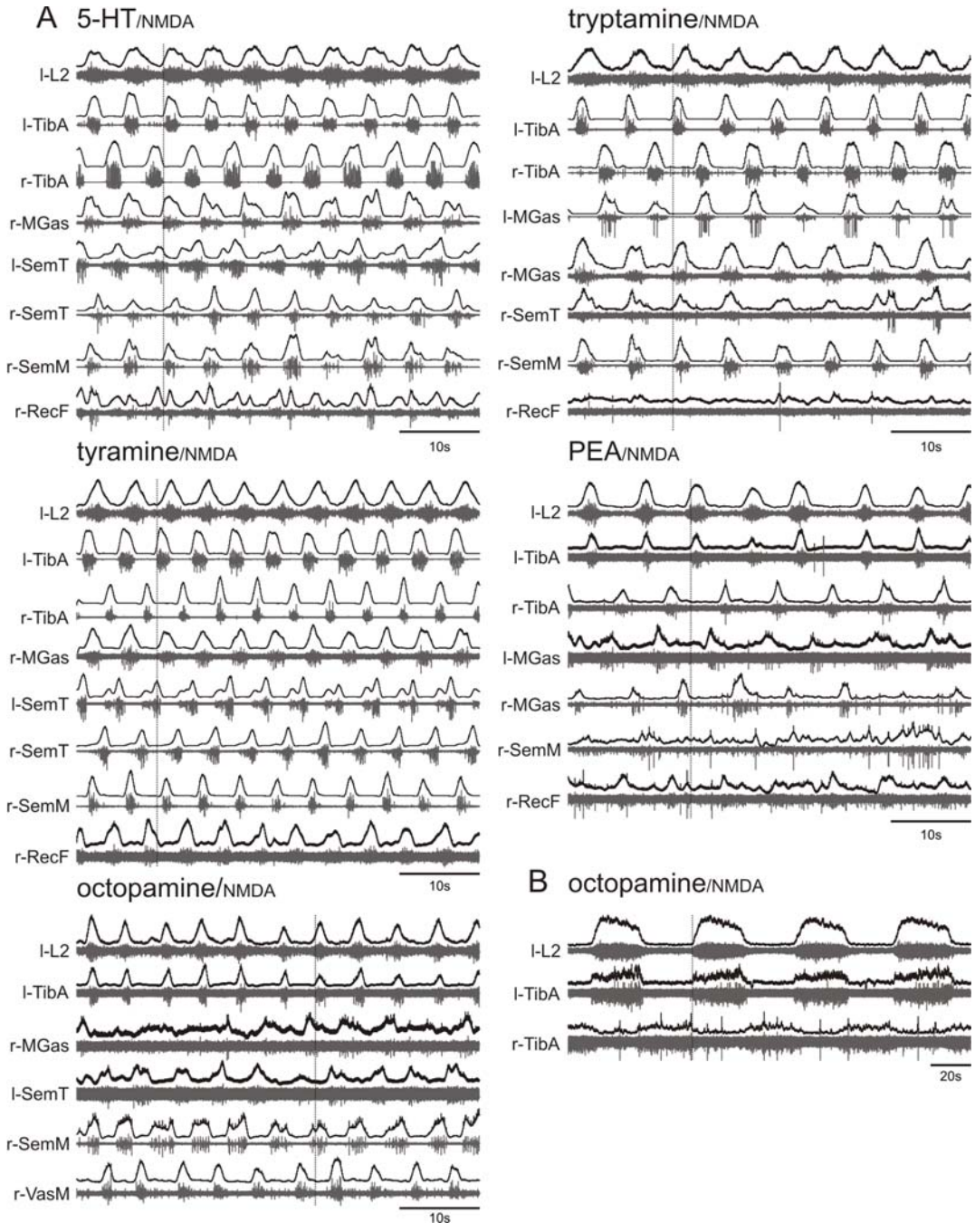

Figure 5.1: Trace amines and 5-HT induced locomotor-like activity

A. 5-HT, tryptamine, tyramine, PEA, and octopamine can all produce a LLA pattern in the presence of NMDA. The pattern and the phase are similar. **B.** Octopamine and PEA also produce a slower locomotor-like activity pattern. In this and subsequent figures, the upper traces in black for each trace has been rectified and low-pass filtered. Vertical dotted lines are provided as a visual aid to examine motor coordination in this and subsequent figures. Abbreviations in this and the following figures are as follows: left L2 ventral root (l-L2), tibialis anterior (TibA; ankle flexor), medial gastrocnemius (MGas; ankle extensor), semitendinosus (SemT; knee flexor / hip extensor), semimembranosus (SemM; knee flexor / hip extensor), vastus medialis (VasM; knee extensor), rectus femoris (RecF; knee extensor / hip flexor), and adductor magnus (AddM; hip adductor).

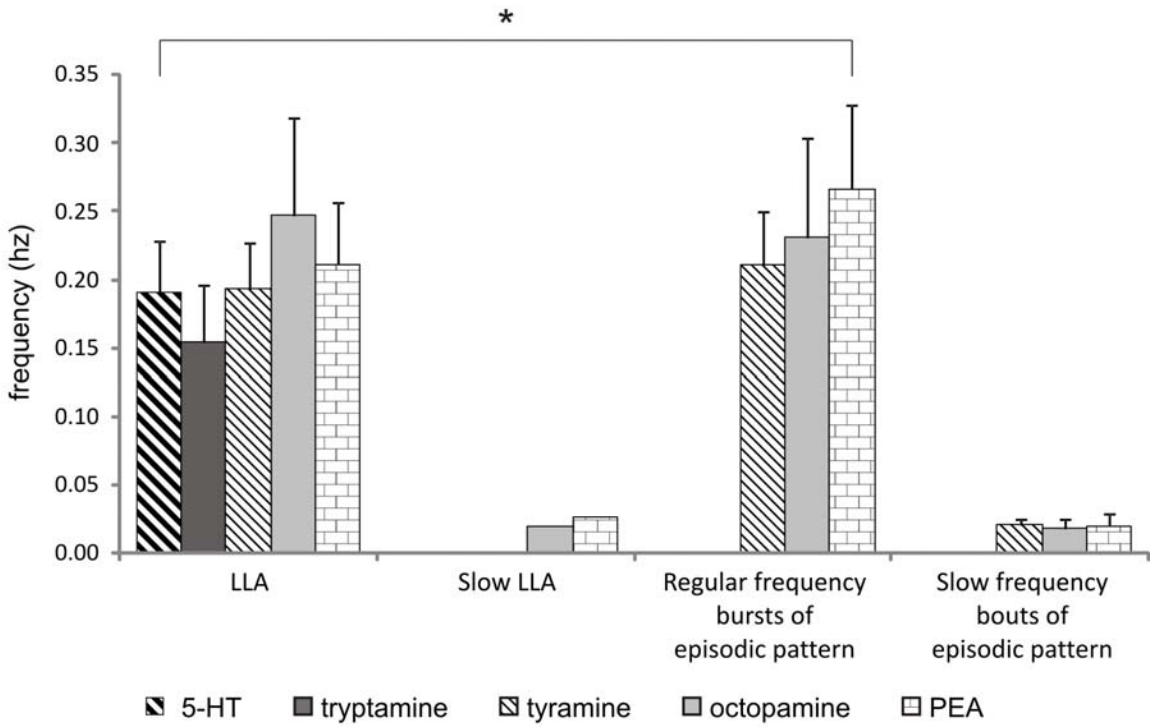

Figure 5.2: Frequencies vary depending on the type of bursting

Note 5-HT and tryptamine only produced LLA, while tyramine, octopamine, and PEA produced other types as well. The regular and slow frequency parts of episodic LLA are different components of the same experiments. LLA stands for locomotor-like activity. The slow LLA and the slow frequency bouts of episodic pattern were significantly slower than the LLA and Regular frequency bursts of episodic pattern. (* indicates $p<0.05$)

bouts of bursts with quiescent periods between them (**Figure 5.3**). The frequencies of the bursts within the bouts for tyramine and octopamine were statistically indistinguishable from 5-HT LLA, similar to the isolated spinal cord (**Figure 5.2**, middle right); however, PEA was statistically faster than the frequency of 5-HT ($p < 0.01$). When the frequencies were compared to the isolated spinal cord, tyramine and octopamine were statistically indistinguishable with values that were numerically slightly higher, while PEA was statistically faster ($p<0.01$). As in the isolated spinal cord, the frequencies of the bouts were very slow in comparison to the locomotor rhythm (**Figure 5.2**, right). The mean frequency values were numerically greater than reported in the isolated spinal cord, but not statistically different.

As in the isolated spinal cord, episodic bursting was characteristically variable. Overall there were 1-26 bursts within the bouts of activity and a break of 5-120 seconds between bouts during the quiescent periods. Another aspect that was variable was the patterning. The episodic pattern can be subdivided into distinct varieties. The most common pattern observed involved concurrently active bouts of LLA with the quiescent periods on ventral root and the muscles (**Figure 5.3A** and **Figure 5.3C**, right). This was seen in tyramine (n=5/5), octopamine (n=4/5), and PEA (n=5/8). Seen less commonly was alternation of the bouts between the ventral root and the muscles on the right and left sides of the body (**Figure 5.3B**). This was seen for tyramine (n=1/5), octopamine (n=2/5), and PEA (n=2/8). There were a couple other notable varieties of episodic patterning. In one experiment, the left L2 ventral root rhythmicity was continuous yet the r-SemM muscle was episodic (**Figure 5.3C**, left side). In another experiment with slow LLA, multiple higher frequency bursts replaced a single slow burst seen on the other

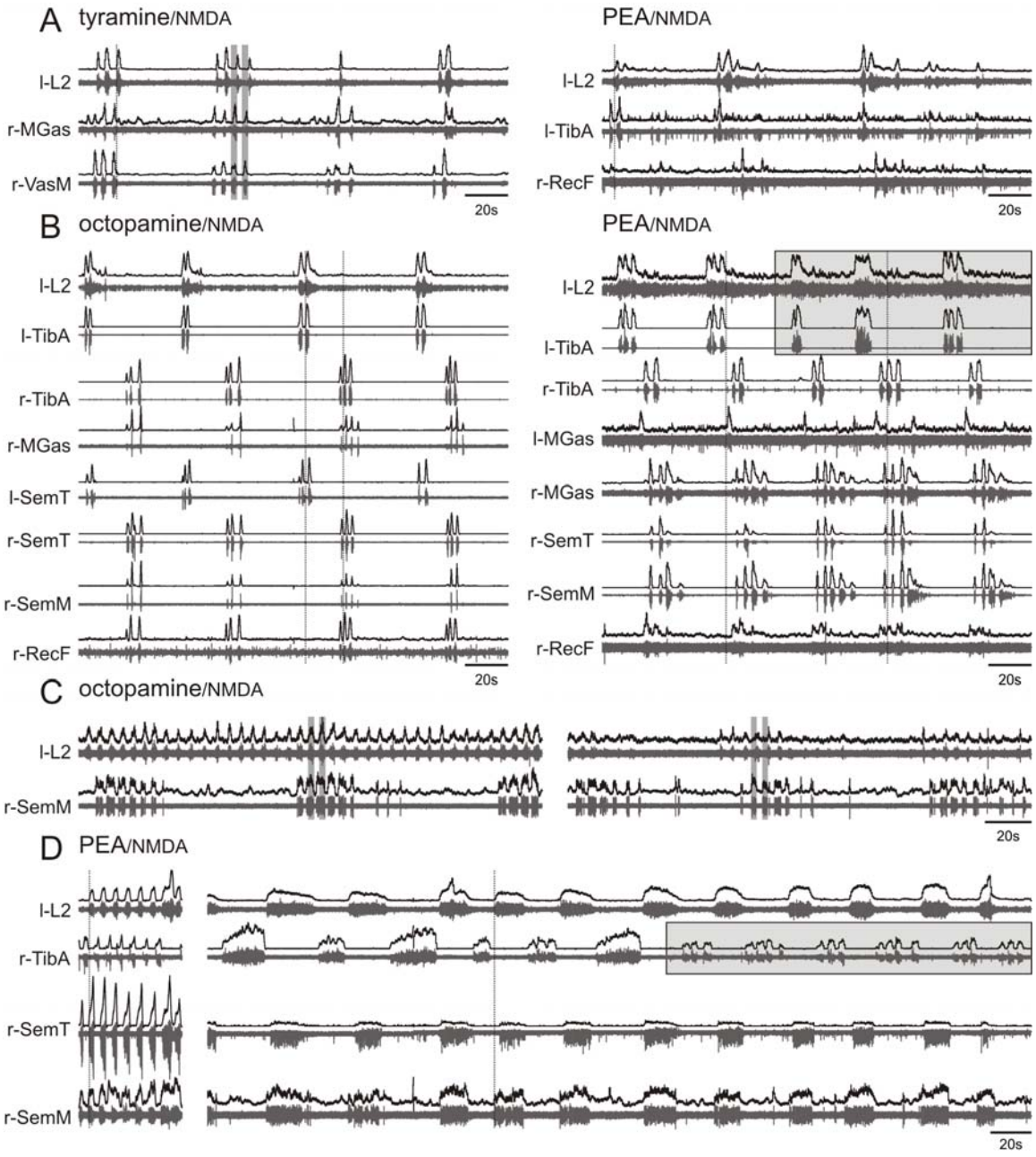

Figure 5.3: Tyramine, PEA, and octopamine produce episodic rhythmic motor bursting patterns These episodic patterns have bouts of locomotor-like bursting that were interrupted by relatively quiescent periods. **A.** Typically, when tyramine, PEA, and octopamine produced an episodic pattern, the bouts were concurrently bursting on the ventral root and muscles (also see the right half of C). **B.** Occasionally, octopamine and PEA produced episodic bouts of bursting that alternated on the right and left side. **C.** Once, for octopamine there was continuous bursting on one channel and episodic bursting on another. **D.** Once, for PEA there was a slow LLA pattern where one channel, r-TibA, had bursting within the episodes during the time of the long bursts on the other channels (highlighted). **C&D.** For tyramine, octopamine and PEA there were a few experiments where the pattern changed over time with no additional intervention. The upper traces in black for each trace have been rectified and low-pass filtered.

channels (**Figure 5.3D**, highlighted). In another the opposite occurred, where a slow burst replaced a bout of higher frequency bursting replaced (**Figure 5.3B**, highlighted).

5.4.1.1.4 Trace amines induced bursting could change phenotypes over time

The pattern of motor rhythmicity spontaneously changed phenotype over time (**Figure 5.3C and D**). This was seen in tyramine (n=2/14), octopamine (n=2/10), and PEA (n=2/14). This included patterns that started out as continuous and changed to episodic or vice versa, as well as patterns that start out continuous and changed to slow LLA (**Figure 5.3D**).

5.4.1.2 Phasing of muscles in 5-HT and the trace amines

Overall, the 5-HT/NMDA phasing was consistent with what has been found previously for 5-HT, where tibialis anterior, an ankle flexor, was active during the flexor phase, and medial gastrocnemius, an ankle extensor, was active during the extensor phase (**Figure 5.4A**). Semimembranosus and semitendinosus, both knee flexor and hip extensors, were usually both active during the extensor phase, while rectus femoris, a knee extensor and hip flexor was active during the flexor phase. The only difference observed from an earlier report was that adductor magnus was active during either the extensor (n=1/2) or flexor phase (n=1/2) rather than just the extensor phase (Kiehn and Kjaerulff 1996).

Phase diagrams revealed differences in phasing during continuous locomotion of the TAs as compared to that observed for 5-HT, showing that different muscles were affected more by certain TAs (**Figure 5.4B**). It should be first emphasized that the left L2 ventral root did not have any significant changes in phasing for any of the TAs, and thus provided a strong standard for comparisons between muscles under the different

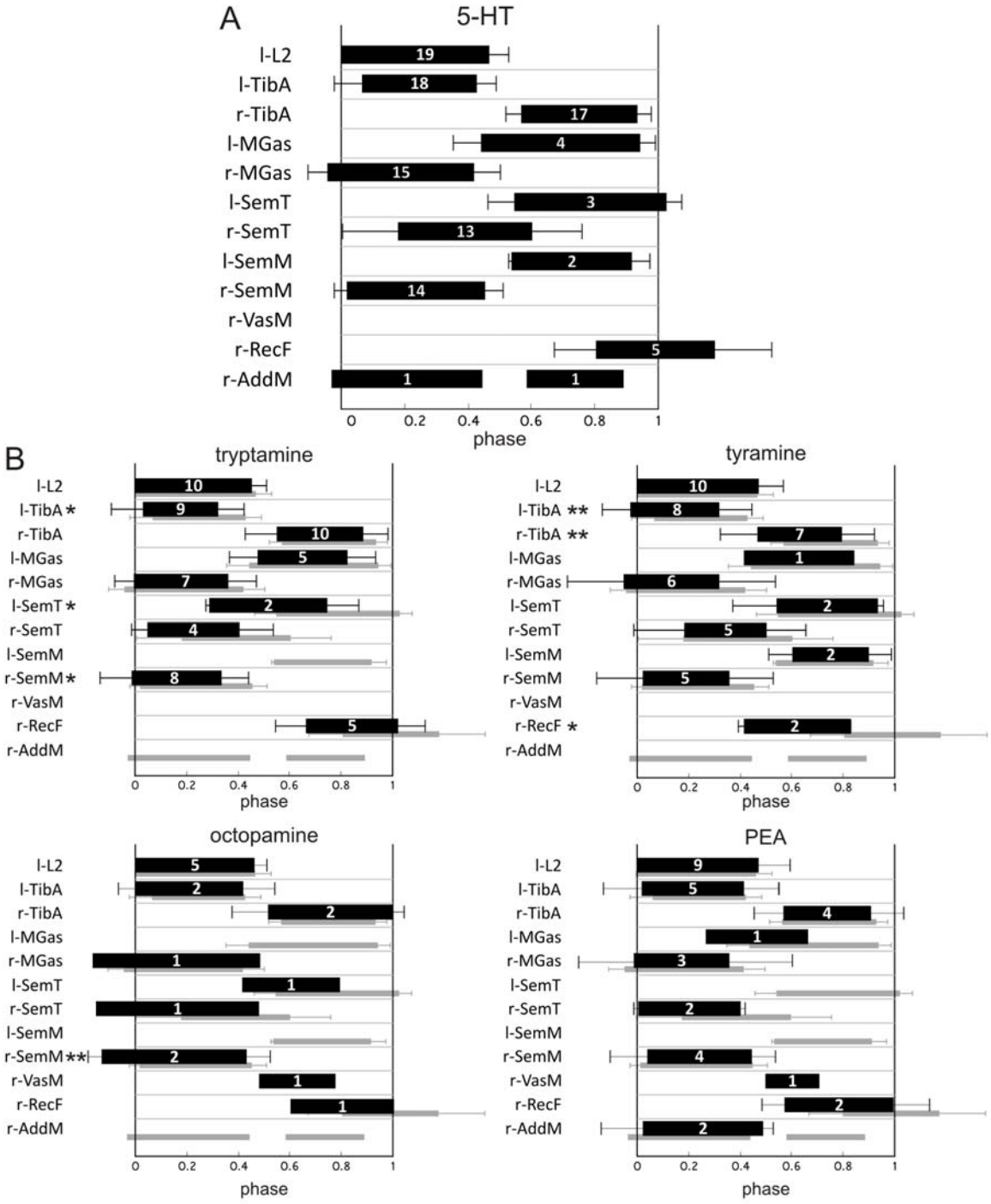

Figure 5.4: Phase diagrams for 5-HT and the trace amines

A. 5-HT. **B.** Tryptamine, tyramine, octopamine and PEA. The phase diagrams show the relationship between the phases for regular LLA patterns. All phases are in reference to the onset of the l-L2 ventral root. Bars represent the average burst duration of muscles normalized to cycle duration. Burst onset is on the left and burst termination on the right for the TA compared to 5-HT. The numbers of rats used to obtain the diagrams are given in the middle. The phase diagram for 5-HT underlays the phase diagrams of the TAs in B. Significant shifts in the average phase of the TAs compared with 5-HT are indicated as * for $p<0.05$ and ** for $p<0.01$.

neurochemical conditions. In comparison to 5-HT, all TAs preserved the muscles in their respective flexor and extensor phase without evidence of phase conversions (i.e. between flexor and extensor activity phases); however, there were shifts in the average phase within the dominant activity period. In all cases, these were leftward shifts towards earlier in the phase. For tryptamine this occurred with tibialis anterior, semitendinosus, and semimembranosus. For tyramine, there were shifts in phase for tibialis anterior and rectus femoris. For octopamine, there were shifts in semitendinosus and semimembranosus. There were no significant shifts observed with PEA.

Unlike for continuous locomotion, TA muscle activity phase conversions were observed in a given TA during episodic patterning compared to continuous TA locomotion. For example, during tyramine induced episodic bursting, the extensor right medial gastrocnemius muscle can shift into the flexor phase (**Figure 5.3A**), and for octopamine, the right semimembranosus muscle is shifted almost entirely into the flexor phase rather than being almost entirely in the extensor phase (**Figure 5.3C**, right). There did not appear to be changes in phase for the episodic bursting when there was alternation of the bouts between the right and left sides for octopamine and PEA (**Figure 5.3B**).

5.4.1.3 Amplitude of TAs compared to 5-HT

In order to examine the relative motor pool recruitment of the TAs compared to 5-HT, the percent difference in peak amplitude in the same preparation was examined. Overall, the TAs appeared to have decreased amplitudes with tryptamine exhibiting the least difference (**Figure 5.5**). Pooling continuous and episodic bursting together, all four of the TAs had significantly decreased amplitudes for left L2 ventral root when compared to 5-HT in the same experiments. Examining tibialis anterior, there was a trend for

decreased amplitude for tyramine, octopamine, and PEA. This was significant for left tibialis anterior and right tibialis anterior during tyramine and right tibialis anterior during octopamine induced LLA (**Figure 5.5**). Overall the sample sizes were too small to individually compare most of the muscles. When grouping all of the muscles together, EMG amplitudes were lower for all four TAs compared to 5-HT. This difference was significant for tyramine ($p<0.001$, $n=13$), octopamine, ($p<0.05$, $n=9$), tryptamine ($p<0.05$, $n=10$), but not PEA ($p= 0.15$, $n=14$).

5.4.2 Trace amines modulate ongoing locomotor activity

Aromatic-L-amino acid decarboxylase (AADC) is the rate limiting enzyme in the production of TAs, but not the monoamines. This highly regulated enzyme can undergo rapid changes in its activity via phosphorylation, which can changes the levels of the TAs, but not the monoamines, allowing minute to minute regulation of TA synthesis (Berry et al. 1996). In Chapter 3, I found that AADC expression is widespread throughout the spinal cord, meaning that TA synthesis is also widespread. Unlike the monoamine, the TAs are produced in the spinal cord, but not typically stored in vesicles, leading to a high turnover rates (Berry 2004; Burchett and Hicks 2006). This high level of TA regulation makes it an ideal candidate for modulating motor activity. It is possible that the TAs act as an additional modulatory layer to further alter ongoing monoaminergic based activity in the spinal cord. To examine this I tested whether the TAs can modulate ongoing 5-HT/NMDA LLA. As will be described below, I found that the TAs altered locomotor patterning, frequency, and amplitude. The most striking changes were to subgroups of tyramine and octopamine, where dramatic changes to patterning were observed.

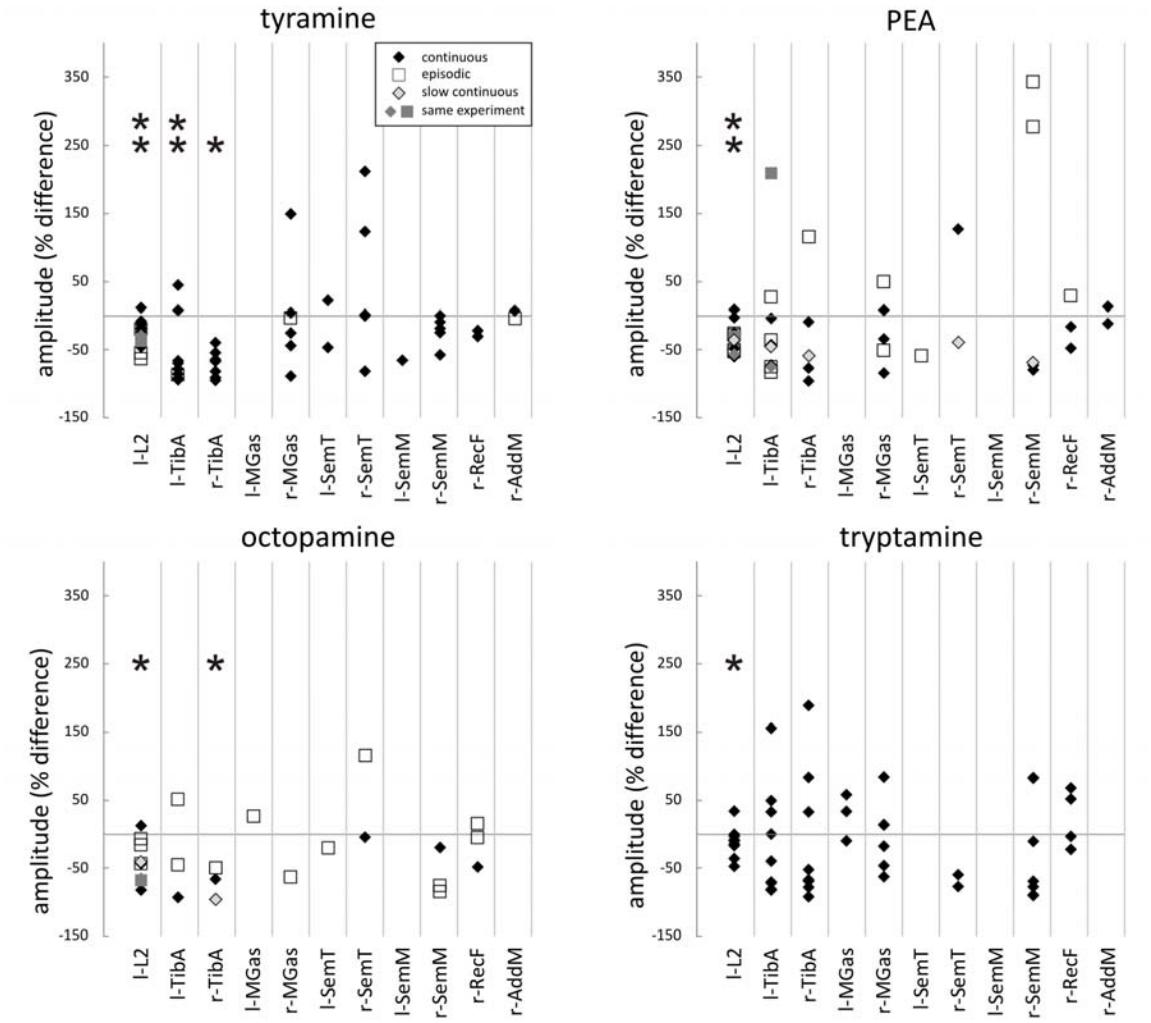

Figure 5.5: Differences in burst amplitude of the trace amines compared to 5-HT

The percent change in left L2 ventral root (l-L2) ENG and muscle EMG amplitude of the TAs/NMDA compared with 5-HT/NMDA in the same experiment was calculated and are shown for individual experiments as either diamonds for continuous LLA or squares for episodic LLA. The decreases in amplitude of the l-L2s were significant for all of the TAs (tyramine, octopamine, PEA, and tryptamine). * indicates $p<0.05$, and ** indicates $p<0.01$.

The trace amines either maintain locomotor-like activity or produce episodic patterning when added to ongoing locomotion

Adding the TAs to ongoing 5-HT/NMDA LLA led to two main responses. First, the continuous locomotor-like pattern produced by 5-HT/NMDA was maintained after the addition of the TAs (**Figure 5.6**). All TAs could have this response. In fact for tryptamine (n=12/12) and PEA (n=11/11), this was the only response seen. However, for tyramine (n=8/12) and octopamine (n=3/7) maintaining the continuous pattern was not the only response.

The second response was the conversion from continuous locomotor-like pattern produced by 5-HT/NMDA to and episodic pattern due to the addition of tyramine (n=4/12) and octopamine (n=4/7) (**Figure 5.7**). Thus, tyramine and octopamine could convert the phenotype of 5-HT locomotion. This is interesting in relation to the section above describing the TA induced patterning in the absence of 5-HT. Here, the incidence of episodic patterning was similar for all TAs except PEA. For PEA, the episodic activity that was previously evoked (8/14 animals) was now completely suppressed in the presence of 5-HT (0/11).

5.4.2.1.1 Trace amines alter the frequency and amplitude of ongoing 5-HT locomotor-like activity

For ongoing continuous 5-HT/NMDA LLA monitored at the L2 ventral root, PEA increased frequency (11±12%; $p<0.05$) without changing amplitude ($p=0.19$), while tryptamine increased amplitude (10±11%, $p<0.01$) without changing frequency ($p=0.6$) (**Figure 5.8, and Figure 5.9**). For octopamine and tyramine, when continuous and episodic patterns were pooled, octopamine increased the 5-HT-evoked frequency

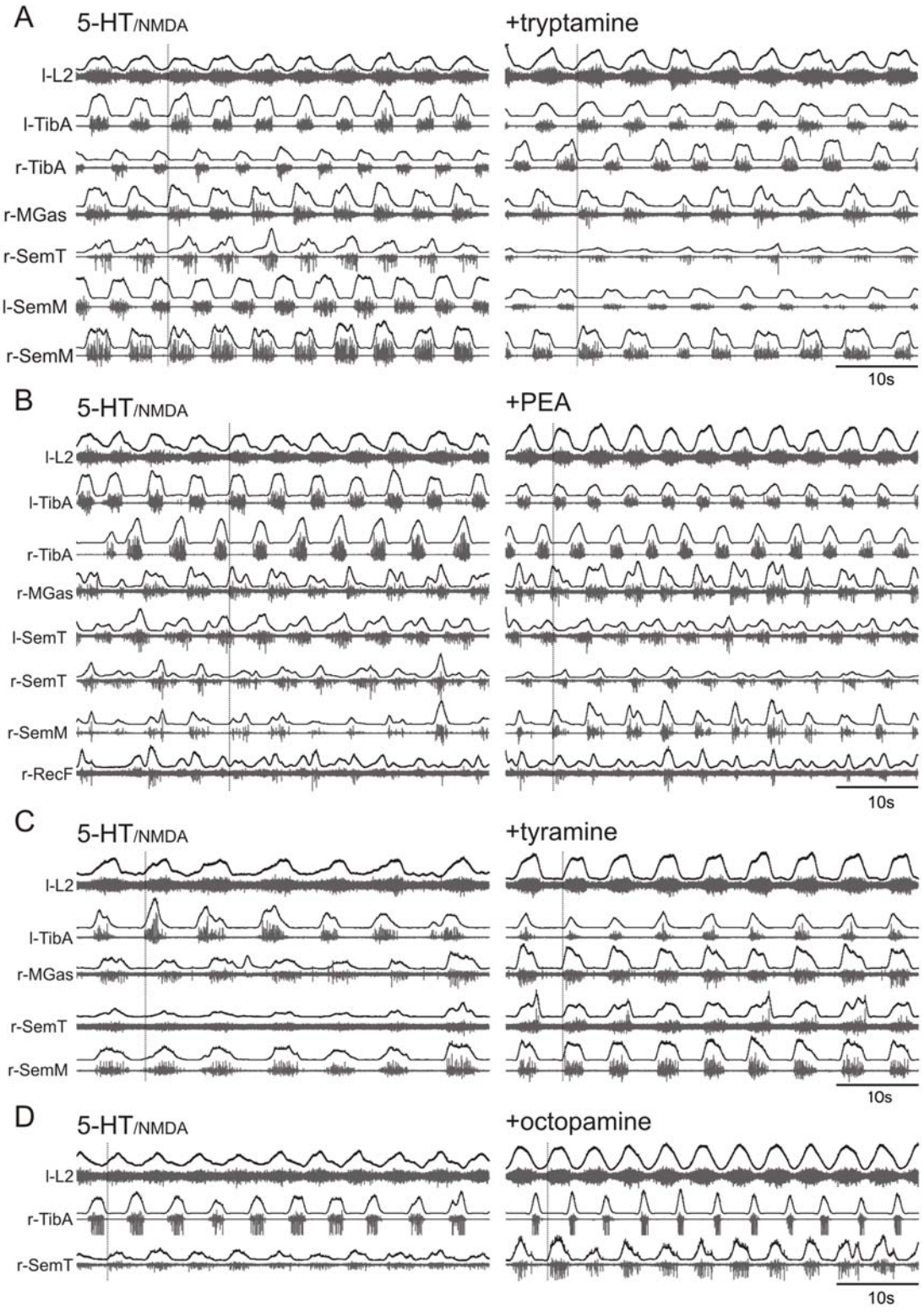

Figure 5.6: Effects of the trace amines on normal 5-HT evoked locomotor-like activity
When the TAs were added to ongoing LLA, continuous LLA can be maintained with the pattern and the phasing between recorded muscles remaining largely comparable. Note however that for any given muscle, addition of the TA can lead to overt changes in EMG amplitude.

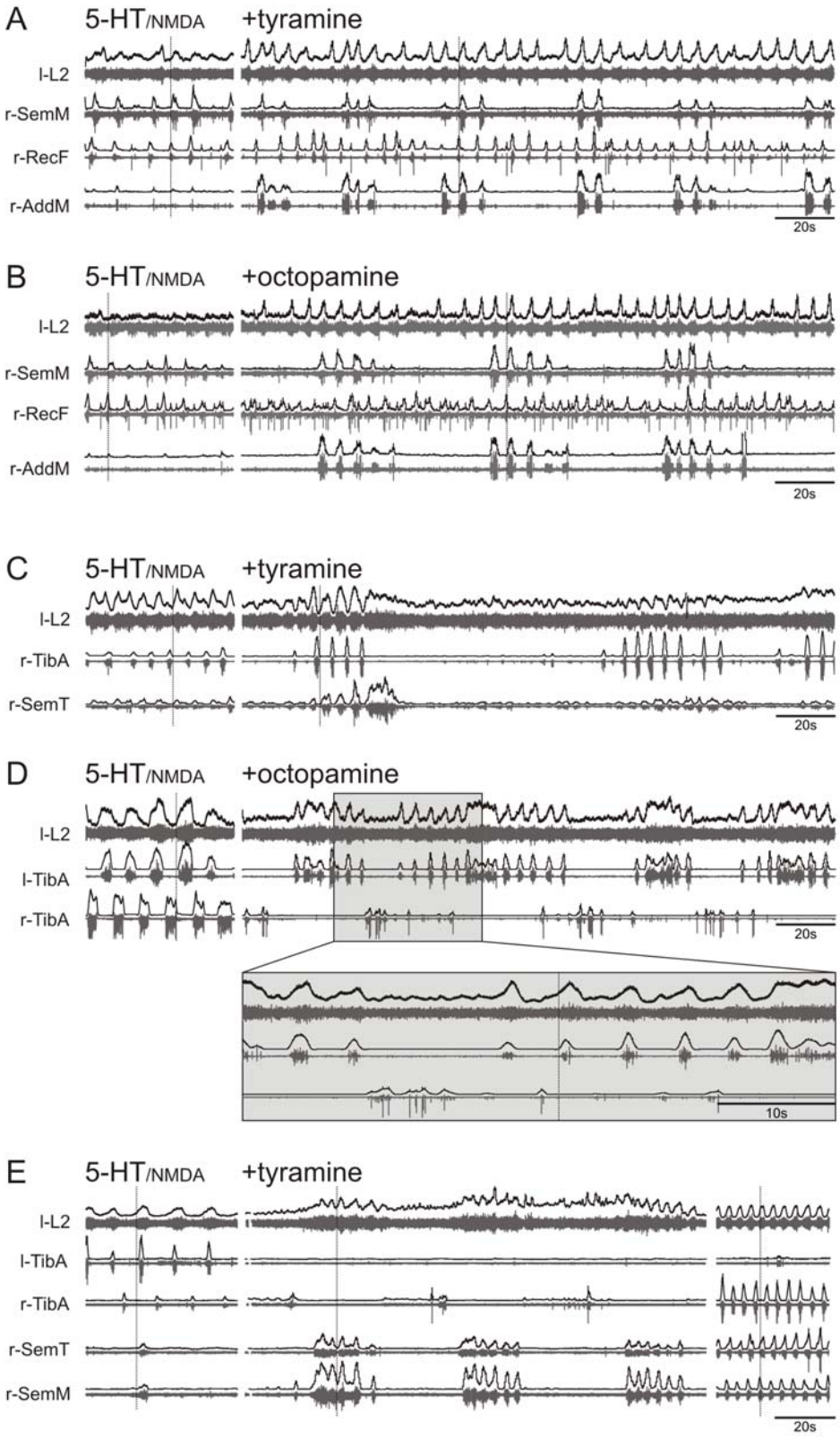

Figure 5.7: Tyramine and octopamine can convert continuous 5-HT locomotion into the episodic pattern

Figure 5.7: continued

These episodic patterns have bouts of locomotor-like bursting that were interrupted by relatively quiescent periods. **A&B.** For tyramine and octopamine, the most common pattern was where some of the muscles and/or ventral root converted to episodic bursting while others maintained a more continuous form of bursting. **C.** For tyramine, there was the conversion to concurrent episodic locomotor bursting. **D.** For octopamine, there was episodic bursting that alternated between the right and left sides. **E.** For tyramine, continuous 5-HT/NMDA LLA may be modulated into episodic bursting by tyramine and then back to continuous bursting with no further intervention

(61±42%; $p<0.01$) with tyramine exhibiting a similar trend for increase (48±65%; $p=0.12$) (**Figure 5.8**). Tyramine and octopamine also increased the amplitude of the left L2 ventral root when added to ongoing 5-HT/NMDA LLA (14±12%, $p<0.05$; and 11±12%, $p<0.05$; respectively) (**Figure 5.9**).

Assuming L2 ventral root amplitude reflects flexor muscle activity, this implies that the TAs, tryptamine, tyramine and octopamine, facilitated flexor activity (Kiehn and Kjaerulff 1996). Based on this, I would conclude that the TAs generally facilitate flexor activity with a clearly significant facilitation from tyramine and tryptamine during continuous locomotion (**Figure 5.9**). However, EMG amplitude for the ankle flexor tibialis anterior actually decreased in amplitude with tyramine ($p<0.05$) with a similar trend observed with tryptamine ($p=0.08$). One explanation for this discrepancy is that the large majority of tibialis motoneurons exit from the L3 spinal segment (Nicolopoulos-Stournaras and Iles 1983). On the other hand, hip and knee flexors appear to exit L2 (iliacus, rectus femoris, pectineus, and gracilis). Of these, I occasionally recorded from rectus femoris (knee extensor/hip flexor) which was always active during the flexor phase of locomotion. While the sample sizes are too small to make statistical comparison, rectus femoris did appear to increase in amplitude in the presence of tryptamine and tyramine supporting the notion that these TAs increase hip flexions while concomitantly decreasing ankle flexion (i.e. tibialis anterior).

For tryptamine, muscles that can act as knee extensors and active during the extensor phase (semimembranosus and semitendinosus) also underwent a significant reduction in amplitude when pooled together ($p<0.05$).

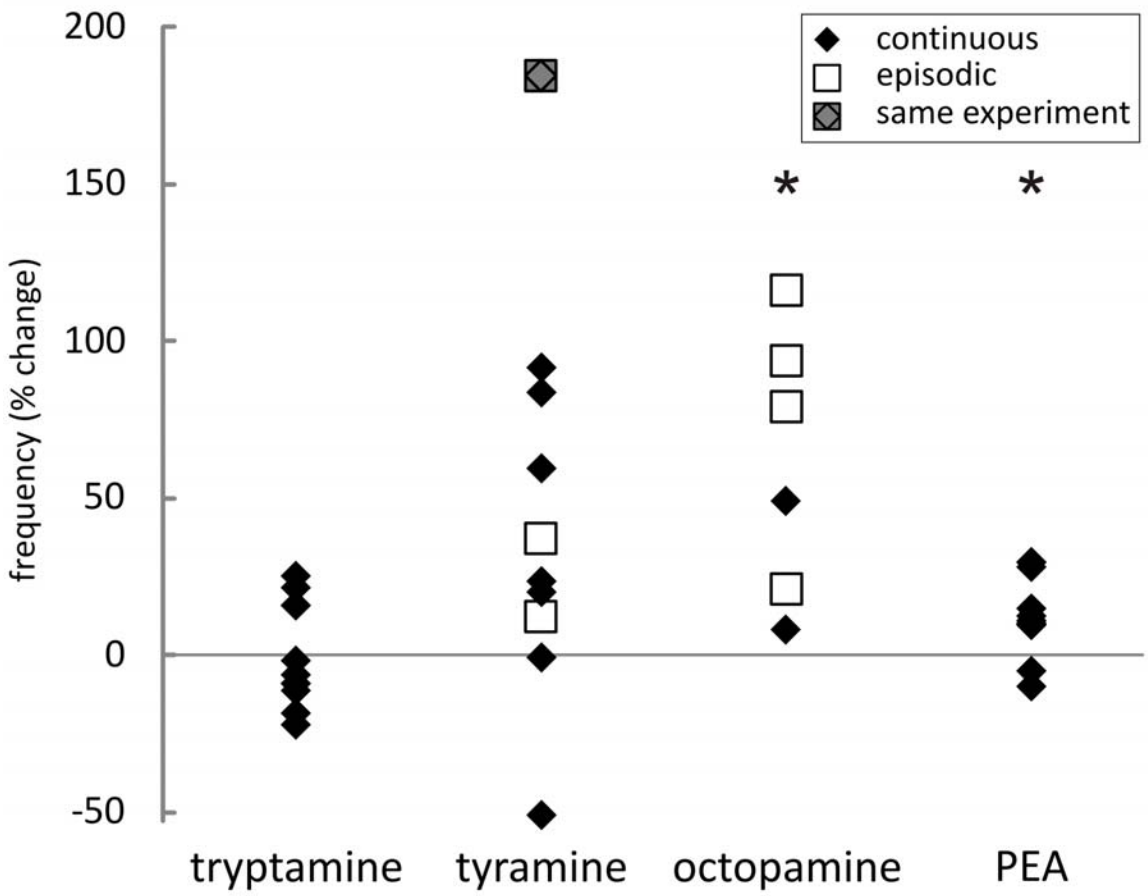

Figure 5.8: The frequency of the motor rhythm increases significantly when octopamine and PEA are added to ongoing 5-HT locomotor like activity

Tyramine also tended toward increases in frequency, while tryptamine has no affect. In one of the tyramine experiments (labeled same experiment above), episodic LLA eventually converted to continuous LLA. The frequency of this experiment was the average of the episodic and continuous LLA and was denoted by a grey diamond inside of a grey square. * indicates a $p<0.05$.

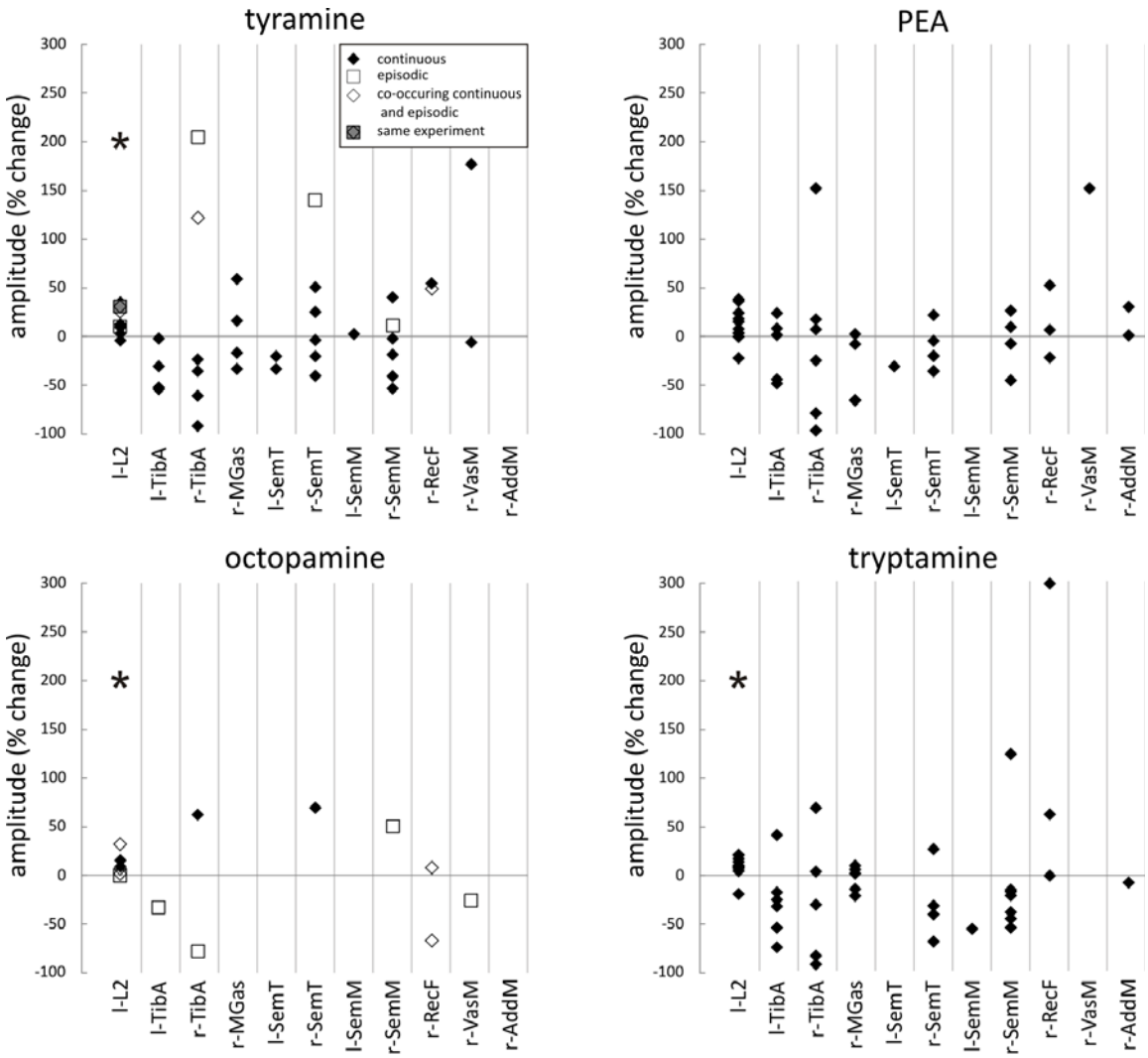

Figure 5.9: Percent change in burst amplitudes after adding the TAs to ongoing 5-HT locomotor-like activity

The increases in amplitude of l-L2 ventral root ENG were significant for tyramine, octopamine, and tryptamine. * indicates a $p<0.05$.

5.4.2.1.2 Tyramine and octopamine convert continuous locomotor-like activity to episodic patterns

In a subset of experiments, tyramine (n=4/12), and octopamine (n=4/7) converted continuous 5-HT/NMDA LLA into episodic bursting (**Figure 5.7**). The patterns produced were similar to those observed when the TAs and NMDA were applied alone (**Figure 5.3**). There were a number of different ways that this pattern manifested itself.

First, the most common pattern observed was where some muscles and/or ventral root converted to episodic bursting while others maintained a more continuous form of bursting. This was seen for both tyramine (n=2/4) and octopamine (n=3/4). For example, while the left L2 ventral root and right rectus femoris bursting are continuous, right semimembranosus and right adductor magnus have episodic bursting (**Figure 5.7A and B**).

Second, the simplest behavior seen with tyramine (n=1/4) was the conversion to concurrent episodic locomotor bursting (**Figure 5.7C**). This was by far the most common episodic activity pattern seen when the TAs were applied without 5-HT in the section above (see **Figure 5.3**).

Third, episodic bursting that alternated between the right and left sides was seen only once (with octopamine and had some LLA overlap during transition periods; **Figure 5.7D**). When the TAs were applied without 5-HT as reported above, this form of bursting was observed for tyramine (1/5), octopamine (2/5), and PEA (2/8), and in the isolated cord, it was observed for octopamine (2/9) and PEA (1/7).

Finally, a pattern never observed previously was seen once for tyramine (1/4). There were alternating bouts that were not associated with flexor/extensor or left/right

coordination (right tibialis anterior alternating with left L2 ventral root, right semimembranosus, and right semitendinosus; **Figure 5.7E**, middle). Additionally, the type of pattern could change over time. This was seen for tyramine (n=2/4). For example, continuous 5-HT/NMDA LLA may be modulated into episodic bursting by tyramine and then back to continuous bursting with no further intervention (**Figure 5.7E**).

5.4.2.2 Trace amines maintain the locomotor phase relations established by 5-HT

Adding the TAs to ongoing 5-HT/NMDA did not significantly change the phasing for any muscle compared for either the continuous or episodic result (**Figure 5.10).**

5.5 Discussion

In this, study, I examined the effects of the TAs on muscle activity recorded in the *in vitro* isolated rat spinal cord with intact hindlimbs. I found that consistent with the patterns observed using ventral root recordings in the isolated spinal cord, the TAs recruited the same predominant patterns: regular continuous locomotion, a slow rhythmic activity whose coordination pattern was consistent with recruitment of locomotor circuits, and episodic patterns that included locomotion. One difference, however, was that the episodic patterns produced were more numerous in the attached hindlimb preparation. Since I hypothesized that the TAs would have distinct differentiable actions on motor pool recruitment during rhythmic motor behaviors, I examined differences in recruitment and found that indeed the TAs did have differences, especially in phasing compared with 5-HT.

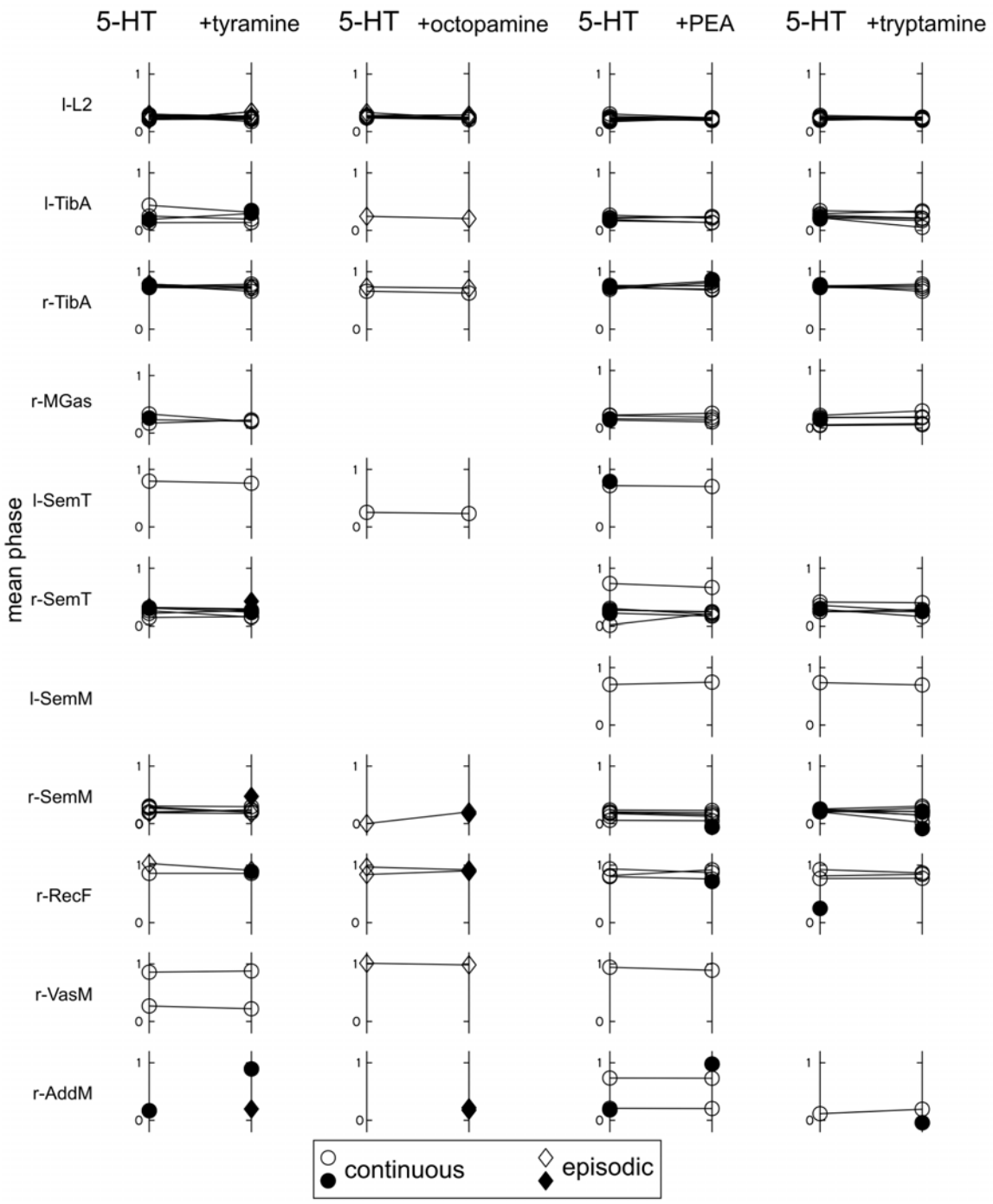

Figure 5.10: Summary of phasing before and after adding the trace amines to ongoing 5-HT induced locomotor-like activity

Adding the TAs to ongoing 5-HT/NMDA LLA did not significantly change the phasing. The mean phase is shown on the y-axis. On the left is the phase for 5-HT/NMDA LLA, and on the right is the phase once the TA has been added to the ongoing 5-HT/NMDA LLA. Open circles or open diamonds indicate an experiment where the root or muscle was present before and after the TA was added; there is a line between the circles or diamonds to demonstrate the change for that experiment. When muscle EMG activity was present only before or emerged only after, it is indicated by a filled in circle or diamond. All phases are in reference to the onset of the l-L2 ventral root. Experiments without a l-L2 are not included.

Further, I examined the ability of the TAs to modulate ongoing 5-HT/NMDA locomotor-like activity, since the TAs may predominantly be acting as modulators of ongoing locomotion. I demonstrated that the TAs modulated the ongoing pattern, and that there were differences between the TAs. All TAs but tryptamine increased locomotor frequency, and all TAs but PEA increased the L2 ventral root amplitude. The most pronounced effects to motor coordination produced with 5-HT locomotion by the TAs were to a subgroup of tyramine and octopamine applications where dramatic changes in patterning were observed.

Overall, given the widespread expression of the synthesis enzyme for the TAs and their high turnover rates, I think that the TAs act to locally modulate the neurons in which they are currently being made. Unlike the monoamines, which are not produced in the spinal cord, the TAs can react to the cellular environment to change the output and add flexibility to motor control mechanisms. It is possible that the widespread range of effects seen by bath application of the TAs does not reflect endogenous actions occurring during normal behavior; however, they give clues to how the TAs ca act as modulators in the spinal cord, even if exaggerated. In this regard, the possibility that they could be used pharmacologically for therapeutic strategies could be explored.

5.5.1 TAs can produce locomotor activity

5.5.1.1 Comparison the output produced by the isolated spinal cord preparation with and without attached hindlimbs

Here, the TAs in the presence of sub-locomotor concentrations of NMDA produced patterns consistent with those observed using ventral root recordings in the isolated spinal

cord. The same predominant patterns were observed; namely, regular continuous locomotion, a slow rhythmic activity whose coordination pattern was consistent with recruitment of locomotor circuits, and episodic patterns that included locomotion.

Despite having an almost identical *in vitro* experimental arrangement, there were several major differences between isolated and the hindlimb-intact rhizotomized preparations. First, the proportions of the different patterns were different. Unlike in the isolated spinal cord where the slow rhythmic pattern was produced 5 of 24 times by tyramine, it was never produced in the attached hindlimbs preparations. Conversely, octopamine and PEA, which never produced the pattern in the isolated spinal cord, each produced the pattern once in the attached hindlimb preparation. This difference is less surprising, given the low occurrence. Additionally, while episodic patterning was dominant for octopamine and PEA in the isolated spinal cord, its expression incidence was equal to regular continuous locomotion in the attached hindlimbs. Second, there was a trend towards higher frequencies in the attached hindlimb preparation, with PEA being significantly faster. Third, the episodic patterns produced in the attached hindlimb preparation were more numerous than in the isolated spinal cord, which could be partially reflecting the number of channels from which I was recording. By adding the complexity of muscles, the output could be increased, since it has been previously suggested that use of ventral root activity may not reflect recruitment of individual muscles (Cowley and Schmidt 1994b). A prime example being where I found that the ventral root was continuously bursting while at least one muscle was having different actions (**Figure 5.3C**, left)

That there were differences between the preparations is interesting given that there was no sensory feedback from the muscles since the dorsal roots were cut. An interesting and perhaps decisive difference in experimental set-up between the isolated spinal cord and the attached hindlimb preparation was the addition of a superfusion system rather than static bath, which likely increased tissue oxygenation by increasing the flow rate. Increasing flow rate has been shown to affect the locomotor-like activity (Wilson et al. 2003).

5.5.1.2 Trace amines produce continuous locomotor-like activity pattern

The general phasing of muscle characteristics of 5-HT and TA induced continuous locomotion were similar to that seen in adult rat (de Leon et al. 1994; Gruner and Altman 1980; Gruner et al. 1980) and in previous work in the isolated spinal cord with one hindlimb attached (Kiehn and Kjaerulff 1996). While there were sometimes distinct amine-dependent shifts in the timing within the phase, tibialis anterior, vastus medialis, and rectus femoris were active at some point during the flexor phase, and medial gastrocnemius, semitendinosus and semimembranosus were active during the extensor phase. The major differences in phasing between 5-HT and tyramine were to muscles acting during the flexor phase (tibialis anterior, an ankle flexor, and rectus femoris, a knee extensor / hip flexor), which were both active earlier in the phase than during 5-HT. This presumably causes the leg to be move forward earlier. The biggest differences with octopamine were to muscles active during the extensor phase (semitendinosus and semimembranosus, both knee flexor/hip extensor), which were both active earlier in the phase than 5-HT. This presumably causes the leg to pull into the equivalent of stance phase earlier. Tryptamine also shifted muscles to earlier in the stage both flexors (tibialis

anterior) and extensors (semitendinosus and semimembranosus, both knee flexor/hip extensor).

The exact behavior represented by each of these shifts in patterns is not clear. With the limited behavior of the neonate, there are very few patterns to which these results can be compared. Previous work has suggested that late flexion of rectus femoris observed in 5-HT rhythms is most consistent with swimming. Of the patterns examined, this appears to be the pattern to which the TAs are most similar (de Leon et al. 1994; Kiehn and Kjaerulff 1996). However, all of the TAs have rectus femoris active earlier in the flexor phase rather than later as seen with swimming. DA has been observed to be the most similar to walking (de Leon et al. 1994; Kiehn and Kjaerulff 1996). Galloping is another interesting behavior to consider; however, there is almost co-contraction of tibialis anterior and gastrocnemius, which is not seen in TA induced patterning. In neonatal rats, crawling is actually the dominant pattern until the middle of the second week postnatal since the rats are not strong enough to pull their bellies off of the ground (Westerga and Gramsbergen 1990); however, there are not enough muscles to make meaningful comparisons with the available EMGs (Juvin et al. 2005).

5.5.1.3 Tyramine, octopamine, and PEA produce episodic activity patterns

The more intriguing pattern to reflect on is the episodic pattern. I do not know the significance of these various patterns of activity or if they are actually relevant, but there are a number of interesting possibilities to consider. First, they could represent simply the starting and stopping of locomotion. Interestingly, normal rodent locomotion is not continuous as seen with 5-HT, but periodic as observed with the TAs (De Bono et al. 2006; Rodnick et al. 1989). Second, it could be part of a different behavior such as

scratch, copulation, or orgasm (Carro-Juarez and Rodriguez-Manzo 2005; 2006; Robertson et al. 1985). Third, the TAs may be playing a role in development. Episodic spontaneous bursting has been observed during development in chick, mouse, and rat many facilitate the formation of functional synaptic connections (Bekoff et al. 1975; Hanson and Landmesser 2003; Nakayama et al. 2004; O'Donovan et al. 2008; Whelan et al. 2000). Interestingly, in the developing rat embryo, AADC positive neurons are observed on day 12 of development in all cells of the notochord and in neuroepithelial cells of the ventral neural tube. AADC was enzymatically active, and these cells were tyrosine hydroxylase negative, which indicates that it is likely that the TAs are being produced in these cell and could be playing a role in development (Teitelman et al. 1983).

5.5.1.4 Amplitude of trace amines compared to 5-HT

As compared to that observed with 5-HT, I observed a decreased amplitude of the left L2 ventral root for all of the TAs and tibialis anterior for tyramine and octopamine. While Kiehn and Kjaerulff (1996) did not observe consistent changes in amplitude between DA and 5-HT (Kiehn and Kjaerulff 1996), Klein et al. (2010) did see higher amplitude for adductor magnus in cauda equine stimulation evoked patterns than 5-HT/NMDA evoked patterns (Klein et al. 2010) consistent with my work that there can be changes.

The observed smaller EMG amplitude for the TAs compared with 5-HT may be related to the excitability of the motoneuron pool. Indeed, 5-HT is known to depolarize motoneurons (Hochman and Schmidt 1998). In Chapter 4, I showed that while tryptamine and tyramine directly depolarize motoneurons, the percent increase over baseline was much smaller than 5-HT. Also, PEA and octopamine do not significantly

change the activity of motoneurons. Therefore, while 5-HT increases excitability in the motoneurons and the neurons at the level of the CPG, the TAs appear to more preferentially CPG neurons. This could easily explain why the TAs have decreased amplitudes compared with 5-HT. If PEA and octopamine are more selective recruiters of the CPG, these may be preferred in clinical strategies clinically where motoneurons are already over-excited and there is the desire to just recruit the CPG.

5.5.2 TAs modulate ongoing locomotor activity

5.5.2.1 The TAs can alter the frequency and amplitude of ongoing locomotor-like activity

When the TAs are added to ongoing 5-HT/NMDA LLA, I observed consistent increases in the frequency for tyramine, octopamine, and PEA which implies that they are having modulatory effects on interneurons that control frequency. The increase in frequency supports facilitation at the level of the rhythm-generator CPGs (Rybak et al. 2006b). One possible group of interneurons that has been identified are the V1 interneurons, which when either inactivate or deleted lead to an inability to burst or step at higher frequencies (Gosgnach et al. 2006). Since tryptamine does not change the frequency of ongoing 5-HT locomotion, it is presumed that it is not acting on these interneurons.

PEA does not have actions on left L2 ventral root amplitude. Unlike the other TAs, PEA never had an effect on motoneurons as reported in the last chapter, and therefore, it is plausible that it would not act to increase amplitude here. Surprisingly, there were no significant changes in amplitude for any of the muscles after the addition of the TAs. This is likely due to the small sample size. The addition of tryptamine and tyramine led

to trends towards increases in amplitude for rectus femoris, supporting the notion that these TAs increase hip flexions. Trends towards decreases were seen for tibialis anterior (ankle flexor) and semimembranosus and semitendinosus (both knee extensors).

5.5.2.2 Tyramine and octopamine produce episodic bursting patterns

Tyramine and octopamine dramatically modulated on-going 5-HT locomotor-like activity by converting it to episodic bursting. Like with the TAs alone, there is a huge amount of variability in the patterning produced. This is potentially a good thing. Variability and flexibility allow the spinal cord to adapt depending on the neuromodulatory state of the system and the requirements of the system (Klein et al. 2010). The modulatory state of the TAs is readily changed by varying the amount of aromatic amino acids, the activity of AADC, or the activity level of the monoamine oxidases, and this variability could account for some of the different patterns seen. Many of the different possibilities as to what could be happening are discussed above.

I demonstrated that one important advantage of the isolated spinal cord with hindlimbs attached is that it provides a more accurate representation of the distinct motor patterns produced by the TAs than ventral root recordings. I showed examples where ventral root recordings alone did not accurately report what was happening in the hindlimb muscles. For example, continuous L2 ventral root activity could be coincident with episodic EMG (**Figure 5.7A and B**). Interestingly this was the dominant pattern in the presence of 5-HT, yet was only seen once with the TAs applied without 5-HT. As a result of the EMG the more complex nature of the TAs was revealed. Similarly, it is likely that the unique pattern produced by DA seen in Chapter 4 could demonstrate important complexities in the DA motor pattern.

5.5.3 Conclusions

This chapter provides evidence that overall, the TAs are capable of facilitating ongoing locomotor output. The TAs can produce unique patterning as well as alter motor patterns produced by 5HT. The most pronounced effects on coordination to 5-HT locomotion by the TAs were to a subgroup of tyramine and octopamine applications where dramatic changes in patterning were observed. While it remains to be seen whether these effects are seen normally, the TAs do appear to be produced endogenously, and therefore controlling their release and/or receptor activation may provide new therapeutic strategies for the management of spinal cord dysfunction after spinal cord injury, and therefore, warrants further study to determine their efficacy.

CHAPTER 6

DISCUSSION AND CONCLUSIONS

6.1 Summary

Trace amines (TAs) named for their low endogenous concentrations in mammals, are related to the classical monoamine transmitters, but have been understudied and thought of as false transmitters. They share structural, physiological, pharmacological, and metabolic similarities with the monoamines, including synthesis by the aromatic-L-amino acid decarboxylase (AADC) enzyme. In 2001, a new class of receptors preferentially activated by the TAs, termed trace amine-associated receptors (TAARs), was discovered establishing a mechanism for TA actions independent of classic monoaminergic mechanisms. While the TAs and some of their receptors are present in the mammalian central nervous system (CNS), their physiologic role remains uncertain. I hypothesized that the TAs are found intrinsically in the spinal cord and that they are able to modulate spinal neural networks.

Using immunohistochemistry, numerous spinal neurons were identified that express AADC, the TAs (octopamine, tryptamine, and tyramine), and TAARs (TAAR1 and TAAR4). Similar results were seen for AADC and TAAR1 with *in situ* hybridization. The most consistent observation was for labeling D cells associated with the central canal and in motoneurons. Overall, these results provided evidence for the presence of an anatomical substrate onto which the TAs could have intrinsic biological actions in the spinal cord.

Using exogenous application of the TAs in the isolated spinal cord *in vitro*, and *in vivo* in the mid-thoracic chronically spinalized, I showed that the TAs could induce rhythmic locomotor-like activity. TA injection-induced hindlimb motor rhythms observed in chronic spinalized animals, supports TA spinal actions independent of the descending monoaminergic systems. In the presence of NMDA, TA applications recruited a variety of rhythmic motor patterns in the isolated spinal cord. This ranged from locomotor activity indistinguishable from 5-HT/NMDA induced locomotion to complex patterns including an episodic form of locomotion where there were locomotor bouts with intervening quiescent periods.

TA actions of pattern generating circuits: (i) had slower kinetics of activation than 5-HT and NA, (ii) were attenuated in the presence of monoamine transport inhibitors, and (iii) had increased intracellular labeling even when incubated in a nominally Na^+-free solution. Together these results suggest that the TAs required transport into neurons to exert their actions, and that transport occurred by Na^+-dependent monoamine transporters as well as additional Na^+-independent transporters.

Finally, I used the *in vitro* isolated spinal cord with attached hindlimbs to record electromyographic (EMG) activity from various hindlimb muscles: (i) to compare the relationship between the TAs and serotonin (5-HT) evoked motor coordination, and (ii) to examine the ability of the TAs to modulate ongoing 5-HT and NMDA locomotor-like activity. The TAs produced both the continuous and episodic patterns on muscles as observed in ventral root recordings, but EMG recordings provided more detailed insight into specific muscle actions. The TAs also generally increased both frequency and

amplitude of ongoing 5-HT locomotor frequency, with tyramine and octopamine also particularly able to alter 5-HT motor coordination patterns.

6.2 Mechanisms involved in trace amine modulation of spinal cord motor function

6.2.1 Trace amines are intrinsically produced in the spinal cord

My immunohistochemistry results confirmed previous analysis of tissue samples (Boulton et al. 1977; Juorio 1988; Karoum et al. 1979; Spector et al. 1963) and demonstrated that the TAs are present in the spinal cord. Two not mutually exclusive options explain their presence. First, some TAs could arise from dietary sources (Gardner et al. 1996; Ghozlan et al. 2004) with subsequent transport across the blood brain barrier via transporters. This is known for PEA and tryptamine but not tyramine (Oldendorf 1971). Indeed, the L-type amino acid transporters (LATs), which transport PEA, were found at the blood-brain barrier (Segawa et al. 1999). Octopamine transport has not been studied.

A second and probably more widespread mechanism is via endogenous synthesis in the spinal cord by AADC from the aromatic amino acid precursors, which were also found in many foods (Gardner et al. 1996). It has been shown that meals cause physiologic-size changes in aromatic amino acid concentrations (Fernstrom 1990) that cross the blood brain barrier (Oldendorf 1971), likely via LATs (Segawa et al. 1999). The raised levels of the aromatic amino acids in the brain increase the synthesis of the monoamine transmitters (Fernstrom and Wurtman 1971; Wurtman et al. 1974) and so, while not explicitly studied, must also be increasing the synthesis of the TAs as the same essential synthesis enzyme is involved (AADC).

With AADC as the rate-limiting enzyme for the synthesis of TAs (Berry et al. 1996; Dyck et al. 1983), events that regulate AADC activity should alter TA levels. Indeed, changes in AADC activity produced proportional changes in TA production (Jones et al. 1983; Juorio 1982; Juorio et al. 1991a; Juorio et al. 1991b). Thus, rapid changes in AADC activity by phosphorylation can change TA levels and fine tune their actions, thus allowing minute to minute regulation (Berry et al. 1996). My results demonstrated widespread AADC labeling in the spinal cord, indicating that the TAs can be produced all over the spinal cord, thus giving a substrate for widespread TA production.

TA labeling was located in the same locations as AADC, including labeling in central canal cells, ventral stream cells emanating from the central canal, ventral funiculus, ventral horn interneurons, and motoneurons. The observed labeling pattern varied between animals, the most common pattern being a widespread diffuse labeling. Nonetheless, there was a subset of animals with more selective and intense labeling in ventral horn interneurons. With TA synthesis dependent on the dietary availability of aromatic amino acids and the modifiable levels of AADC activity in different cells based on their phosphorylation status, the observed variability in TA labeling would be expected. If there were both a high level of aromatic amino acids and increased activity of AADC, there should be a higher level of the TAs in the spinal cord. However, a low supply of aromatic amino acids and/or low activity of AADC would lead to less TA synthesis and consequent labeling. This variability could be an advantage for the modulation of spinal motor function.

In addition to AADC, octopamine further requires DBH, which is located only in descending neurons, implying that octopamine is only produced in descending

noradrenergic neurons. However, another enzyme, called monooxygenase, DBH-like 1(Moxd1) has comparable function (Chambers et al. 1998; Xin et al. 2004) and so could also theoretically convert tyramine to octopamine. Moxd1is strongly expressed in the mouse spinal cord including in presumed motoneurons and around the central canal (**Appendix A.1**) (Allen_Spinal_Cord_Atlas 2009). Further studies will be required to determine if Moxd1 does in fact produce octopamine, and hence, if octopamine is producing intrinsically in the spinal cord.

6.2.2 Aromatic-L-amino acid decarboxylase positive neurons represent a intrinsic trace aminergic system

As stated above, I found widespread AADC labeling in the spinal cord, including expression around the central canal, the ventral funiculus, and in ventral neurons, including motoneurons. The strongest labeling was consistently found around the central canal in cells consistent with D1 cells (Jaeger et al. 1984a; Jaeger et al. 1983). Co-labeling of the TAs with AADC in the central canal cells provides strong evidence that the D cells are in fact trace aminergic as had been previously hypothesized (Berry 2004; Jaeger et al. 1983).

D1 cells project at least one of their processes into the lumen of the central canal, which makes them part of a group of CSF-contacting neurons (Jaeger et al. 1983; Vigh et al. 2004). The specific role of the D cells and whether they are specifically involved in locomotion will need further investigation, although one intriguing hypothesis is that D1 cells function to monitor and transport aromatic amino acids from the CSF and then synthesize TAs to intrinsically increase their activity (via TAs intracellularly activating G_s-coupled TAARs) . This increased activity could then be synaptically relayed to the

motor system. Indeed, it should be noted that CSF-contacting cells present in zebrafish larva can initiate slow swimming by optogenetic stimulation and that these neurons provide the necessary tone for spontaneous forward swimming (Wyart et al. 2009). Importantly, I showed that D cells and their white matter projection system were maintained after chronic spinalization demonstrating that that the white matter tract is part of an intrinsic spinal aminergic system.

The presence of other $AADC^+$ neurons elsewhere in the spinal cord, based on both *in situ* hybridization and immunohistochemistry, demonstrated that there are other populations of cells in the spinal cord also capable of synthesizing TAs. This includes motoneurons.

6.2.3 Trace amine-associated receptors provide a substrate for trace amine actions in the spinal cord

I found that both TAAR1 and TAAR4 were widely expressed in the neonatal rat spinal cord, including in the central canal, ventral interneurons, and motoneurons. TAAR expression was found in the same locations as AADC and the TAs, providing a mechanism though which the TAs can produce intrinsic modulatory actions. As has been observed before for TAAR1 in HEK cells (Bunzow et al. 2001; Miller et al. 2005), I observed cytoplasmic labeling for TAAR1 and TAAR4, both of which are activated by the TAs. Tyramine and PEA activate TAAR1, while PEA and tryptamine activate TAAR4 (Borowsky et al. 2001). The cytoplasmic location of the ligand and the receptor (e.g. tyramine and TAAR1) supports intracellular activation of signal transduction pathways, as suggested previously (Miller et al. 2005). Since the TAARs are intracellular, access to them is limited. Hence, in order to have actions on them, the TAs

must either be produced in the cells from their aromatic amino acids precursors, or be taken up by transporters (described below). Importantly, this form of intrinsic neuromodulation does not require release from synaptic vesicles, and could explain why the TAs do not appear to be concentrated in vesicles. It could also explain the relatively trace amounts of TAs found in the CNS, as TA actions would not require storage as long as their production was in the vicinity of the intracellularly located TAARs. This also means that access to the TAARs in cells not producing TAs would be limited by cell membrane transporters, and would explain the low potency of the TAs for TAAR4 expressed in cell lines not containing such transporters (e.g. HEK cells in (Borowsky et al. 2001). An exclusive intracellular location of TAARs may also explain why other studies failed to identify TAAR ligands despite the prediction that they must be small molecular weight compounds structurally similar to the TAs (Lindemann et al. 2005; Lindemann and Hoener 2005). Almost all assays have assumed cell surface expression of the receptor.

Currently, the role of the TAARs in the spinal cord is unknown. However, it is known that they that they are $G\alpha_s$-coupled proteins able to activate cyclic adenosine monophosphate (cAMP) (Borowsky et al. 2001; Bunzow et al. 2001). There are a number of classes of downstream substrates which cAMP signaling can activate including transcription factors, voltage-gated ion channels, ion pumps, and neurotransmitter receptors, all of which are candidates after TAAR activation (Greengard 2001).

6.2.4 Evidence of trace amine actions on descending monoaminergic terminals

In my work, one possible mechanism by which the TAs could exert their action then, is by transporter-mediated TA uptake at descending monoaminergic terminals. It has been reported that TAs can facilitate monoamine transmitter efflux in the CNS via reverse transport (Paterson et al. 1990; Sulzer et al. 2005). This monoamine efflux could contribute to the bursting I see in my experiments. TAs uptake is facilitated by monoamine transporters, which are greatly enhanced by TA activation of TAAR1 (Xie et al. 2007). Further, PEA activation of TAAR1 is required for monoamine efflux via the monoamine transporters, suggesting an important modulatory role of TAAR1 in monoamine transporter function (Xie and Miller 2008). In my experiments, I observed that inhibition of monoamine transport significantly attenuated motor actions for the TAs but not for 5-HT or NA (**Figure 4.6 and 4.7**). This suggests then that a significant component of TA-evoked actions is likely due to monoamine transmitter efflux from descending terminals. However, TA induced motor rhythmicity remained after monoamine transporter block indicating that TAs are binding at sites at least partly independent of descending monoamine presynaptic terminals (**Figure 4.11**).

6.2.5 Possible trace amine action at monoamine receptors

One site of TA-evoked actions that are independent of presynaptic descending terminals is on monoamine receptors. Tryptamine directly activates $5HT_2$ and $5HT_7$ receptors (Boess and Martin 1994), the same critical receptors required 5-HT induced locomotion (Liu et al. 2009; Liu and Jordan 2005; Madriaga et al. 2004). This would explain why tryptamine and 5HT evoked similar locomotor patterns with similar sensitivity to block with methysergide. However, in comparison to tryptamine, all other TAs appear to have very low affinity to the monoamine receptors tested, such that the

applied doses would not be expected to have direct actions (Peddi et al. 2003; Shen et al. 1993; U'Prichard et al. 1977). Thus, tyramine, PEA and octopamine must also be acting at additional sites.

6.2.6 Trace amine actions through transporters found in the spinal cord

I showed that there is widespread expression of somatic TAAR1 and TAAR4 receptors. They are located intracellularly consistent with earlier observations for TAAR1 (Bunzow et al. 2001; Miller et al. 2005; Xie et al. 2007). To activate these receptors, the TAs require a transport mechanism. This was demonstrated by (Xie et al. 2007) associated with the monoamine transporters. As monoamine transporters are not found in spinal neurons (**Appendix A.4**), in order for the TAs to exert their actions alternate transport systems must be present. I showed that the TAs can be transported into spinal neurons via a Na^+-independent mechanism. Multiple Na^+-independent plasma membrane transporters have been shown to transport PEA, tyramine, and tryptamine and are widely expressed in the spinal cord (**Appendix Figure A.5 and Figure A.6**), providing an intrinsic substrate for independent actions within spinal neurons (**Figure 4.11**). These plasma membrane transporters were the L-type amino acid transporters (LATs), the organic cation transporters (OCTs), and the plasma membrane monoamine transporter (PMAT). PEA can be transported via LATs, tyramine via OCTs and PMAT, and tryptamine via PMAT. Currently, octopamine transport has not been tested at these transporters. Importantly, since these transporters have bidirectional transport, the TAs can be synthesized in one neuron, and transported into nearby neurons. This allows modulatory actions via TAARs in neurons not endogenously synthesizing TAs (i.e. AADC negative). In my experiments, it is reasonable to assume that exogenous

application of relatively high TA concentrations would be sufficient to activate TAAR1 and TAAR4 in spinal neurons (Borowsky et al. 2001; Bunzow et al. 2001). Given their widespread distribution and the similarly widespread presence of required Na^+-independent transporters, broad modulation of spinal circuits would be expected.

6.2.7 Differences in the trace amines

While the TAs are similar to each other in many ways, there are differences in structure, synthesis, storage, transport, receptor activation, and degradation. Many of these differences are highlighted in the **Table 6.1** below.

Table 6.1: Differences between the trace amines

	tryptamine	tyramine	octopamine	PEA
structurally similar to 5-HT	yes	no	no	no
structurally similar to DA and NA	no	yes	yes	yes
immunohistochemical evidence that the TA is found in the spinal cord	yes	yes	yes	no antibody available
1st synthesis enzyme	AADC	AADC	AADC	AADC
2nd synthesis enzyme	none	none	DBH (or Moxd1?)	none
synthesized in descending terminals	yes	yes	yes	yes
synthesized in blood vessels	yes	yes	no	yes
synthesized in spinal cord neurons	yes	yes	unknown (Moxd1?)	yes
found in vesicles	no	small amounts	yes	no
transported via monoamine transporters	yes	yes	yes	yes
immunohistochemical evidence that transported into spinal cord neurons	yes	yes	yes	no antibody available
transporter known to transport the TA is found in the spinal cord	PMAT	OCTs and PMAT	unknown	LATs
activates TAAR1, which is found in the spinal cord	yes	yes (highest affinity)	yes	yes (2nd highest affinity)
activates TAAR4, which is found in the spinal cord	yes	no	no	yes
increases in cAMP with co-expression of monamine transporters and TAAR1 over just monoamine transporters	yes	yes	yes	yes
actions on 5-HT receptors	yes	no	no	no
action on catecholamine receptors	no	no	no	no
degraded by MAOA	yes	yes	yes	no
degraded by MAOB, which is not found in spinal cells	yes	yes	yes	weakly

6.2.8 Mechanisms associated with TAAR activation and signaling

Previous reports suggest that the monoamine receptors, 5-HT_2, 5-HT_7, D_1, and α_1, are involved in mediating actions on the locomotor network (Gabbay and Lev-Tov 2004; Liu et al. 2009; Liu and Jordan 2005; Madriaga et al. 2004). An interesting and very plausible role for the anatomical substrate created by the widespread expression of AADC, the TAs, and the TAARs would be as an activity amplifier / reinforcement mechanism. In this scenario, descending monoamine transmitters known to promote locomotion activate G-proteins, leading to downstream signal transduction pathways capable of phosphophorylating AADC (**Figure 4.11**).

5-HT_7 and D_1 both activate the $G_s\alpha$ subunit, which stimulates adenylate cyclase (AC) to produce cyclic adenosine monophosphate (cAMP) from adenosine triphosphate (ATP). cAMP, a second messenger, activates protein kinase A (PKA, cAMP-dependent protein kinase) (Gervasi et al. 2007), and PKA phosphorylates AADC (Duchemin et al. 2000).

5-HT_2 and α_1 both activate the $G_q\alpha$ subunit, which stimulates phospholipase C (PLC) to hydrolyzes phosphatidylinositol 4,5-biophosphate (PIP_2) into two second messengers, diacyl glycerol (DAG) and inositol triphophate (IP_3). IP_3 causes the release of calcium from intracellular stores, leading to the activation of Calcium/calmodulin-dependent protein kinase type II **(**CaMKII) (Dash et al. 2007). CaMKII phosphorylates AADC (Neff and Duchemin 2002).

As many of my *in vitro* experiments also used NMDA, intracellular cascades following NMDA receptor activation could also potentially increase TA levels. NMDA receptor activation-induces Ca^{2+} entry into the neurons, which activates CaMKII and nitric oxide synthase (NOS) (Matsumura et al. 2010) (**Figure 4.12**). CaMKII

phosphorylates AADC (Neff and Duchemin 2002). NOS produces nitric oxide (NO), which can lead to inhibition of MAO activity (Muriel and Perez-Rojas 2003), and thereby reducing TA degradation. Further, NO leads to the activation of cGMP-dependent protein kinase (PKG) (Schlossmann and Hofmann 2005), which can then phosphorylate AADC (Duchemin et al. 2010).

The aforementioned phosphorylations of AADC increase its activity and cause an increase in the levels of TAs (Berry 2004; Duchemin et al. 2000; Duchemin et al. 2010; Neff and Duchemin 2002), leading to activation of the TAARs. This could then transform the externally-generated increase in neuronal excitability to a self-sustaining intrinsic mechanism where TAAR induced increases in G proteins produce a positive feedback continued phosphorylation of AADC and TA production. In this scenario, activity is amplified and self-sustaining until terminated by G_i-coupled pathways (e.g. $5HT_1$ receptors). Thus, the TAs and their receptors may act as an activity switch which leads to cellular changes in excitability based on the environment, AADC activity, and receptor activation. These details support a tight interdependence of the TAs with the monoamines on spinal motor function. If true, the downstream role of the TAs suggests a refinement of monoamine-induced activity ongoing locomotor pattern, not necessarily the initiation of it. This implies that the monoamines and the TAs work together, but that each has its own role.

It is important to emphasize that in this scenario, other pathways independent of the monoamine and their receptors that also co-activate the same signal transduction pathways could also recruit the TAARs leading to the unique intrinsic modulatory actions of the TAs.

6.2.9 TA actions in the spinal cord on a network level

Tryptamine, tyramine, octopamine, and PEA all recruited locomotor-like patterns similar to that observed for 5-HT as well as more complex episodic locomotor rhythms. Based on flexor/extensor and left/right coordination during the continuous locomotor-like pattern, the TAs are likely acting at the level of the central pattern generator (CPG) to produce the patterning (**Figure 4.10**).

Both continuous and episodic locomotor phenotypes could be observed within individual animals indicating that the episodic pattern involves activation of additional cellular/network interactions that influence the output of the spinal locomotor central pattern generator. Assuming that these different modulatory actions were due to actions at distinct spinal cord sites, there are three possible levels of network neurons that could be affected by tyramine, octopamine, and PEA to give the unique episodic patterns (**Figure 4.10**). In all cases, it was assumed that the CPG level produced the regular locomotor-like activity pattern, which was then further modulated to produce the unique episodic bursting. The first level where the modulation could be occurring would be neurons that project onto the CPG. These neurons could produce a slow alternation of excitatory and inhibitory drive to the CPG, thus causing the waxing and waning of activity. The second level where the modulation could be occurring would be in neurons within the CPG where two events co-exist with distinct time courses. For example, one could be associated with synaptic network interactions occurring at the locomotor frequency while a second could be via non-synaptic biochemical pathways occurring at a slower frequency (Katz and Clemens 2001). As the slow rhythm changes, locomotor frequency changes (e.g. **Figure 4.4E**). The third level where the modulation could be

occurring would be in neurons downstream of the CPG (e.g. motoneurons). In this case, locomotor frequency would be unchanged during the rhythm, but amplitude would alternate between more excitable and less excitable states. I think that since there appears to be coordination between the bouts of bursting across flexors/extensors and left/right alternation, that the first two possibilities are more likely.

6.3 The trace amines function as neuromodulators

Neuromodulation is a substance that alters the cellular and synaptic properties of a neuron and alters the subsequent neurotransmission, so that even with the same input, the output will be different (Katz 1999; Katz 1995; Katz and Frost 1996). Thus, it allows the circuit to become more flexible (Katz 1995). As a general guideline rather than a firm definition, neuromodulation is mediated by metabotropic receptors (Katz 1999; Katz 1995).

The first type is extrinsic neuromodulation. It is usually thought to originate from sources extrinsic to the circuit being affected and therefore not a part of the circuit itself. This is considered the 'conventional' form (Katz 1995)and is typified by the descending classical monoaminergic neurons as they project to the spinal cord and potently modulate spinal sensory, autonomic, and motor activity (Hochman et al. 2001; Millan 2002; Schmidt and Jordan 2000).

A second type of neuromodulation is intrinsic neuromodulation and not as widely recognized. During intrinsic neuromodulation, neuromodulatory substances can also be released by neurons that are intrinsic to a circuit, and can affect other neurons and synapses within the same circuit. One functional consequences of intrinsic neuromodulation is that it operates whenever the circuit is active because it is an integral

part of the circuit and as such its actions are proportional to the amount of activity within the circuit itself (Katz and Frost 1996).

A third type of neuromodulation has been referred to as biochemical integration, which modulates the neuron in which it acts. During biochemical integration, the biochemical intracellular signals (e.g. second messengers like cAMP) are integrated to affect the cellular and synaptic properties of the neuron, often across different time scales (Katz and Clemens 2001).

I have demonstrated that the TAs are intrinsic to the spinal circuits and can affect the activity of spinal motor circuits. The possibility must be considered that that TAs act as both intrinsic neuromodulators as well as a biochemical integrators.

Under normal conditions, I hypothesize that the TAs act as part of a biochemical activity monitor that sets the excitatory 'tone' of the neuron to regulate its output. This form of biochemical integration may control the excitability of spinal neuronal networks via TAARs, and as such, should be an additional route that can be explored for therapeutic manipulation after spinal cord injury. It may turn out that the TAs are better at temporally modulating the spinal function or easier to manipulate than the monoamines.

6.4 Relevance of the trace amines an intrinsic neuromodulatory system

While the TAs are potentially important modulators of CNS function, most studies aimed at understanding their functional role on the neural systems were undertaken decades ago in the mammalian spinal cord. The seminal discovery in 2001 of a new family of G-protein coupled receptors preferentially activated by TAs (Borowsky et al. 2001; Bunzow et al. 2001) rekindled interest in this dying field (Berry 2004; Branchek

and Blackburn 2003; Davenport 2003; Kim and von Zastrow 2001; Premont et al. 2001). However, without an identifiable circuitry for these traces substances, there was little real physiological experimentation that can be accomplished. Perhaps this has prevented the predicted resurgence of studies on TAs (Berry 2004; Branchek and Blackburn 2003; Davenport 2003; Kim and von Zastrow 2001; Premont et al. 2001), leaving their physiological role as speculative (Berry 2004).

The research presented here was one step towards establishing the TAs as bona fide endogenous neuromodulators with their own actions. These studies improve our understanding of the TAs by establishing a spinal cord substrate for TA to have intrinsic biological actions on the spinal motor circuitry.

The TAs appear to be the first irrefutable amine neuromodulatory system intrinsic to the mammalian spinal cord. Since the TAs can modulate sensory (Bowman et al. 1964; Reddy et al. 1980) and motor systems, control of their release and/or receptor activation may provide new therapeutic strategies for the management of spinal cord dysfunction, including after loss of descending monoaminergic systems as occur after spinal cord injury.

6.5 Future studies

In order to further establish the how the TAs are functioning in the spinal cord, future studies are necessary.

(i) To demonstrate that endogenous production of the TAs can produce spinal cord actions, in preliminary work I have shown that blocking TA degradation with MAO inhibitors caused rhythmic motor activity similar to endogenous application of the TAs (see **Appendix B**).

(ii) To further establish the requirement of transporters in spinal neurons in spinal motor function transport inhibitors for the LATs, OCTs, and PMAT should be used. Recently, transport inhibitors were tested for PMAT, and sertraline was found to be the most potent (Haenisch and Bonisch 2010).

(iii) To demonstrate that dietary alterations in aromatic amino acids have functional consequences on motor circuits, in preliminary work I observed that applications of the aromatic amino acids could increase motor activity and that phenylalanine could activate rhythmical activity (not shown).

(iv) To investigate behavioral relevance of TAs on spinal motor behavior in adult rats to more closely approximate spinal cord injuries in adults, injections of the TAs into spinalized adult rats *in vivo* should be investigated to determine if they too have rhythmic locomotor hindlimb movements that could suggest clinical application.

(v) Interestingly, I found that dopamine was similar to the TAs both by having a unique pattern as well as its longer time for burst initiation. A new study in PLoS one just came out showing that A11 is likely L-dopa-ergic (Barraud et al. 2010), which opens the possibility that L-dopa is released from A11and transported into other neurons to make dopamine intracellularly.

(vi) Unfortunately, TAAR antagonists are currently unavailable; however, they should be tested when they do become available.

(vii) Also, future studies should address the role of the TAs during development. With only AADC activity and no TH activity early in development, the TAs could be playing an important role in development (Teitelman et al. 1983).

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APPENDIX A

ALLEN SPINAL CORD ATLAS

The Allen Spinal Cord Atlas (http://mousespinal.brain-map.org) provides a comprehensive searchable RNA *in situ* hybridization database of gene expression of the mouse spinal cord at postnatal days 4 and 56 (Allen_Spinal_Cord_Atlas 2009). Spinal cord *in situ* hybridization expression patterns obtained from the Allen Spinal Cord Atlas of the synthesis enzymes, degradative enzymes, trace amine-associated receptors, and transporters associated with the monoamine and putative trace amine neuromodulators are in the following figures. Expression for each gene is marked as absent or present based on the annotation index designation. However, on several occasions, overt reaction product were observed in some neurons even though tagged as absent.

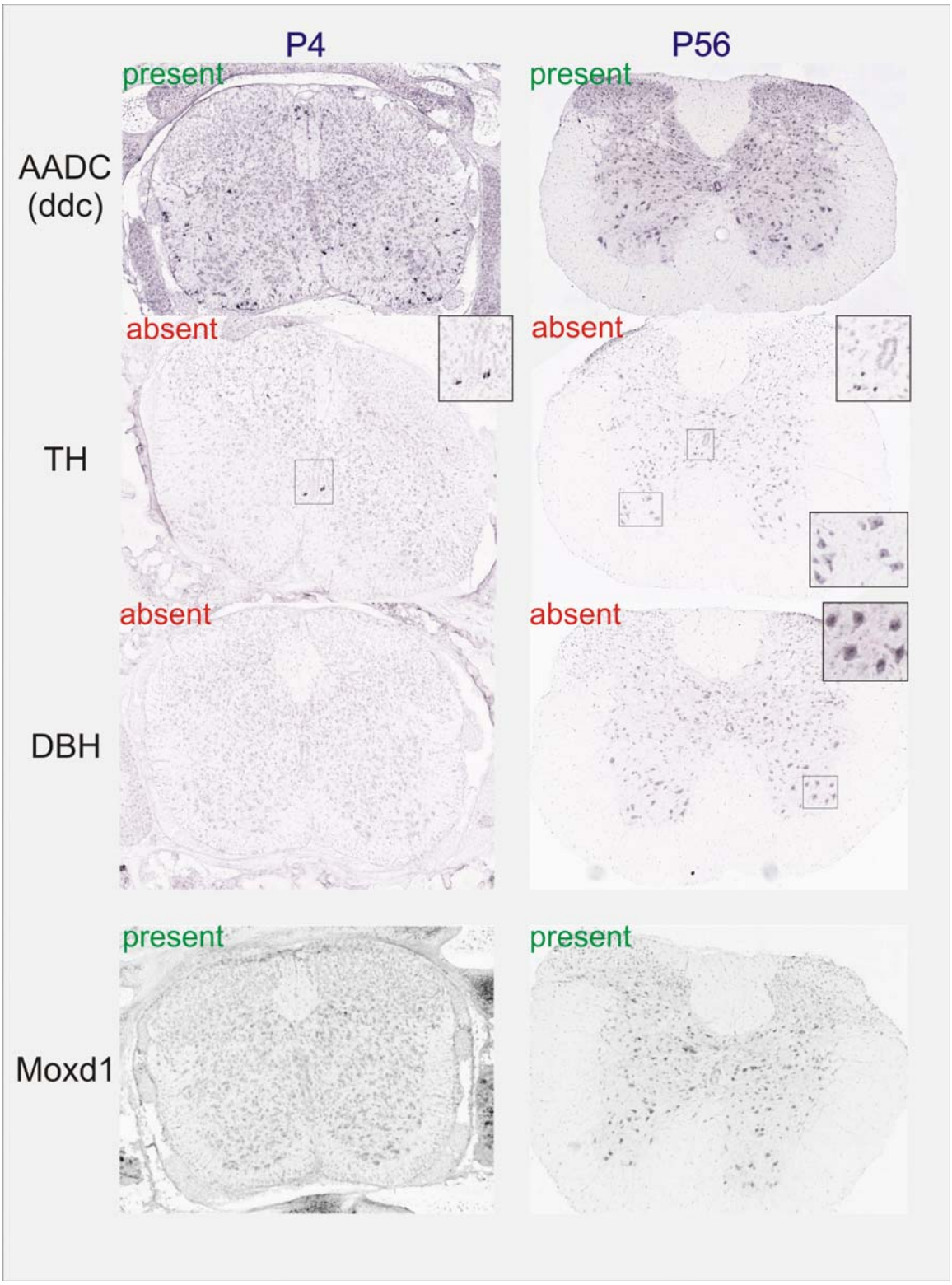

Figure A.1: Allen Spinal Cord Atlas *in situ* hybridization of synthesis enzymes
AADC, aromatic amino acid decarboxylase (also called dopa decarboxlase); TH, tyrosine hydroxylase; DBH, dopamine b-hydroxylase; Moxd1, monooxygenase, DBH-like 1.

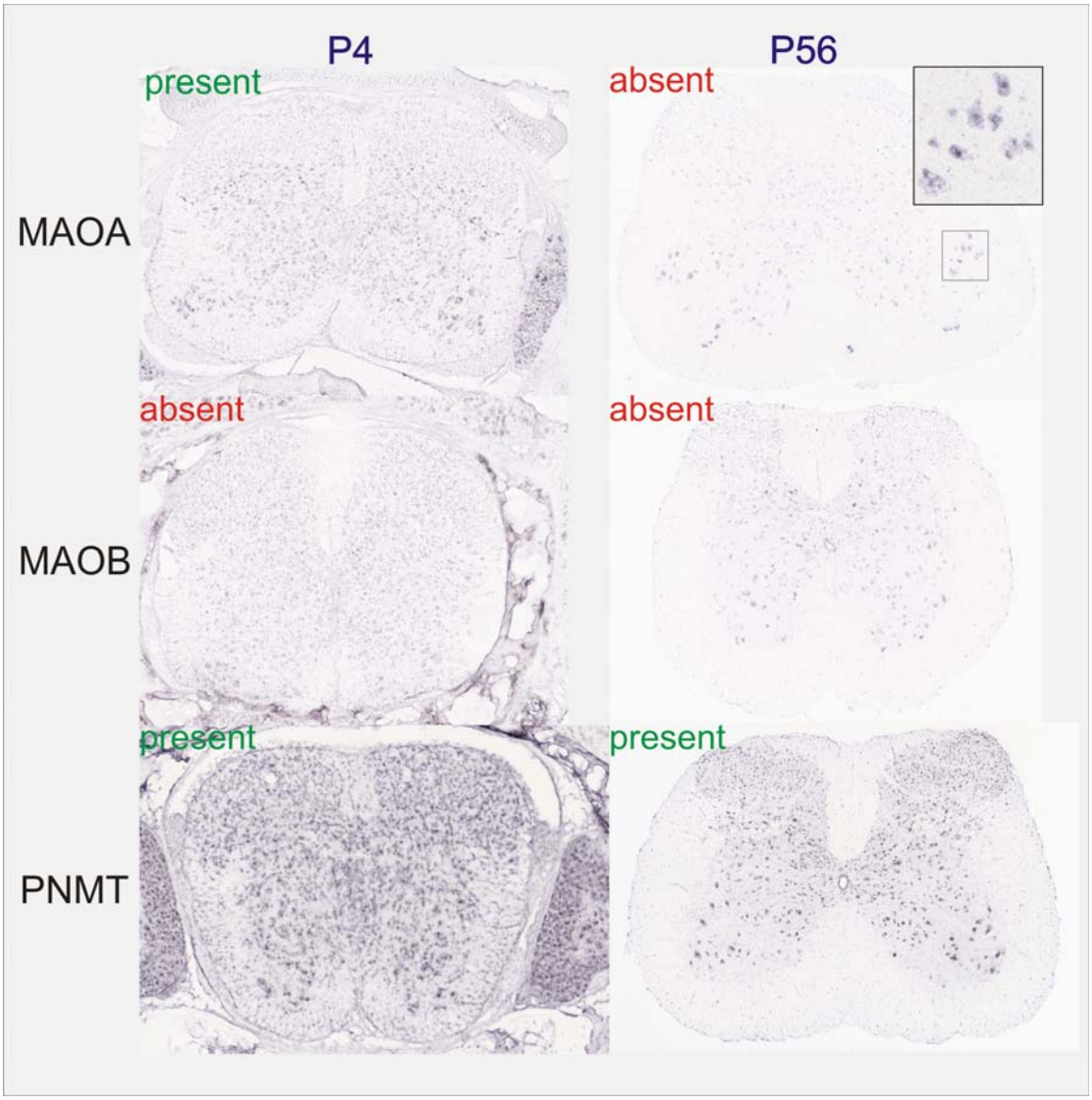

Figure A.2: Allen Spinal Cord Atlas *in situ* hybridization of degradation enzymes
MAOA, monoamine oxidase A; MAOB, monoamine oxidase B; PNMT, phenylethanolamine n-methyl transferase.

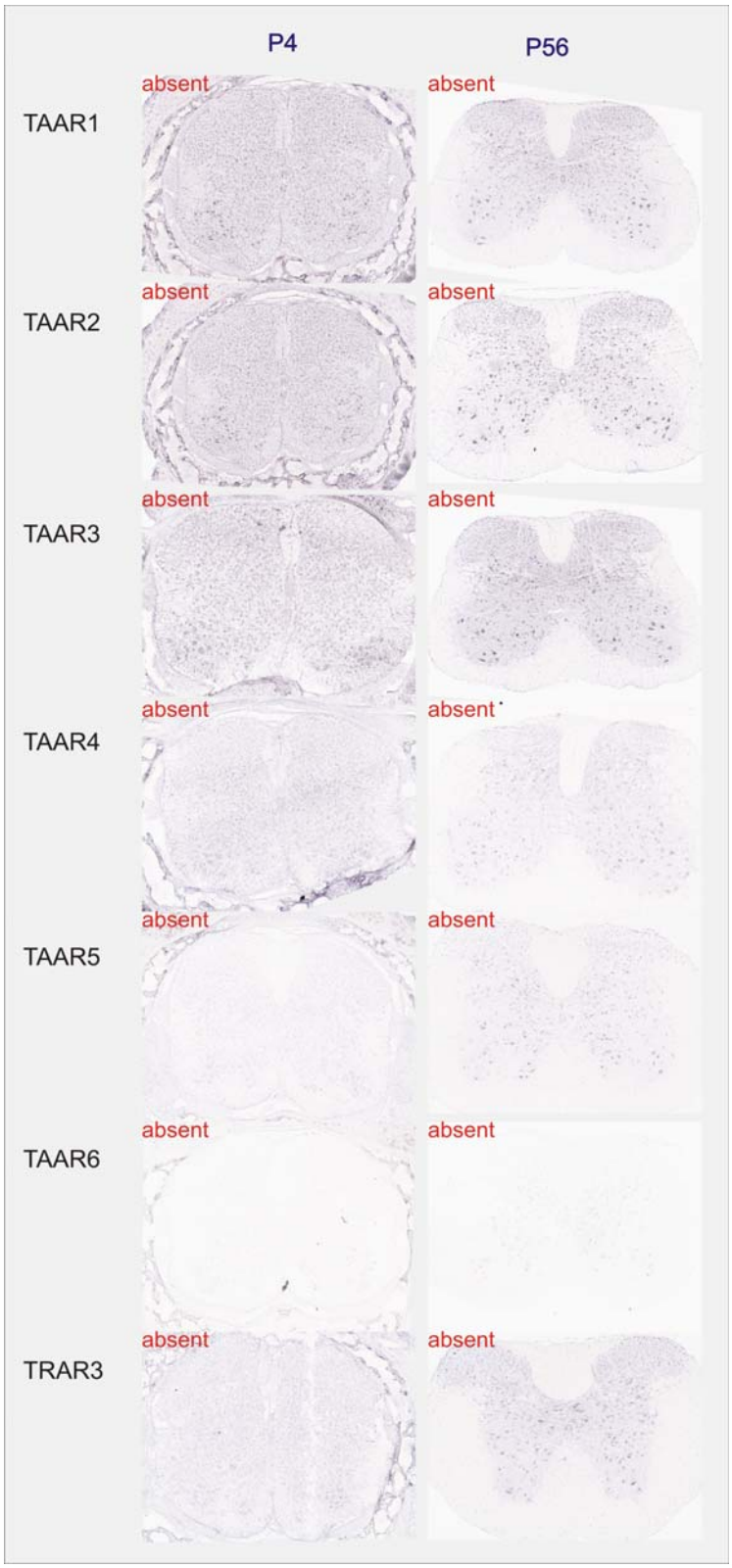

Figure A.3: Allen Spinal Cord Atlas *in situ* hybridization of trace amine-associated receptors and trace amine receoptor 3

TAAR, Trace amine associated receptor

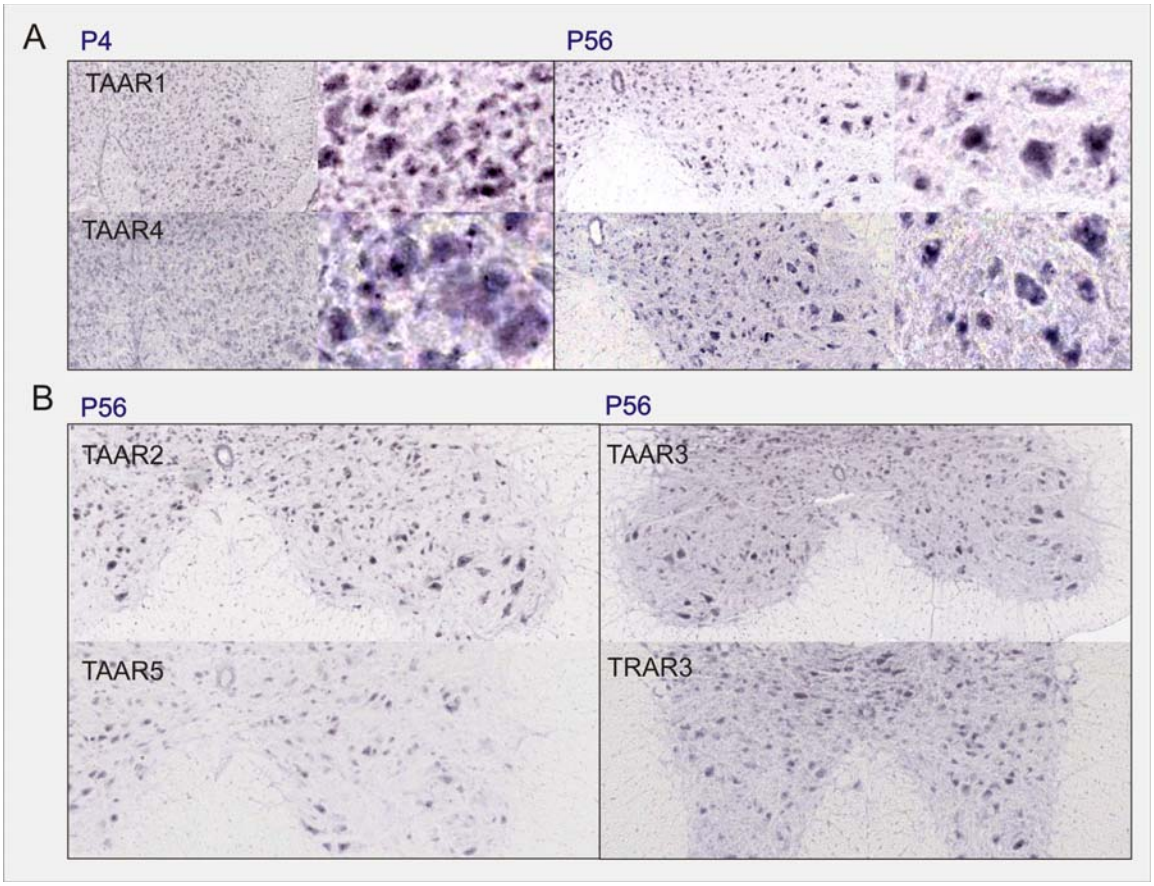

Figure A.4: Higher magnification of select TAARs

A. Higher magnification and contrast enhanced images of TAAR1 and TAAR4 show clear reaction product in motoneurons at both P4 and P56. **B.** In addition, weak labeling for TAAR2, TAAR3, TAAR5, and TRAR3 may be present in the adult ventral horn.

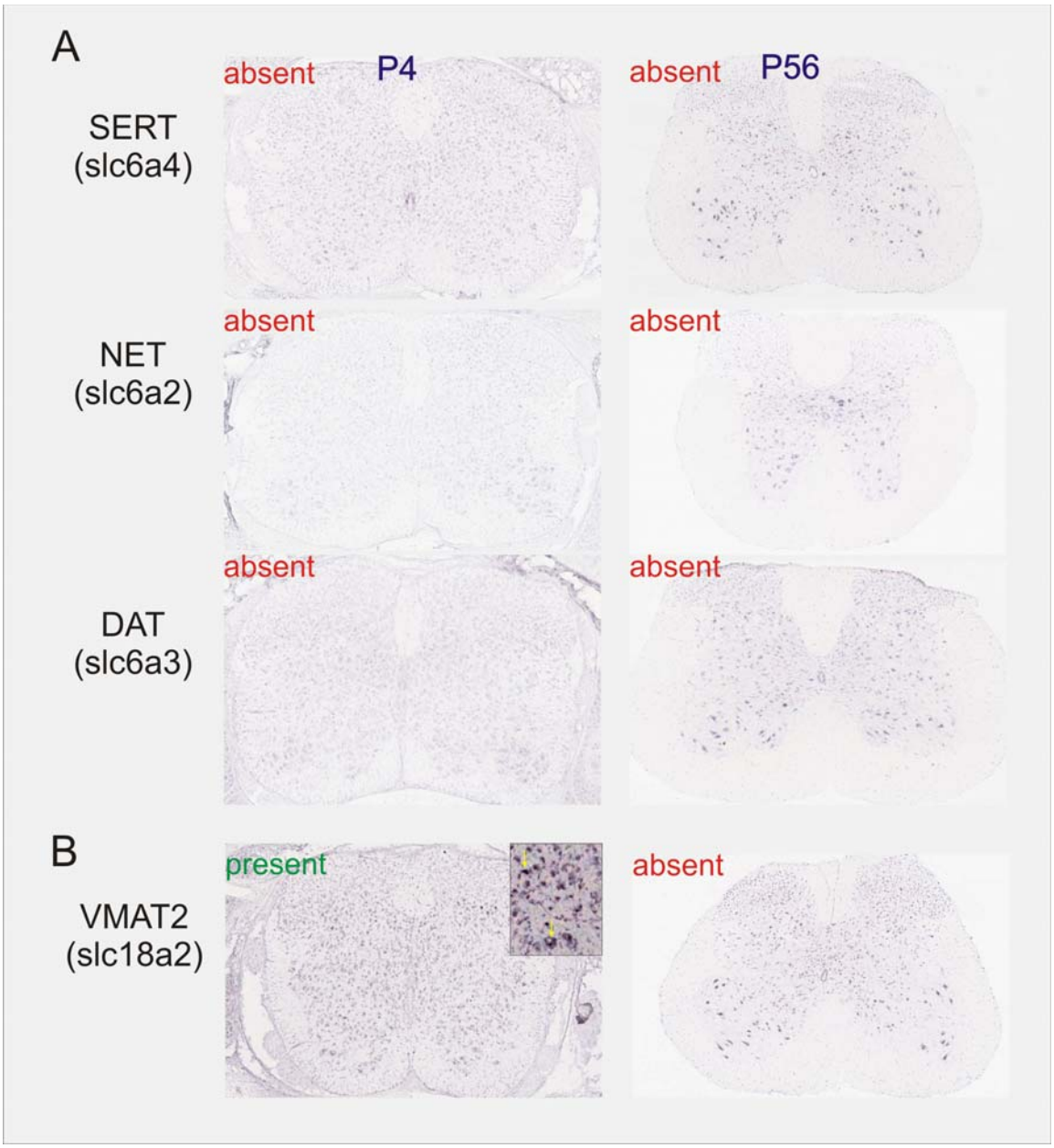

Figure A.5: Allen Spinal Cord Atlas *in situ* hybridization of monoamine and vesicular transporters
A. Expression results for the high affinity monoamine transporters SERT, NET and DAT. B. The vesicular monoamine transporter 2 (VMAT2) is expressed in P4 spinal cord but reported as absent in the adult. Labeling at P4 Higher magnification and contrast enhanced image of VMAT2 in the neonate shows labeling in motoneurons and other spinal neurons (arrows).

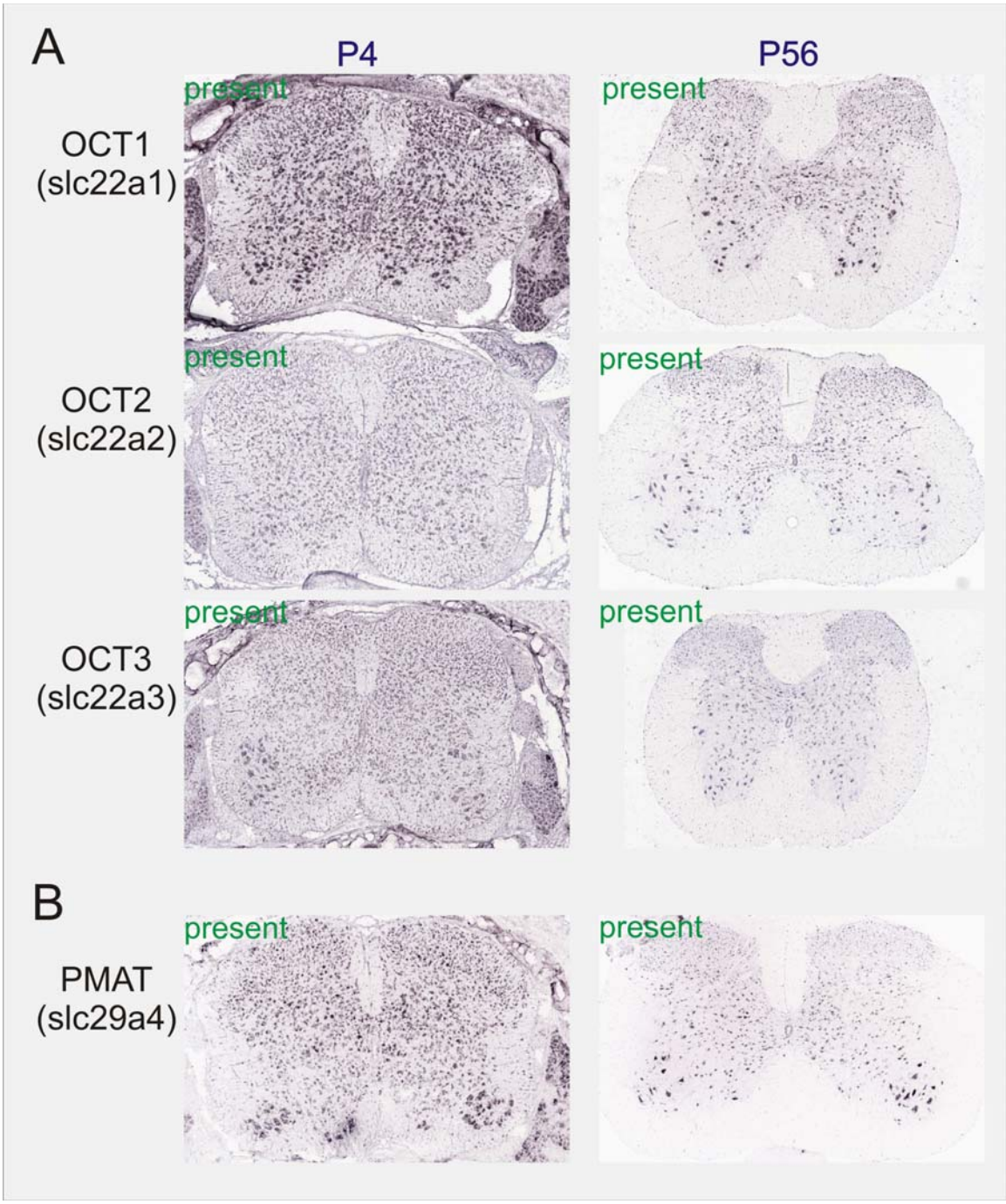

Figure A.6: Allen Spinal Cord Atlas *in situ* hybridization of organic cation transporters and the plasma membrane monoamine transporter

A. The organic cation transporters, OCT1-3, are widely expressed in spinal neurons in both neonate and adult mouse spinal cord. **B.** The low affinity plasma membrane monoamine transporter is preferentially expressed in intermediate gray and ventral horn regions in both neonate and adult mouse spinal cord implicating a predominant role in motor function.

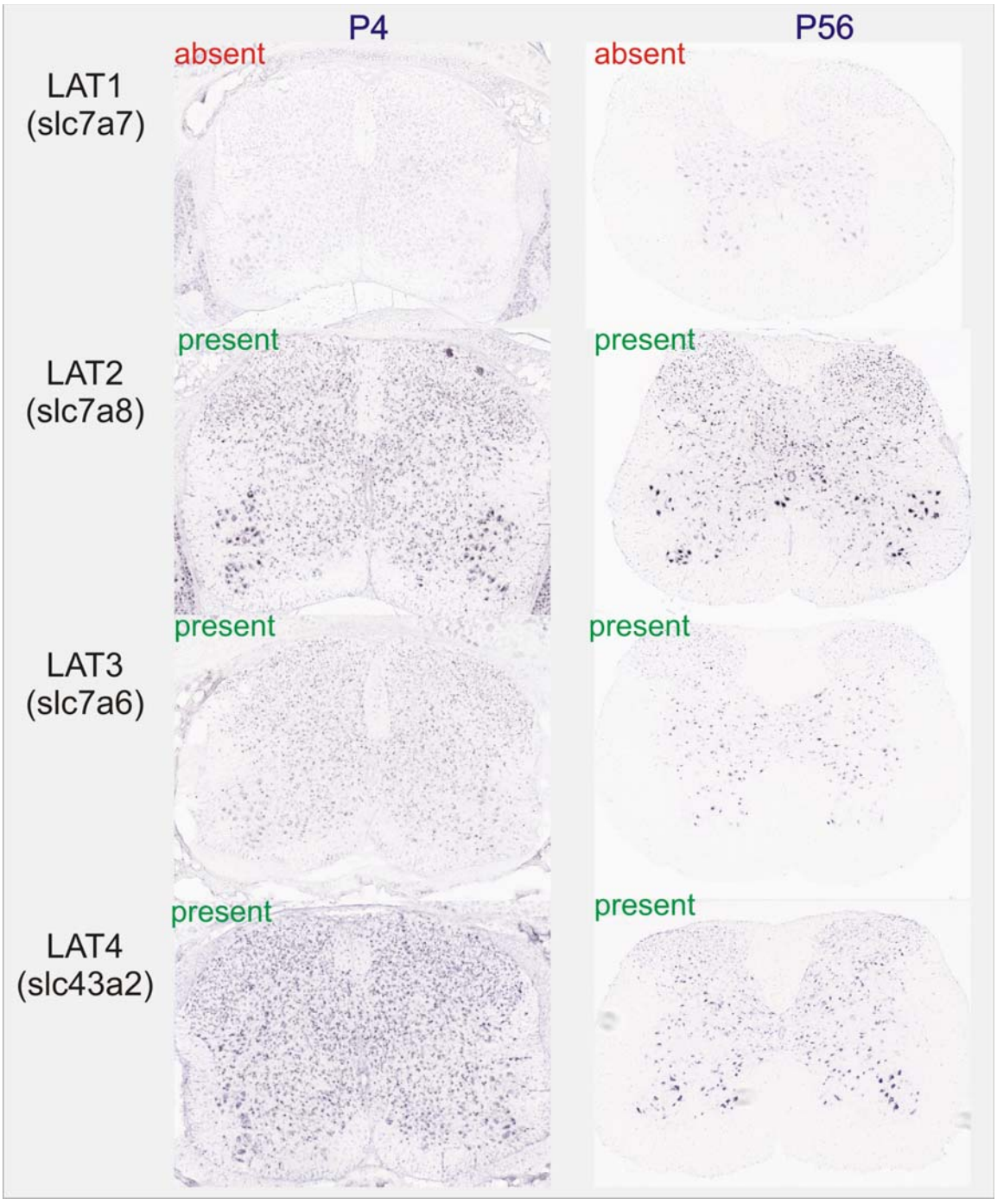

Figure A.7: Allen Spinal Cord Atlas *in situ* hybridization of L-amino acid transporters
L-amino acid transporters (LATs) 2-4 are widely expressed in the mouse spinal cord.

APPENDIX B

EVIDENCE FOR TRACE AMINE PRODUCTION IN THE SPINAL CORD

B.1 Introduction

TAs are synthesized from aromatic amino acids via aromatic amino acid decarboxylase (AADC) and further by dopamine-b-hydroxyase (DBH) for octopamine. A defining feature that makes TAs 'trace' amines is their lack of sequestration in synaptic vesicles and consequent rapid degradation by monoamine oxidases (MAOs), their presumed primary mechanism for termination. Two different types of MAO, named A and B, have been characterized. Both MAOA and MAOB are found in the spinal cord, with MAOA found in motoneurons (Luque et al. 1995; Saura et al. 1992). MAOB has a high affinity for PEA, while the other TAs are degraded by both (Bortolato et al. 2008; Shih et al. 1999)

The MAOs do not have short-term effects on the release of the classical monoamine transmitters (Berry 2004; Cragg et al. 2000; Houdouin et al. 1990) and therefore, can be used to study the faster effects of increased intracellular TAs on neural function.

B.2 Methods

All experimental procedures complied with the NIH guidelines for animal care and the Emory Institutional Animal Care and Use Committee.

B.2.1 Immunohistochemistry

The spinal cord was isolated from rats who had been intraperitoneally injected with Fluorogold 24 hours prior to sacrifice to retrogradely label most spinal motoneurons (Ambalavanar and Morris 1989) (Merchenthaler 1991).

Isolated spinal cords were incubated in one of four different treatments for 2 hours: (1) control, (2) in the presence of aromatic amino acid precursors phenylalanine, tyrosine, and tryptophan (all at 100 μM), (3) the TAs (all at 100 μM), and (4) the TAs plus MAOA and MAOB inhibitors clorgyline and deprenyl, respectively (both at 100 μM). Subsequent tyramine immunolabeling and densitometry was performed on lateral MNs to measure changes in expression.

The spinal cords were then post-fixed for 1 hour in Lana's fixative than cryoprotected in 10% sucrose, 0.1M PO_3 until sectioned into 10 um thick sections on a cryostat and processed for immunohistochemistry. All incubations and washes were performed in 0.1M PO_3-buffered saline containing 0.3% triton X-100 (PBS-T). Tissue was washed overnight in PBS-T at 4°C followed by incubation in primary antibody for 48-72 hours. Slides were then washed three times for 30 minutes and incubated in secondary antibody. The primary antibody was following Rabbit anti-tyramine 1:1000 (Chemicon) and the secondary antibody was cy3 anti-rabbit (Jackson Immunoresearch).

Densitometry was performed on lateral MNs using the Neurolucida Software at a scale of 1700x magnification. Omission and absorption controls were blank.

B.2.2 Electrophysiology

B.2.2.1 General setup

Sprague-Dawley rats postnatal (P) day 0-5 were decapitated, eviscerated, and placed in a bath containing oxygenated (95% O_2, 5% CO_2) artificial cerebral spinal fluid (aCSF)

containing the following (in mM): 128 NaCl, 1.9 KCl, 1.2 KH_2P0_4, 26 $NaHCO_3$, 2.4 $CaCl_2$, 1.3 $MgSO_4$, and 10 glucose (pH of 7.4). The spinal cord was exposed by a ventral vertebrectomy and carefully dissected out of the body cavity leaving the dorsal and ventral roots attached. The spinal cord was secured with insect pins to a chamber with Sylgard (Dow) on the bottom. Glass suction electrodes were applied to dorsal and/or ventral roots, after which the preparation was allowed to recover for at least 1 hour before experimentation at room temperature. The ventral root electroneurographic activity was amplified (10,000x), band-pass filtered at 10-3,000 Hz and digitized at 5kHz (Digidata 1321A, 16-bit; Axon Instruments). Band-pass filter frequency settings were selected with consideration to observed frequency components with the low-pass filter set ant mush greater than the Nyqist frequency. Data was captured on a computer with the pCLAMP acquisition software (v8-9, Molecular Devices; Union City, CA).

B.2.2.2 Neurochemicals

Neurochemicals, which were stored in 10mM or 100mM stock solutions at -20°C, were added to the bath (typically 30mL) to achieve the final concentration in the chamber. Neurochemicals were obtained from Sigma-Aldrich (St. Louis, MO), including NMDA (5 μM) and tyramine (10μM).

The following monoamine oxidase inihibitors were used: nialimide (non-specific MAOA and MAOB inhibitor; usually at 10 mM), clorgyline (MAOA inhibitor; up to dose of 100 μM), and deprenyl (MAOB inhibitor; up to a dose of 100 μM).

The aromatic amino acids were used: phenylalanine (100 μM), tyrosine (100 μM), and tryptophan (100 μM).

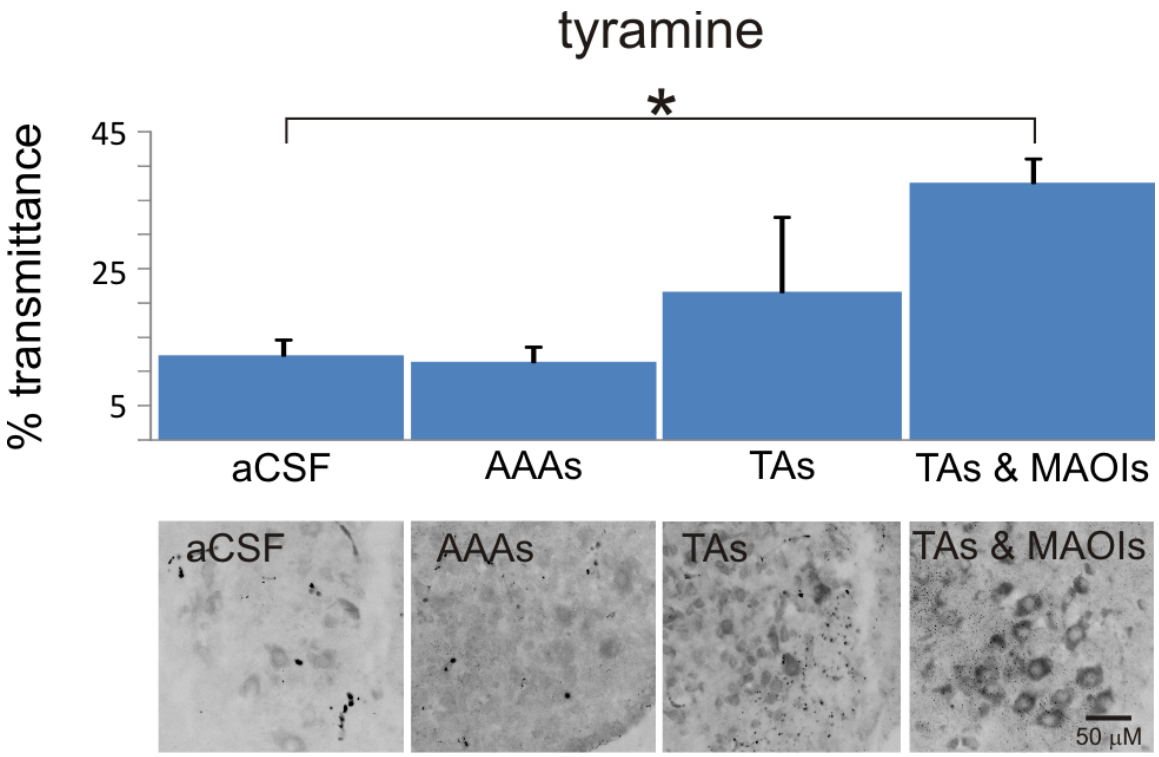

Figure B.1: MAO inhibitors can increase endogenous trace amines
Tyramine expression was increased in motoneurons following pre-incubation in the presence of MAO inhibitors. Changes were quantified using densitometry in lateral motoneuronal tyramine labeling after incubations in control, aromatic amino acids, the TAs, and the TAs plus the MAOs inhibitors clorgyline and deprenyl. (* indicates $p<0.01$).

B.3 Results

B.3.1 Monoamine oxidase inhibitors can cause an accumulation of tyramine

To examine the effects of metabolism on tyramine expression, spinal cords were incubated in control aCSF, aromatic amino acids, TAs, and TAs plus MAO inhibitors (**Appendix Figure B.1**) with subsequent tyramine immunolabeling and densitometry to measure changes in expression. Tyramine expression did not increase in the presence of aromatic amino acids suggesting that their concentration was not rate limiting (n=2). Tyramine increased, albeit not significantly, after incubation in the TAs alone (n=2). TAs and MAO inhibitors led to a significant increase in tyramine immunolabeling (n=2; $p<0.01$), indicating that tyramine was accumulating in motoneurons since its degradation was being blocked.

B.3.2 Actions of monoamine oxidase inhibitors support an endogenous role for the trace amines

Since MAO inhibitors can cause tyramine to accumulate in motoneurons and, importantly, they do not have short-term effects on the release of the classical monoamine transmitters (Berry 2004; Cragg et al. 2000; Houdouin et al. 1990), the MAO inhibitors can be used to study the faster effects of increased intracellular TAs on neural function. Deprenyl, clorgyline, and nialamide were tested since they are used clinically, so any observed actions on spinal circuits may have clinical relevance.

When applied alone to the bath, the MAO inhibitors were able to produce slow burst of activity with nialamine (n=1/4) and sudden transient increase in spontaneous activity lasting several minutes with clorgyline and deprenyl (n=3/5). These observations suggest

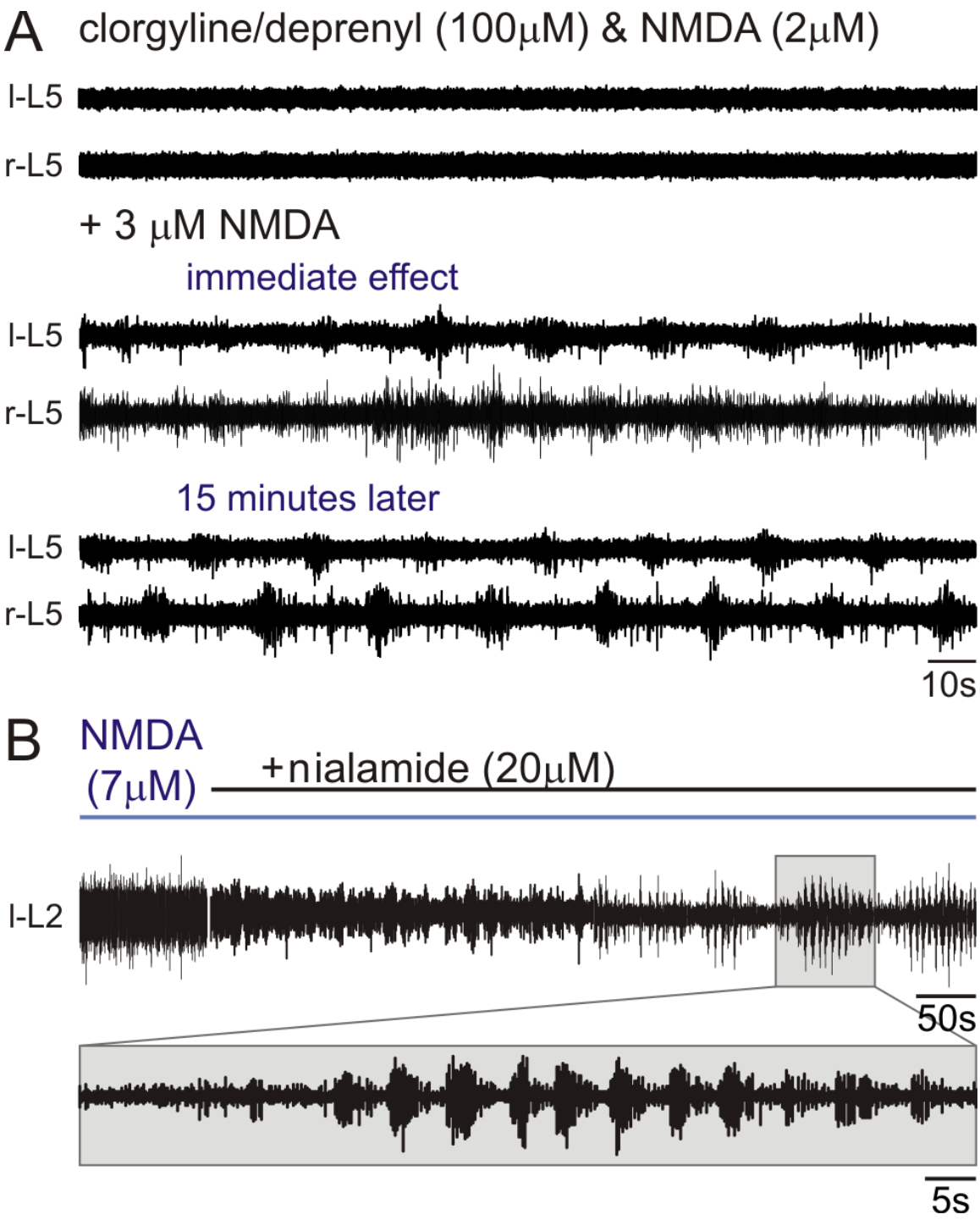

Figure B.2: MAO inhibitors can increase neuroactivity

A. The bath was incubated in 100 μM clorgyline, 100 μM deprenyl, and 2 μM NMDA. Subsequent addition of 2 μM NMDA resulted in the expression of long-lasting L5 left-right alternating ventral root activity. The L2 ventral roots remained silent, suggesting selective recruitment of the extensor half-center. **B.** The MAOA and MAOB inhibitor, nialamide, converted NMDA-evoked spontaneous ventral root activity into episodic LLA. Only the l-L2 channel is shown due to poor recording quality of the other channels.

that amplifying endogenous concentrations of TAs promote motor activity but are insufficient to recruit locomotion.

In the presence of NMDA at doses that did not evoke consistent activity, clorgyline and deprenyl produced stable left/right L5 alternation that lasted for the 25 minute duration of the recording (0.1 Hz; n=1/1; **Appendix Figure B.2A**). Similarly, nialamide recruited rhythmical motor activity (n=2/4): one displaying right/left L2 flexor alternation (0.075 Hz) that lasted over 1 minute and the other episodic LLA lasting over 15 minutes at 0.21 Hz (**Appendiz Figure B.2B**). Intriguingly, in the two animals where nialamide with NMDA did not produce rhythmicity, application of the aromatic amino acid precursors (tyrosine, tryptophan, and phenylalanine) led to pronounced increases in motor activity; in one, clear busting events lasted for the 25 minute duration of the recording. This did not happen in the absence of nialamide implying that increased levels of aromatic amino acids are capable of synthesizing TAs via AADC, which in the absence of degradation, can elevate TAs levels to exert a biological action on the motor system.

B.4 Discussion

B.4.1 MAO inhibitors increase endogenous trace amines and can increase neuroactivity

As MAOA is distributed throughout the P4 mouse spinal cord (**Appendix Figure A.2**), and AADC also has widespread expression, the application of MAO inhibitors should increase TA levels. Indeed, the incubation experiments show that MAO inhibitors do increase the levels of TAs. Also, application MAO inhibitors alone were able to increase activity. That they do not always increase activity, could be due to other factors

in the spinal cord, including not enough aromatic amino acids available to make more or not enough AADC activity to convert the aromatic amino acids to TAs.

Further, the MAO inhibitors were capable of recruiting rhythmic motor activity in the presence of NMDA including the wax and wane episodic activity so characteristic of tyramine, octopamine, and PEA bath application. To support the idea that the state of the spinal cord matters, adding aromatic amino acids to the bath after MAO inhibitors where unable to produce rhythmic activity did produce bursting in one cord. Together these actions strongly support endogenous production of the TAs in the spinal cord, and that at high enough local concentrations they are able to affect motor function.

Interestingly, while both MAOA and MAOB are found in the in spinal cord using immunohistochemistry and radioligands to look at expression of the enzymes (Luque et al. 1995; Saura et al. 1992), examining the *in situ* hybridization in the Allen Spinal Cord Database shows that only MAOA, and not MAOB, is expressed in the spinal cord, meaning MAOB is likely found in the descending monoaminergic terminals (**Appendix Figure A.2**) **(Allen_Spinal_Cord_Atlas 2009)**. This has implications regarding especially the local concentration of PEA, which is preferentially degraded by MAOB (Bortolato et al. 2008; Shih et al. 1999). If PEA is produced in, say motoneurons, where there is no MAOB, it must be transported out of the neuron and into another neuron where it can be degraded, possibly implying a higher concentration in the cell that typically assumed.

APPENDIX C

SPINALMOD ALGORITHM FOR BURST DETECTION

```
function [begmarks,endmarks] = BurstDetection(filtered, BurstThresh, ...
   MinBurstDur, MinPeriod, BurstStart, MinInterBurst,EndMin, sampfreq)


%These formulas convert the time inputed by the user into sample points
%more easily used by the computer.
if BurstStart==0
   BurstStart=1; %Prevents the program from stopping b/c it can't handle 0
else
   BurstStart=BurstStart*sampfreq;%Start time of the 1st burst
end
if MinPeriod==0
   MinPeriod=1;
else
   MinPeriod=MinPeriod*sampfreq;%Minimum period btw bursts
end
if MinBurstDur==0
   MinBurstDur=1;
else
   MinBurstDur=MinBurstDur*sampfreq;%Minimum burst duration
end
```

```
if MinInterBurst==0
   MinInterBurst=1;
else
   MinInterBurst=MinInterBurst*sampfreq;%Min. time btw bursts
end
if EndMin==0
   EndMin=1;
else
   EndMin=EndMin*sampfreq;
   %Minimum time btw fluctuations at the end of the burst
end

%Cuts off all the filtered data before the beginning of the burst,
%thus reducing the number of false positives for the start of the burst
cutfiltered=filtered(BurstStart:end);
%Checks whether the filtered data is above the Burst Threshold and
%returns the indicies of the filtered data that are above threshold
y=[find(cutfiltered>BurstThresh)];%Indicies of points above threshold
%Creates arrays of the filtered data to be compared with each other to
%find the points that cross threshold and indicate the start or end of a
%burst
abovethresh=cutfiltered(y);%All values that are above the threshold
```

```
%The following will test the to see if the points before and after the
%indicies above threshold exist
q=find(cutfiltered);
if q(1)==y(1)
   %Filltered data one point before the threshold points
   testbeg=[cutfiltered(y(1)) cutfiltered(y(2:end)-1)];
   %Filtered data one point after the threshold points
else
   testbeg=cutfiltered(y-1);
end
if q(end)==y(end);
   testend=[cutfiltered(y(1:end-1)+1) cutfiltered(y(end))];
else
   testend=cutfiltered(y+1);
end
%ii=1;iii=1; I=1;Burst=false;endmarks=[];%Used for initialization


%The following finds the bursts.
%For the onset, it checks all points above threshold with the point
%immediately before it.  If the point before is below threshold, it is
%marks an onset, and then, it starts to look for the end of the burst.  It
%checks all points above threshold with the point immediately after it.  If
%the point after is below threshold, it marks a potential end.  It then
```

```
%takes a number of end points to see if the difference btw them is greater
%than a user inputted EndMin, the minimim distance btw the burst and the
%next point to cross the threshold, which hopefully is hte next burst.
%MinInterBurst is the minimum distance btw the end of the burst and the
%beginning of the next.  If so, it marks the first point as end. Then, it
%goes back to find the next onset making sure the value is greater than the
%user inputted MinPeriod, the minimum distance btw bursts. To do this, it
%goes back a number of points so that it does not miss the first point.
if BurstStart==1
   BurstStart=0;
else
   BurstStart=BurstStart-1;
end


begmarks=[];
endmarks=[];
ii=1;iii=1; I=1;Burst=false;endmarks=[];a=[];
for i=1:length(y)
   %The following code looks for the beginning of a burst
   if (Burst==false) && (abovethresh(i)>testbeg(i)) &&...
         (testbeg(i)<BurstThresh) && (ii==1 ) %this looks for the first burst's
beginning
      begmarks(ii)=y(i)+BurstStart;
```

```
    ii=ii+1;
    Burst=true;%true indicates that the beginning of a burst has been marked
elseif (Burst==false)&&(abovethresh(i)>testbeg(i))&&...
        (testbeg(i)<BurstThresh)&&...
        (y(i)-(begmarks(ii-1)-BurstStart) > MinPeriod) &&...
        (y(i)-(endmarks(iii-1)-BurstStart) > MinInterBurst)
    begmarks(ii)=y(i)+BurstStart;
    ii=ii+1;
    Burst=true;
end

%The following code looks for the end of a burst
if (Burst==true) && (abovethresh(i)>testend(i)) &&...
        (testend(i)<BurstThresh) &&...
        ((y(i)-(begmarks(ii-1)-BurstStart)) > MinBurstDur)
    em(I)=y(i)+BurstStart;
    if I>1 && ((em(I)-em(I-1))>=EndMin)
        endmarks(iii)=em(I-1);
        a=find(y==(endmarks(iii)-BurstStart));
        iii=iii+1;
        Burst=false;%false indicates that the end of a burst has been marked
        I=1;
    else
```

```
        I=I+1;
    end
end

%The following code goes back and check the point for the beginning of
%a burst
if isempty(a)==0
    for b=a:i
        if (Burst==false)&&(abovethresh(b)>testbeg(b))&&...
                (testbeg(b)<BurstThresh)&&...
                ((y(b)-(begmarks(ii-1)-BurstStart)) > MinPeriod) &&...
                ((y(b)-(endmarks(iii-1)-BurstStart)) > MinInterBurst)
            begmarks(ii)=y(b)+BurstStart;
            ii=ii+1;
            Burst=true;
        end
    end
    a=[];
end

%The following code double checks for the end of a burst
%This portion of the code was added later to fix the EMG problem...
if (Burst==true) && (abovethresh(i)>testend(i)) &&...
```

```
        (testend(i)<BurstThresh) &&...
        ((y(i)-(begmarks(ii-1)-BurstStart)) > MinBurstDur)
      em(I)=y(i)+BurstStart;
      if I>1 && ((em(I)-em(I-1))>=EndMin)
        endmarks(iii)=em(I-1);
        iii=iii+1;
        Burst=false;%false indicates that the end of a burst has been marked
        I=1;
      else
        I=I+1;
      end
    end
end
```

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