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Neurotransmitter Plasticity
Guest editor: Davide Dulcis

Journal of CHEMICAL NEUROANATOMY

Aims and Scope

Journal of Chemical Neuroanatomy is a vehicle for the publication of scientific reports relating the functional and biochemical aspects of the nervous system with its microanatomical organization. The scope of the journal concentrates on reports which combine microanatomical, biochemical, pharmacological and behavioural approaches.

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In the cover The confocal images show dopaminergic neurons (green) in the adult rat hypothalamus labeled for other neurotransmitters (in red) such as, vasopressin (top left), neuropeptide Y (top right), and somatostatin (bottom panels). Hypothalamic dopaminergic neurons display neurotransmitter plasticity in response to altered photoperiod and contribute to stress responses. *Courtesy of Davide Dulcis.*

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Special issue

Neurotransmitter Plasticity

Guest Editor

Davide Dulcis

Introduction

The ever-changing brain: Clinical implications

Perception of the external world and behavioral responses, complex feelings associated with emotions and memories, and even human intelligence all depend on the appropriate synthesis and release of specific neurotransmitters in the brain. Since the discovery of chemical synaptic transmission it has been thought that transmitters are fixed and invariant throughout life. At the beginning of the nineteenth century, Henry Dale discovered that each neuron secretes one transmitter at all its terminals and formulated, for the first time, the concept of chemical identity of each neuron. This concept was widely interpreted to mean that, according to Dale's Principle, each type of neuron only released a single transmitter. Despite this dogma, scientists have found in the past few decades clear evidence of multiple transmitters synthesized and released by the same neuron. This special issue dedicated to activity-dependent neurotransmitter plasticity reviews some of the most recent studies in the field showing how the embryonic nervous system is assembled and the adult brain is modified following both genetic and experience-dependent environmental instructions. Experiences such as learning and memory can in fact radically change the brain wiring or neuronal function. In this issue, the authors will discuss studies that have provided a new look at how neuronal wiring is assembled in the developing brain and the impact the environment has on continually shaping the brain later in life. The pathophysiological processes associated with neurological disorders and aging could turn out to be "unwanted" aspects of such ongoing brain plasticity.

Recent studies demonstrated that changes in electrical activity rapidly and reversibly reconfigure the transmitters and corresponding postsynaptic receptors that neurons express. Induction of *de novo* transmitter expression can be achieved by selective activation of afferents recruited by a physiological range of sensory input or via transcranial magnetic stimulation (TMS) and/or optogenetics. Strikingly, neurons acquiring an additional transmitter project to appropriate targets prior to transmitter re-specification in some cases, indicating the presence of reserve pools of neurons that can boost circuit function. The potential clinical value of circuit-specific neurotransmitter plasticity for treatments of neurological disorders in the nervous system is gradually becoming apparent in the field. The ability to manipulate this novel form of neuroplasticity could provide the basis for novel non-invasive treatment of disorders of transmitter and receptor metabolism and neurodegenerative diseases in the developing, adult, and aging brain.

The discovery that changes in electrical activity dynamically regulate neurotransmitter expression and release both in the developing and mature brain is the first step toward the

understanding of this form of neuroplasticity. Neuronal networks are identifiable by their overall organization, neuronal composition, anatomical circuit connectivity, physiological input and output, and molecular signatures of transcription factors, ion channels and neurotransmitters, both during development and in the adult. Sufficient information has been obtained to design experiments aimed at expanding the list of neuronal circuits that would respond to activity manipulations by changing neurotransmitter expression. Restoration of neuronal circuit function by transmitter plasticity is an attractive prospect for several reasons. Manipulation of activity differs from other treatments in several significant regards. The first is that both application and onset of detectable effects can be achieved in a relatively short period of time. A second appealing aspect of neurotransmitter plasticity is the prospect for non-invasive treatments for neurological disorders. Finally, it is becoming clear that selective circuit-activation can induce striking results in restoring function to diseased circuits. Optogenetics has been used to achieve optical deep brain stimulation of afferent axons projecting to the subthalamic nucleus of hemiparkinsonian rats. Although this method is invasive, these experiments demonstrate that circuit-specific activation can lead to enhanced therapeutic effects in the adult mammalian brain with fewer nonspecific effects than obtained with classical deep brain stimulation (DBS).

Current clinical treatments of neurotransmitter-related disorders include: pharmacology to restore appropriate levels of neurotransmitter or modulate neurotransmitter receptor activation, embryonic stem cell transplantation to replace degenerated cells, surgical removal of brain nuclei to reduce excitation in epilepsy, electroconvulsive therapies, DBS, and TMS. Activity-dependent neurotransmitter plasticity induced by selective activation of brain circuits might provide spatial refinement of current clinical treatments. Integration of neurons with the correct identity in damaged circuits via induction of neurotransmitter plasticity within identified circuits could increase the precision and effectiveness of future clinical approaches. In the postembryonic nervous system, where neurogenesis is restricted to a few regions in the brain and the extracellular environment no longer provides appropriate cues to guide axonal pathfinding of ectopically and surgically implanted embryonic stem cells, the activity-mediated recruitment of reserve pool neurons to neurotransmitter plasticity opens new hope for a functional rescue of damaged neural circuits.

Activity-dependent neurotransmitter plasticity in adult and aging neuronal circuits that share the same target with the diseased circuits would have a clinical impact on patients with

neurodegenerative disorders such as Parkinson's and Alzheimer's as well as in subjects with specific functional impairments due to neural tissue damage caused by stroke or aging. Similarly, neurotransmitter plasticity could be induced to compensate for altered function by reestablishing the balance of excitation and inhibition in affected brain regions. This could be particularly relevant in subjects affected by schizophrenia, depression and other mood disorders, developmental disorders, and neurological conditions due to substance use and addiction.

Neurotransmitter plasticity may provide a mechanistic understanding of effectiveness of some of the current treatments involving activity manipulation. Future research will reveal the molecular mechanisms and the genes involved in these novel

forms of activity-dependent neurotransmitter plasticity during development and their stability in the aging adult.

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Crosstalk among electrical activity, trophic factors and morphogenetic proteins in the regulation of neurotransmitter phenotype specification

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ABSTRACT

Morphogenetic proteins are responsible for patterning the embryonic nervous system by enabling cell proliferation that will populate all the neural structures and by specifying neural progenitors that imprint different identities in differentiating neurons. The adoption of specific neurotransmitter phenotypes is crucial for the progression of neuronal differentiation, enabling neurons to connect with each other and with target tissues. Preliminary neurotransmitter specification originates from morphogen-driven neural progenitor specification through the combinatorial expression of transcription factors according to morphogen concentration gradients, which progressively restrict the identity that born neurons adopt. However, neurotransmitter phenotype is not immutable, instead trophic factors released from target tissues and environmental stimuli change expression of neurotransmitter-synthesizing enzymes and specific vesicular transporters modifying neuronal neurotransmitter identity. Here we review studies identifying the mechanisms of catecholaminergic, GABAergic, glutamatergic, cholinergic and serotonergic early specification and of the plasticity of these neurotransmitter phenotypes during development and in the adult nervous system. The emergence of spontaneous electrical activity in developing neurons recruits morphogenetic proteins in the process of neurotransmitter phenotype plasticity, which ultimately equips the nervous system and the whole organism with adaptability for optimal performance in a changing environment.

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1. Introduction

The genesis of a neuron starts with the neural progenitor exiting the cell cycle followed by the first phases of neuronal differentiation and the specialization of the newborn neuron. For a long time it was believed that the neurotransmitter phenotype was predetermined with the specification of the neural progenitor and that this fate was sealed and unique, meaning that the neuron born from the specified progenitor will permanently express a specific and single neurotransmitter phenotype. However, many studies spanning through the last decades have challenged these dogmas, demonstrating that neurotransmitter phenotypes may be multiple for a single neuron and that the identity of these phenotypes may change developmentally and upon changes of the intrinsic and extrinsic environments through adulthood (Spitzer, 2012, 2015).

The specialization of neural progenitors consists in the combinatorial expression of a specific set of transcription factors which will control expression of target genes related to the identity of the developing neuron, including genes associated with neurotransmitter phenotype. The expression of a specific neurotransmitter identity in the differentiating neuron depends on the transcriptional regulation of the biosynthetic and release machinery necessary for implementing the specific transmission in the chemical synapse. However, progenitor cells and developing neurons are sensitive to a myriad of signaling mechanisms that are spatiotemporally dynamic and may add to the genetic program triggered in progenitors, intercept it or even switch it.

Here we review studies in diverse species ranging from zebrafish and *Xenopus* to mice and rats that identify the mechanisms of neurotransmitter specification through neural progenitor specialization and neuronal differentiation with particular emphasis on the findings that demonstrate that acquisition of neurotransmitter identity is plastic and subjected to dynamic changes. We focused on classical neurotransmitters: acetylcholine, biogenic amines and the amino acid transmitters.

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The review is centered on the role of morphogenetic proteins and trophic factors in the specification of neurotransmitter identity and their interaction with electrical activity when mediating the changes in neurotransmitter phenotype.

1.1. Catecholaminergic phenotype

1.1.1. Preliminary specification

Expression of the noradrenergic and dopaminergic phenotypes starts with the recruitment of specialized progenitors. The sympathetic noradrenergic neurons originate from neural crest-derived progenitors that become fate-restricted mostly by bone morphogenetic protein (BMP) signal (Howard, 2005). Transcription factors necessary for the expression of dopaminergic and noradrenergic phenotypes include Mash1, Phox2a, Phox2b, Hand2 and Gata2/Gata3 (Stanke et al., 1999; Goridis and Rohrer, 2002). Regulatory regions in genes encoding the biosynthetic enzymes for catecholamines, tyrosine hydroxylase (TH) and dopamine β -hydroxylase, contain binding sites for these transcription factors. Alternatively, some of them are upstream of those transcription factors that bind to the neurotransmitter identity target genes like neurotransmitter biosynthetic enzymes and vesicular transporters, becoming necessary for the expression of the catecholaminergic phenotype. For instance, BMP2 supports the persistent expression of Mash1 in neural progenitors from the fetal rat gut (Lo et al., 1997), and Mash1, in turn, promotes the expression of proneuronal genes, but Mash1 expression terminates after neuronal differentiation when other transcription factors take over to promote expression of noradrenergic and dopaminergic phenotypes (Lo et al., 1999, 2002; Goridis and Rohrer, 2002).

In the central nervous system, another morphogenetic protein, Wnt, specifically regulates the number of progenitors specified for the dopaminergic phenotype of diencephalic neurons, early during neural ectoderm patterning in zebrafish (Russek-Blum et al., 2008). Wnt activity restricts the number of dopaminergic neurons in the developing diencephalon by negatively regulating expression of the transcription factor Fezl (Russek-Blum et al., 2008), which in turn regulates the development of monoaminergic neurons (Levkowitz et al., 2003). In zebrafish forebrain and anterior hindbrain catecholaminergic neuron specification depends strongly on Nodal signaling and to a lesser extent on Sonic hedgehog (Shh) and fibroblast growth factor (FGF) 8 (Guo et al., 1999; Holzschuh et al., 2003). Specification of midbrain dopaminergic neurons is strongly regulated by Shh signaling through the transcriptional regulation of target genes at different stages of these neurons' development (Abeliovich and Hammond, 2007). The recruited transcription factors include the canonical Shh pathway effectors Gli, which are required for neural progenitor proliferation (Zervas et al., 2004) and the expression of Phox2a (Blaess et al., 2006) as demonstrated in the developing mouse midbrain. Lmx1a is necessary for chick and mouse midbrain dopaminergic neuron specification and its expression is also dependent on Shh signaling (Andersson et al., 2006). On the other developmental end, expression of Nurr1 also mediated by Shh in postmitotic midbrain precursors induces TH expression allowing for the maturation of the midbrain dopaminergic phenotype (Wallen and Perlmann, 2003). Many other transcription factors contribute to the specification of the midbrain dopaminergic phenotype and it is through the complex interaction among these factors that the mature phenotype is established (Abeliovich and Hammond, 2007; Panman et al., 2011).

1.1.2. Plasticity

The catecholaminergic phenotype has become a classic paradigm for the switch in neurotransmitter identity. Noradrenergic sympathetic axons innervating the rat sweat glands experience a

switch to the cholinergic phenotype when they reach their target (Landis and Keefe, 1983; Schotzinger and Landis, 1990; Francis and Landis, 1999). This target-dependent switch in neurotransmitter phenotype is dependent on the expression of gp130 receptor in mouse sympathetic neurons and cytokine release from the sweat glands (Stanke et al., 2006). The transcriptional mechanism of this switch involves the upregulation of the expression of Satb2, chromatin architecture protein, when noradrenergic nerves contact the rat sweat glands, which in turn binds to responsive elements in the choline acetyltransferase (ChAT) locus becoming necessary and sufficient for the switch from noradrenergic to cholinergic phenotype (Apostolova et al., 2010). Moreover, Satb2 expression is regulated by the mitogen-activated protein kinase p38 α / β activity, which is necessary for the upregulation of the cholinergic phenotype in noradrenergic sympathetic rat neurons grown *in vitro* and in mice *in vivo* (Loy et al., 2011). The participation of p38 both in neurotransmitter phenotype switching (Loy et al., 2011) and activity-dependent synaptic plasticity (Thomas and Huganir, 2004) poses it as a key signaling factor in the integration of short-term transcription-independent and long-term transcription-dependent plastic responses to the changing environment, challenging the presumed rigidity of the early neurotransmitter phenotype specification.

Indeed, the role of electrical activity in the specification of the catecholaminergic phenotype manifests in the central nervous system. A change in environmental stimuli changes the number of neurons expressing the dopaminergic phenotype of the ventral suprachiasmatic nucleus in *Xenopus* larva (Dulcis and Spitzer, 2008) and in the adult rat brain (Dulcis et al., 2013), suggesting a universal mechanism. In the amphibian the switch to the dopaminergic trait results in the expression of a dual neurotransmitter phenotype, NPY- and TH-expressing neurons (Dulcis and Spitzer, 2008). In the rat the increase in number of dopaminergic neurons happens at the expense of the decrease in the somatostatin phenotype (Dulcis et al., 2013).

The role of morphogens in contributing to the neurotransmitter phenotype specification continues beyond morphogenesis and early patterning. The transcription factors Engrailed 1 and 2 necessary for the midbrain dopaminergic phenotype are first expressed during mouse brain patterning by the action of FGF8, and then they induce expression of this morphogen to maintain the dopaminergic phenotype in already differentiated neurons (Alberi et al., 2004; Simon et al., 2004). Also, BMP4 induces the dopaminergic phenotype in cultured GABAergic neurons derived from the mouse cortical striatum during a sensitive period *in vitro* (Stull et al., 2001). Considering that BMPs (Swapna and Borodinsky, 2012), Shh (Belgacem and Borodinsky, 2011), Wnts (Varela-Nallar et al., 2010) and FGF modulate Ca²⁺ dynamics and kinase activity in developing neurons together with the electrical activity-dependent plasticity of neurotransmitter phenotype, the potential role of morphogenetic proteins in postmitotic neurons participating in neurotransmitters respecification through Ca²⁺-mediated signaling becomes apparent.

1.2. GABAergic/Glutamatergic phenotypes

1.2.1. Preliminary specification

GABAergic interneurons represent a diverse population in the central nervous system. Differentiation of GABAergic phenotypes is thought to be a default fate of differentiating neuronal precursors (Furmanski et al., 2009), which depends on the expression of particular transcription factors. For instance, the Dlx transcription factors promote differentiation of olfactory GABAergic interneurons in mice by regulating the expression of Wnt5a (Paina et al., 2011). Interestingly, the Wnt signaling switches from canonical, β -catenin-mediated, to non-canonical,

Ca^{2+} /PKC-mediated, signaling in the transition from proliferating neural precursors to differentiating neurons by the action of different Wnt ligands (Paina et al., 2011). In the mouse cerebellum the transcription factor Ptf1a is necessary and sufficient for driving GABAergic neuron differentiation of the cerebellar ventricular zone precursors (Hoshino et al., 2005), presumably through Shh action (Huang et al., 2010; Fleming et al., 2013). In the forebrain, the Nkx2.1-expressing medial ganglionic eminence progenitors, specified by the Shh signaling, generate GABAergic or cholinergic neurons but the expression of Lhx6, which feeds forward to promote neuronal production of Shh (Flandin et al., 2011), shifts them towards the GABAergic phenotype (Zhao et al., 2003; Manabe et al., 2005; Flandin et al., 2011). In the chick embryonic diencephalon, the thalamus develops into a rostral population of GABAergic neurons and a caudal region of glutamatergic thalamic neurons. These are patterned by different Shh levels through the regulatory control of Pax6 and Irx3 expression; Pax6 is necessary for the expression of the glutamatergic phenotype and inhibits the GABAergic phenotype (Robertshaw et al., 2013). In the dorsal spinal cord, Tlx1 and Tlx3, transcription factors upregulated by Wnt signaling (Kondo et al., 2011), act as selectors of the glutamatergic over the GABAergic phenotype in chick and mouse embryos (Cheng et al., 2004), while Lbx1 inverts the selection inducing the specification of the GABAergic phenotype in these spinal neurons (Cheng et al., 2005). BMPs are also involved in the differentiation of the GABAergic phenotype in mice. BMP2 enhances the expression of the GABA transporter gat1 by recruiting transcription factors Smad4 and YY1, which directly bind to the transporter regulatory region (Yao et al., 2010).

1.2.2. Plasticity

Many of the genetic programs summarized in the previous section are sensitive to electrical activity, thus enabling the process of glutamatergic and GABAergic phenotype specification to be activity-dependent. Ca^{2+} spikes in developing spinal neurons of *Xenopus tropicalis* phosphorylate the transcription factor cJun that represses transcription of the glutamatergic/GABAergic transcription factor selector Tlx3, promoting the specification of the GABAergic phenotype over the glutamatergic one (Marek et al., 2010). In the rat hippocampus, the expression of the GABAergic phenotype is induced by depolarizing stimuli in glutamatergic granule cells (Gomez-Lira et al., 2005). Interestingly, during development the GABAergic and glutamatergic phenotypes overlap in the frog spinal cord (Root et al., 2008) and in the rat dentate gyrus (Gutierrez et al., 2003) and they become restricted to segregated neuronal populations as development progresses and the nervous system matures (Gutierrez, 2003; Root et al., 2008). Similarly, in the auditory system the GABAergic/glycinergic neurons of the medial nucleus of the trapezoid body that synapse in the rat lateral superior olive also express the glutamatergic phenotype, most prominently during the developmental peak of synapse elimination (Gillespie et al., 2005) that is important for acquiring precision of tonotopy in the inhibitory auditory pathway (Noh et al., 2010).

Neurotrophins BDNF and NGF promote the acquisition of the GABAergic and cholinergic phenotypes of mouse basal forebrain neurons *in vitro* and *in vivo*. The neurotrophin-induced GABAergic phenotype is non-cell autonomous, mediated by factors released from p75 receptor-expressing cholinergic neurons (Lin et al., 2007). The promotion of the GABAergic phenotype by neurotrophins is not dependent on proliferation since the total number of neurons remains the same. Instead it seems to recruit cells that are non-cholinergic and non-GABAergic to change their transmitter specification (Lin et al., 2007).

These changes in neurotransmitter specification do not appear to be dissociated from the earlier developmental processes and

cues that regulate specification of progenitors. Morphogens also participate in the respecification of neurotransmitter identity by recruiting Ca^{2+} -mediated activity in their signaling. For instance, Shh switches from the canonical to non-canonical signaling during *Xenopus laevis* spinal cord development, transitioning from the proliferative Gli-dependent pathway to the Ca^{2+} spike activity-mediated neuronal differentiation (Belgacem and Borodinsky, 2011, 2015). This change in pathways results in an increase in number of GABAergic neurons in the developing spinal cord mediated by the Shh- Ca^{2+} spikes signaling axis (Belgacem and Borodinsky, 2011). Remarkably, the intercalation of Ca^{2+} activity in Shh signaling represses Gli activity, thus inverting Shh action on its own canonical pathway (Fig. 1) (Belgacem and Borodinsky, 2015).

Fig. 1. Interplay between Shh and Ca^{2+} spike activity regulates neurotransmitter specification in developing spinal neurons. Shh binds to Patched (Ptc) releasing the constitutive inhibition on the coreceptor Smoothened (Smo) which activates phospholipase C (PLC) that increases inositol triphosphate (IP3) and diacylglycerol (DAG) levels thus enhancing Ca^{2+} spike activity through the activation of transient receptor potential channel 1 (TRPC1), voltage-gated Na^+ and Ca^{2+} channels (Na_v , Ca_v) and Ca^{2+} release from stores. Enhanced Ca^{2+} spike activity intercalates in Shh canonical pathway, inverting Shh action and leading to Gli2 cytosolic localization, Gli2 and Gli3 processing into repressor forms, repression of Gli1 transcription and an overall downregulation of Gli activity. In contrast, Ca^{2+} spikes activate transcription factors cAMP-responsive element binding protein (CREB) and cJun, which promote the expression of the GABAergic over glutamatergic phenotype by regulating expression of the transcription factor selector tlx3. Based on Cheng et al. (2004), Marek et al. (2010), Belgacem and Borodinsky (2011, 2015).

1.3. Cholinergic phenotype

1.3.1. Preliminary specification

The molecular mechanisms of the specification of the cholinergic phenotype are still unclear. Nevertheless, some key players have been identified from studies focused on the specification of vertebrate motor neurons, which are cholinergic. MNR2 is a homeobox gene upregulated by Shh signaling, expressed by chick motor neuron progenitors and transiently by postmitotic neurons that is necessary and sufficient for the expression of ChAT, acetylcholine synthetic enzyme (Tanabe et al., 1998). Also, the cholinergic/motor neuron phenotype can be elicited in mouse embryonic stem cells *in vitro* by recapitulating the morphogenetic environment of motor neuron progenitors *in vivo*, particularly retinoic acid and Shh (Wichterle et al., 2002). In the mouse basal forebrain, the specification of cholinergic neurons is dependent on the expression of the transcription factor Lhx8 (Mori et al., 2004).

Other studies have shown that BMPs, and particularly BMP9, enhance the expression of the cholinergic phenotype in mouse septum and spinal cord neurons, both *in vitro* and *in vivo* (Lopez-Coviella et al., 2000). The mechanisms of BMP-induced cholinergic specification in mice and rat basal forebrain may involve the upregulation of c-Fos expression (Lopez-Coviella et al., 2005), which in turn enhances expression of the cholinergic phenotype genes, ChAT, VACHT and AChE (Kaufer et al., 1998). Trophic factors like NGF also regulate the expression of the cholinergic phenotype in the rat pheochromocytoma cell line (PC12) and in primary neuronal cultures from the mouse embryonic septum, potentially by recruiting the Akt/PI3K pathway and by regulating GSK3 activity (Madziar et al., 2008), which in turn may control CREB activity that also regulates the expression of the cholinergic locus in mouse and rat neurons (Brock et al., 2007; Liu et al., 2008).

1.3.2. Plasticity

The cholinergic phenotype is probably the paradigm of neurotransmitter phenotype respecification. The switch from noradrenergic to cholinergic upon innervation of the footpad sweat glands reviewed above represents the first evidence of the plasticity of the cholinergic phenotype specification. This is not restricted to the peripheral nervous system, mouse and rat glutamatergic hypothalamic neurons induce expression of the cholinergic phenotype when glutamate signaling is inhibited through NMDAR-mediated and Ca^{2+} /CREB/NF- κ B-dependent mechanism (Belousov et al., 2001; Belousov et al., 2002; Liu et al., 2008). In contrast, excessive stimulation of brain excitability by stress or by inhibiting the acetylcholine esterase *in vivo* and in hippocampal slices respectively, downregulates expression of the cholinergic gene locus through a Ca^{2+} and c-Fos-mediated pathway (Kaufer et al., 1998).

Moreover, the cholinergic phenotype in the developing *X. laevis* spinal cord is also sensitive to the levels of Ca^{2+} -mediated electrical activity. Enhancement of Ca^{2+} spike frequency leads to lower number of cholinergic neurons while suppressing Ca^{2+} spike activity increases the number of cholinergic neurons (Borodinsky et al., 2004). These changes in neurotransmitter phenotype follow a homeostatic rule with excitatory neurotransmitter phenotype, glutamatergic and cholinergic, increasing when activity is suppressed and viceversa for the inhibitory neurotransmitter phenotypes, GABAergic and glycinergic (Borodinsky et al., 2004). The changes in the cholinergic phenotype of motor neurons upon changes in activity levels (Borodinsky et al., 2004) are accompanied by corresponding changes in the neurotransmitter receptor expression in the skeletal muscle resulting in non-cholinergic

neuromuscular junctions in the *Xenopus* tadpole (Borodinsky and Spitzer, 2007).

1.4. Serotonergic

1.4.1. Preliminary specification

The generation of serotonergic neurons starts with the specification of neural progenitors of the mouse ventral hindbrain that express the transcription factor Nkx2.2 induced by Shh (Briscoe et al., 1999). Gata-2 is induced by the Shh-Nkx2.2 pathway and is necessary for enhancing expression of Gata-3, Pte-1 and Lmx1b which all participate in the specification of the serotonergic phenotype in the mouse and chick hindbrain (van Doorninck et al., 1999; Cheng et al., 2003; Ding et al., 2003; Craven et al., 2004).

1.4.2. Plasticity

The number of serotonergic, tryptophan hydroxylase-expressing, neurons *in vitro* increases upon serotonin stimulation through the upregulation of BDNF expression and signal transduction through trk-C receptor in embryonic rat raphe-derived cultured cells (Eaton et al., 1995; Galter and Unsicker, 2000). In contrast, the trophic factors CNTF and LIF reduce the expression of the serotonergic phenotype at the expense of increasing expression of the cholinergic phenotype of embryonic raphe nuclei neuronal cultures (Rudge et al., 1996).

In the *X. laevis* tadpole hindbrain the number of serotonergic neurons depends on the level of spontaneous Ca^{2+} spike activity. Enhanced activity downregulates expression of Lmx1b and thus decreases the number of cells expressing the serotonin synthesizing enzyme tryptophan hydroxylase (Demarque and Spitzer, 2010). These changes in serotonergic neuron number modify the swimming pattern of frog larva (Demarque and Spitzer, 2010).

Fig. 2. Intrinsic and extrinsic stimuli change neurotransmitter specification in developing and mature neurons. Preliminary specification of neural progenitors during the embryonic development is instrumental to morphogenesis of different nervous system structures and functional nuclei. However, the identity of developing neurons is not sealed. Instead features like neurotransmitter phenotype are sensitive to the changing internal and external environment; the emerging stimuli and developmental cues crosstalk with morphogenetic proteins to implement plasticity of neurotransmitter phenotype expression necessary for adapting to the ever-changing environment.

1.5. Concluding remarks

Early specification of neural progenitors sets the organizing structural principles of the developing nervous system. However, many aspects of neuronal differentiation are not terminally fated by the specialization assumed by progenitor cells and the neurotransmitter phenotype is a paradigmatic example (Fig. 2). The cues that implement nervous system embryonic patterning do not shut off or disappear but instead they participate from the plastic events driving the changes in neurotransmitter phenotype specification. This is achieved by interactions of morphogenetic proteins with the changing intrinsic and external environment as the nervous system develops and matures. The emergence during neural development of pathways mediated by trophic factors, electrical and calcium activity that in turn are sensitive to persisting morphogenetic protein signaling, orchestrates neuronal differentiation by modulating activity of critical transcription factors. Moreover, the intercalation of the emerging signaling pathways in the maturing neurons allows to switching gear on the morphogenetic protein action and repurposing them for implementing distinct responses to different stimuli.

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The plastic neurotransmitter phenotype of the hippocampal granule cells and of the moss in their *messy* fibers

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ABSTRACT

The granule cells (GCs) and their axons, the mossy fibers (MFs), make synapses with interneurons in the hilus and CA3 area of the hippocampus and with pyramidal cells of CA3, each with distinct anatomical and functional characteristics. Many features of synaptic communication observed at the MF synapses are not usually observed in most cortical synapses, and thus have drawn the attention of many groups studying different aspects of the transmission of information. One particular aspect of the GCs, that makes their study unique, is that they express a dual glutamatergic–GABAergic phenotype and several groups have contributed to the understanding of how two neurotransmitters of opposing actions can act on a single target when simultaneously released. Indeed, the GCs somata and their mossy fibers express in a regulated manner glutamate and GABA, GAD, VGlut and VGAT, all markers of both phenotypes. Finally, their activation provokes both glutamate-R-mediated and GABA-R-mediated synaptic responses in the postsynaptic cell targets and even in the MFs themselves. The developmental and activity-dependent expression of these phenotypes seems to follow a “logical” way to maintain an excitation–inhibition balance of the dentate gyrus-to-CA3 communication.

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1. Introduction

The granule cells of the dentate gyrus (DG) in the hippocampal formation give origin to the bundle of axons to which Ramón y Cajal gave the adjective of “mossy” because of their appearance, in form of ramified clumps, resembling moss. These mossy fibers (MFs) possess unique anatomical, biochemical, biophysical and information transmitting characteristics, which make their study a complicated task. It turns even more complicated and interesting by the fact that these fibers express different information transmission phenotypes. So I take the liberty to confer them a second adjective: “messy” (which definition by the Merriam-Webster's Dictionary is “marked by confusion”). Indeed, their study has to be technically delicate, thorough and rigorous to avoid obtaining data that can bring confusion when interpreting their physiological meaning.

The mossy fibers make 3 types of synapses along their path through the *hilus* and along the *stratum lucidum* of CA3. One with

the pyramidal cells of CA3, which contact is comprised by a giant MF bouton and a dendritic spine of the apical dendrite of the pyramidal cell; second, synapses called “en passant” made by varicosities along the MFs and interneurons and, finally, phylo-podial extensions that originate from the MF giant boutons and that also contact interneurons (Acsády et al., 1998).

In the adult rodent, the stimulation of the MF produces depolarizing monosynaptic responses in their target cells: interneurons and mossy cells in the *hilus*, and interneurons and pyramidal cells in the CA3 region. Thus, granule cells have been considered as glutamatergic. Years of research on MF transmission, particularly in *in vitro* preparations, have shown that the characteristics of neurotransmission are highly dependent on the target cells from which recordings are made, and virtually all these studies have been conducted in the presence of GABA_A-R antagonists to avoid contamination from the stimulation of interneurons scattered in the *hilus* or *stratum lucidum*. So the typical “experimental assumptions” that the neurophysiologists working on the MF synapse have made are: (1) true MF stimulation would not produce monosynaptic inhibitory responses; however, polysynaptic inhibitory responses can be recorded because interneurons can be driven by MF activation and they, in turn, inhibit pyramidal cells and other interneurons as well. Therefore, blocking glutamate receptors prevents both the monosynaptic

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Fig. 1. A₁. The granule cells of the dentate gyrus excite pyramidal cells, through giant boutons, and interneurons, through boutons *en passant* and filopodial extensions. The latter in turn, release GABA to inhibit pyramidal cells and sustain feed-forward inhibition. This scheme depicts glutamatergic-only transmission of the granule cells, after the third week of age. A₂. Stimulation of this arrangement (granule cell 1-to-CA3 pyramidal cell 1) provokes monosynaptic EPSP and polysynaptic GABA_A and GABA_B-dependent responses. B₁. The origin of an inhibitory response in a pyramidal cell to MF stimulation is through the activation of an interneuron. B₂. Thus, stimulation of granule cell 2 that does not innervate the pyramidal cell 2, but instead drives an interneuron connected to this pyramidal cell (2), evokes a polysynaptic IPSP.

excitatory response and, thus, the di- or polysynaptic activation of GABAergic interneurons from which GABA-mediated responses originate. (2) On the other hand, the presence of GABA_A-R antagonists in the extracellular medium blocks monosynaptic inhibition, which could be elicited by direct activation of interneurons when bulk stimulation is given over the *stratum lucidum*, through which the MFs run (Fig. 1).

Up to this point, all synaptic responses obtained by what is presumed to be selective MF stimulation under the aforementioned conditions seem to be accounted for. Not quite. Glutamatergic responses recorded in CA3 cells can be evoked by the activation of one or more of the anatomically distinct fibers that impinge onto them: the recurrent axons from CA3 pyramidal cells themselves, the perforant path, the commissural and finally the MFs. Therefore, responses to the activation of these sources should be told apart and, for this, some conditions have to be met.

2. Electrophysiological criteria to distinguish glutamatergic transmission of MF origin

For years, consistent results were obtained under the above-mentioned assumptions about glutamate-mediated MF transmission. Therefore, obtaining other type of response (for instance, mono-synaptic GABAergic transmission in the presence of glutamate receptor blockers) would mean that something in these experiments was different from those that everybody conducted and that helped to establish the referred assumptions. Alternatively, their interpretation was wrong because it did not comply with such assumptions. And certainly, there were *ad-hoc* explanations if such a response appeared. For instance, if GABA_A-R-mediated responses were elicited, that would mean that the electrode supposedly used to selectively stimulate the MFs, directly stimulated interneurons. Thus, a regular practice to avoid “contamination” from interneuronal transmission when MFs are stimulated is the perfusion of a GABA_A-R antagonist, either bicuculline, or picrotoxin, or gabazine dissolved in the extracellular medium. Even so, selective stimulation of the MF tract over the

stratum lucidum is “tricky” and it does not always produce pure MF responses because commissural axons, as well as collaterals of other CA3 pyramidal cells can be stimulated too (Henze et al., 1997); so synaptic responses had to “follow certain rules” if they are to be considered of MF origin. Briefly, the physiological and pharmacological characteristics of transmission of MF origin are: (1) strong frequency-dependent potentiation (>300%) in response to modest increases in stimulation frequency; (2) robust NMDA-independent LTP and (3) depression (>80%) of the responses by activation of mGluR, which are present in the MFs. Indeed, proving the presence of these physiological and pharmacological characteristics together defines transmission of MF origin. These and several other physiological characteristics of MF transmission and its plasticity are thoroughly reviewed elsewhere (Henze et al., 2000; Urban et al., 2001; Bischofberger and Jonas, 2002; Lawrence and McBain, 2003; Nicoll and Schmitz, 2005; Jaffe and Gutiérrez, 2007; Galván et al., 2011; Ruiz and Kullmann, 2013).

Despite these experimental manipulations that are routinely used to define transmission of MF origin, the only way to undoubtedly isolate responses to MF activation is by conducting paired recordings, whereby the presynaptic granule cell is depolarized to fire an action potential and a synaptic response to each action potential should be recorded in the postsynaptic target cell. Alternatively, pure MF-mediated synaptic responses can be evoked by the selective stimulation of a MF giant bouton. This has been accomplished through a method that implies recording from a presynaptic giant MF bouton while recording the postsynaptic pyramidal cell in a hippocampal slice (Geiger and Jonas, 2000; Bischofberger et al., 2006). A second method that permits to record synaptic responses to selective stimulation of MF giant boutons implies labeling these boutons, then dissociating the pyramidal cells from their network with the MF boutons attached to their apical dendrites, which can then be identified and directly stimulated by means of a patch pipette while recording from the pyramidal cell (Beltrán et al., 2012; Beltrán and Gutiérrez, 2012). Finally, pure granule cell-mediated synaptic responses can be

obtained from cultured granule cells establishing autapses (Rost et al., 2010). Because MF transmission is in many ways target-dependent, this latter method can only provide one type of synapse, i.e., a granule cell impinging onto itself. However, a method was developed in which identified granule cells dissociated from GFP-expressing rats are co-cultured and connected with pyramidal cells and interneurons of wild-type rats, giving the possibility of conducting paired recordings of a presynaptic granule cell (or other GFP⁺ cell) connected to either a pyramidal cell, or to an interneuron of CA3 or to another granule cell with no fluorescence, identifying it as the postsynaptic cell. In these cultures, the physiological and pharmacological characteristics of transmission of MF origin are similar to those observed in slices (Osorio et al., 2013; Fig. 2).

3. Biochemical contents of the granule cells

For a given cell to release its contents, it should produce or incorporate them from the extracellular *milieu* via specific membrane transporters. It is well known that granule cells produce and release various substances: dynorphin (McGinty et al., 1983; Chavkin, 2000), enkephalin (Gall et al., 1981), Zn⁺⁺ (Wenzel et al., 1997; Molnar and Nadler, 2001), brain derived neurotrophic factor (BDNF; Gall and Lauterborn, 1992; Lowenstein and Arsenault, 1996), adenosine (Braas et al., 1986), and other peptides like somatostatin, neuropeptide Y, neurokinin B and cholecystokinin (Tonder et al., 1994; Schwarzer et al., 1995). However, it has been demonstrated that the granule cells produce and release the two most abundant excitatory and inhibitory amino acids for fast neurotransmission, respectively: glutamate (Crawford and Connor, 1973; Storm-Mathisen, 1981; Storm-Mathisen et al., 1983) and GABA (see below).

Studies carried out on MF synaptosomes provided the first neurochemical evidence showing that MF terminals contained and released GABA (Terrian et al., 1988; Taupin et al., 1994a,b). Sandler and Smith (1991) found GABA immunoreactivity in MF terminals that made asymmetric synaptic contact with spines arising from large dendrites of CA3 pyramidal cells of monkey and human hippocampi. Granule cell bodies, however, were not immunoreactive to GABA. These authors also showed the colocalization of GABA and glutamate within the same terminals with electron microscopy. The proposal at that time was that GABA could be synthesized in the granule cells from a route different from the GAD-dependent pathway. This idea, again, was proposed at a time when GAD had not been detected in the granule cells. Sloviter et al. (1996) later demonstrated that GABA and its synthesizing enzyme, GAD₆₇, co-existed in the MFs of rats, monkeys and humans and that seizures produced an up regulation of GAD (see also Schwarzer and Sperk, 1995; Ramirez and Gutiérrez, 2001), moreover, it was also found that the content of GABA itself was increased by seizures (Gómez-Lira et al., 2002; Fig. 3). Quantitative immunogold analysis confirmed the co-existence of these amino acids within single MF terminals, and in close relationship with synaptic vesicles, where GABA concentration was found to be lower than that of glutamate (Bergersen et al., 2003).

The last component of the molecular markers that define the GABAergic phenotype of the granule cells is the presence of GABA-specific membrane and vesicular transporters. Using immunohistological techniques, Frahm et al. (2000) determined the presence of the GABA membrane transporter (GAT-1) in the granule cells and Gómez-Lira et al. (2002) showed that MF terminals capture GABA and nipecotic acid blocks this uptake. The presence of GAT-1 was also corroborated by electrophysiological experiments showing that block of the GAT-1 prolonged GABA_A-mediated synaptic responses of MF origin (Vivar and Gutiérrez, 2005). On the other hand, for a neurotransmitter to be released, it should first be

incorporated into synaptic vesicles via a specific transporter, by using the electrochemical gradient of H⁺ and chloride between the vesicle lumen and the presynaptic cytosol (Ahnert-Hilger and Jahn, 2011; Riazanski et al., 2011). Indeed, several studies have demonstrated that the MFs and their terminals express both the vesicular transporters' RNAs and proteins for both, glutamate and GABA (Lamas et al., 2001; Gómez-Lira et al., 2005; Safulina et al., 2006; Zander et al., 2010; Münster-Wandowski et al., 2013). Interestingly, several papers describe the presence of VGAT in terminals of putative glutamatergic fibers within the hippocampus but not in the MF terminals. The work in which the vesicular GABA transporter was first described showed granule cells that hybridized with the antisense RNA for the transporter (McIntire et al., 1997) but did not determine the presence of the protein in the MF terminals. Chaudhry et al. (1998) observed only a few gold particles in MF terminals in a study using electron microscopic post-embedding immunogold localization of VGAT. The same authors, using immunohistological procedures concluded that despite the MF being enriched with GABA, they were not immunoreactive to VGAT. The same group later studied the expression of VGAT in developing rodents but did not find it in MF (Boulland and Chaudhry, 2012). Because the expression of the markers of the GABAergic phenotype depends on age and activity, it is likely that the variability of these states, as well as subtleties in the methods used (antibodies and/or different tissue fixating techniques) may yield different results. The expression of VGAT in MF needs to be confirmed by other groups and methods.

4. The neurotransmitter phenotype of the granule cells is plastic

As previously described, the granule cells were shown to contain GAD and GABA (Sloviter et al., 1996) but no assumption was made as to whether the MFs could release GABA besides glutamate. The up-regulation of GAD after seizures described by Sloviter et al. (1996) in the rodent and monkey, and confirmed by Schwarzer and Sperk (1995) in the rat, made us hypothesize that MFs could release GABA after seizures. We reported, in an abstract form (Gutiérrez and Heinemann, 1997) and later in a series of articles that the granule cells indeed expressed a GABAergic phenotype and that they could release GABA (Gutiérrez, 2000; Gutiérrez and Heinemann, 2001; Ramirez and Gutiérrez, 2001; Lamas et al., 2001). We found that MF stimulation evoked monosynaptic GABAergic responses of pyramidal cells of CA3 at the same time that GAD67, GABA, VGAT mRNA were up-regulated after kindling epilepsy in the rat. The up-regulation of these GABAergic markers, as well as the GABAergic synaptic responses were transient because neither the GABAergic markers nor the GABAergic synaptic responses could be detected a month after the seizures stopped, but they reappeared when seizures were again kindled. Recent evidence has confirmed the up-regulation of GAD in the mossy fibers of temporal lobe epilepsy patients (Sperk et al., 2012). Walker et al. (2001) independently reported the presence of GABAergic responses with physiological and pharmacological characteristics of transmission of MF origin in the healthy guinea pig. We later demonstrated that the expression of the GABAergic phenotype in the otherwise glutamatergic granule cells followed a developmental and activity-dependent program (Gutiérrez et al., 2003). We described that the granule cells expressed a dual glutamatergic-GABAergic phenotype during the first 3 post-natal weeks, whereby MF stimulation evoked mono-synaptic GABAergic synaptic potentials while all the markers of the GABAergic phenotype were detected. After this period, these GABAergic markers as well as the GABAergic synaptic responses disappeared, with the glutamatergic ones remaining. The GABAergic phenotype would be again induced after seizures and, importantly, even

Signatures of transmission of mossy fiber origin

Fig. 2. A₁. Co-cultures of GFP⁺-GCs with GFP⁻ pyramidal cells and interneurons of CA3 allow paired recordings of connected, identified GCs and target cells after 3 weeks in vitro. Once visually identified, patch electrodes were positioned for recording (dark shadows). A₂. Once the pre-synaptic and the post-synaptic cells are selected by their expression or lack of expression of GFP and morphology, GC are depolarized to fire action potentials, to which the post-synaptic pyramidal cell responded with an EPSP for every pre-synaptic action potential. B₁. EPSPs evoked by a granule cell in a pyramidal cell undergo strong frequency potentiation when the firing frequency of the pre-synaptic cell is raised from 0.1 to 1 Hz, while its membrane potential remains unchanged. B₂. A second signature of transmission of MF origin is the depression of the responses by the activation of group II mGluRs with DCG-IV (1 μ M). The EPSPs were completely blocked by ionotropic glutamate receptors antagonists NBQX + APV. C. Stimuli trains of 2 s at 50 Hz were delivered 3 times for induction of LTP/LTD. Representative example of a single train of action potentials of a GC (green) and the

without seizures after repeated LTP-inducing stimulation. Indeed, it was possible to record from a single cell, where all synaptic responses were blocked by glutamatergic antagonists, the appearance of mono-synaptic GABAergic responses after several successive LTP-inducing stimulation trains were provided (Gutiérrez, 2002); moreover, we were able to prove that the emergence of GABAergic responses coincided with the up-regulation of GAD, VGAT mRNA and that it was dependent on protein synthesis (Gutiérrez, 2002; Romo-Parra et al., 2003; Gómez-Lira et al., 2005; Fig. 3).

Surprisingly, Safiulina et al. (2006) reported that during the first week of life (P0–P6), MFs were exclusively GABAergic. However, from P6 the glutamatergic phenotype is turned on in addition to the GABAergic one enabling simultaneous glutamatergic and GABAergic transmission in a single pathway, which provides an efficient and rapid synergism on their target cells during development (Gutiérrez et al., 2003; Kasyanov et al., 2004). It was also later proved that different fibers or boutons release either glutamate or GABA or both (Beltrán and Gutiérrez, 2012; Fig. 4). The sequence of the development of the initially GABAergic-only, dual GABAergic–glutamatergic, and finally glutamatergic-only phenotype of the MFs is summarized in Fig. 6.

In accordance with the real postulate of Dale (not the “one neuron–one neurotransmitter principle”, which he never postulated) that one neuron is a biochemical unity and that the neurotransmitter(s) that it releases should be released from all its terminals (Gutiérrez, 2009a,b), we and others have shown that interneurons in the *hilus* and in *stratum lucidum* of the CA3 area (Bergersen et al., 2003; Romo-Parra et al., 2003; Galván and Gutiérrez, 2011) also receive the dual glutamate and GABA signal directly from the MFs. One would argue that inhibition of interneurons would result in the disruption of feedforward inhibition in the DG-to-CA3 projection, with the concomitant disinhibition of CA3. Surprisingly, when MF co-release glutamate and GABA this produces a strong shunt inhibition in pyramidal cells, as the interneurons can still be excited by the MF excitatory signal, due to an apparent higher release of glutamate as compared to that of GABA. Therefore, pyramidal cells receive the inhibitory input from both, the MFs and the interneurons driven by them (Treviño et al., 2011). Evidence for a higher amount of glutamate as compared to GABA in MF terminals has also been determined by ultrastructural studies (Bergersen et al., 2003), however, studies establishing differences between giant MF boutons, which impinge on pyramidal cells, and en passant or filopodial extensions, which innervate the interneurons, are still in course (Galván and Gutiérrez, 2011).

All the aforementioned electrophysiological data had strongly established, albeit in an indirect fashion, that the MFs co-release glutamate and GABA. The experimental design of these studies complied with “the rules” of MF transmission. Therefore, if the MFs release more than one neurotransmitter for fast communication, the physiological and pharmacological characteristics that we have previously described should apply to both transmitters. From the data described above, and because both, the GABAergic and glutamatergic transmission comply with the rules that transmission of MF should follow, it is as valid to say that the MFs are glutamatergic as they are GABAergic. Indeed, both signals have been shown repeatedly and by several groups to possess the same physiological and plastic profile, the same latency and, finally, the same pharmacological sensitivity to activation of mGluR. Despite the strong evidence in favor of the hypothesis of co-release of glutamate and GABA from the MFs, Uchigashima et al. (2007) did

not find MF-mediated GABAergic synaptic responses and suggested that the positive evidence observed by others could be related to responses of interneuronal origin. However, they did not prove either that the “false MF-mediated” GABAergic responses were indeed of interneuronal origin or that MF did not release GABA.

Unambiguous, direct evidence that MF boutons corelease glutamate and GABA was recently obtained by stimulating single, identified MF boutons, whereby the post-synaptic pyramidal cell responded with either a glutamatergic-only or a GABAergic-only-mediated current or with a compound current mediated by both glutamate and GABA (Beltrán and Gutiérrez, 2012). Moreover, this evidence was obtained from pyramidal cells, which have been isolated from their network, but that keep MF boutons attached to their apical dendrites. In such a preparation, single, identified MF boutons could be directly stimulated to obtain pure MF responses from the pyramidal cell. Taking advantage of the fact that before day 21 of age the MF are thought to release GABA, besides glutamate, but only glutamate after this age, we demonstrated that the stimulation to a MF bouton evoked NBQX + APV-sensitive, as well as bicuculline-sensitive currents with the same latency and intensity threshold in the post-synaptic pyramidal cell. By contrast, and as expected, GABAergic responses were never observed in older (>22 days) rats. The GABAergic and glutamatergic responses evoked by MF bouton stimulation were both sensitive to mGluR, whereas GABAergic responses evoked in the same cell by stimulating an interneuronal bouton were not (Beltrán and Gutiérrez, 2012; Fig. 4). The release of GABA from granule cells was also shown to occur in autaptic cultures (Cabezas et al., 2012; Valente et al., 2015).

Because granule cells are continuously being generated in the DG, it suggested the possibility that their physiological characteristics during their development parallel those described for granule cells in the post-natal rat (Espósito et al., 2005) and we hypothesized that they would also recapitulate the transition of the phenotype observed in granule cells of developing animals. Indeed, Lara et al. (2012) found that granule cells born in the adult rat transiently express markers of the GABAergic phenotype, although no electrophysiological data was provided to support their use of GABA for fast neurotransmission (Fig. 5F). Whether this is possible remains to be solved. Granule cells co-release glutamate and GABA during the first days of post-natal life, but granule cells born in the adult rodent take some time in establishing connections so as to detect the co-release before the 3 weeks of age (provided that they also co-release both amino acids during their development in the adult). Moreover, there is a possible segregation of GABA in particular boutons (Beltrán and Gutiérrez, 2012), which can make GABAergic transmission from the MF even more difficult to be detected or not observed at all (Toni et al., 2008).

Thus, given all this evidence, the reasons to consider the MFs as GABAergic are as valid as those that make them to be considered as glutamatergic. Furthermore, all the ultrastructural studies of the MF terminals to date recognize the presence of endogenous GABA in healthy, adult rats (Sandler and Smith, 1991; Sloviter et al., 1996; Bergersen et al., 2003), however, MFs do not release GABA in the adult stage. Therefore, the GABAergic phenotype in the adult rodent remains dormant until a calcium- and BDNF-dependent, as well as protein-synthesis-dependent mechanisms are put in play (Romo-Parra et al., 2008; Gómez-Lira et al., 2005), allowing GCs to release GABA. The nature of the molecular determinants of these mechanisms is matter of current investigation.

postsynaptic responses (black) of a pyramidal cell. After 10 min baseline, 3 stimuli trains were delivered to the GC (green arrows in plots) and responses were monitored 30 s after the last train, at 0.033 Hz. GC–PC synapses displayed LTP. Action potentials of the GC and synaptic responses above the graph plot were obtained at the time points indicated by the numbers. At the end of each experiment DCG-IV was perfused and, as expected for transmission of MF origin, the synaptic responses were strongly depressed. Modified from Osorio et al. (2013).

Activity-dependent plasticity of the phenotype of the granule cells in the adult rat

Fig. 3. A₁. MF-evoked synaptic responses in a pyramidal cell in a control rat. Blockage of ionotropic glutamate receptors block all responses, confirming the glutamatergic nature of the monosynaptic excitatory, as well as the polysynaptic nature of the inhibitory responses. A₂. By contrast, in hippocampal slices prepared from rats that underwent seizures or from control rats to which a repeated LTP-inducing protocol was applied over the perforant path in vitro, the blockage of glutamate receptors uncovers a fast IPSP with the same latency of the EPSP. This IPSP could be blocked by GABA_A-R blockers. B₁. Immunohistological experiments on hippocampal slices from control rats revealed GAD₆₇ positive interneurons scattered in the hippocampus and, in the inset, in the border of the hilus and the granule cell layer. B₂. The same experiments performed in slices obtained from epileptic, kindled rats showed that the GCs and MFs are highly immunoreactive to GAD₆₇. C₁. With single-cell PCR we determined the expression of the mRNA of the vesicular transporters for glutamate (VGlut) and GABA (VGAT) in GCs from control and from epileptic rats. Granule cells from control rats express VGlut but not VGAT, while GCs from epileptic rats (C₂) express both VGlut and VGAT mRNAs. Modified from Gutiérrez (2002) (A); Ramírez and Gutiérrez (2001) (B); Gómez-Lira et al. (2005) (C).

5. GABA receptors activated by the release of GABA from the mossy fibers

Bergersen et al. (2003) determined the presence of GABA_A receptors, both apposed MF terminals as well as in the MF themselves. Moreover, they showed that these receptors coexist with glutamate receptors in the post-synaptic site. GABA acting on

GABA_A receptors modulates the excitability of the MFs (Ruiz et al., 2003; Treviño and Gutiérrez, 2005; Franco et al., 2015) and therefore further release of neurotransmitter (Treviño and Gutiérrez, 2005; Yamamoto et al., 2011). On the other hand, it is well known that GABA_B receptors are present in the MF terminals and their activation with the agonist baclophen inhibits the release of GABA from the MFs (Gutiérrez, 2002). Thus, GABA released from

Fig. 4. Stimulation of single MF boutons (MFB) attached to dissociated pyramidal cells reveals corelease of glutamate and GABA in developing rats. (A) A slice of a 15 day old rat shows Zn²⁺-containing MFs (left) labelled with the fluorescent sensor for Zn²⁺, Zinpyr-1, or with dextranamine transported from the granule cell layer to the MFs terminals (right). (B) MFBs can be identified attached to the apical dendrites of the dissociated pyramidal cells by their fluorescent label. Left panel, bright field photograph of a dissociated pyramidal cell with a MFB attached (arrow); right panel shows the MFB under fluorescence (arrow). Other non-fluorescent bulks (putative boutons) can also be observed in the basal dendrite or axons of pyramidal cells (IntB in C). (C) Schematic drawing depicting the experimental set-up. MFBs were localized on the apical dendrite, while an interneuron bouton was localized in the basal dendrite (or axon) for direct stimulation with a patch pipette while recording the synaptic responses using the whole-cell voltage-clamp technique. (D) Electrophysiological identification of the recorded cell. (E) The synaptic current evoked by stimulation of MFs in normal ACSF reversed at 0 mV and, on blockage of ionotropic glutamate receptors, the reversal potential shifted to -60 mV, consistent with currents mediated by GABA_A receptors. In red, the compound inward-outward current evoked by glutamate and GABA corelease. (F) By contrast, the synaptic currents evoked by stimulation of IntBs in normal ACSF and during blockage of iGluRs reversed -64 mV, both consistent with currents mediated by GABA_A receptors. Modified from Beltrán and Gutiérrez (2012).

the MFs acts to regulate further release of neurotransmitter and modulates information transfer of the MFs by acting on GABA_A (Ruiz et al., 2003; Treviño and Gutiérrez, 2005; Franco et al., 2015; Yamamoto et al., 2011), as well as on GABA_B receptors (Gutiérrez, 2002; Safiulina and Cherubini, 2009; Cabezas et al., 2012; Valente et al., 2015).

6. Functional relevance of the dual glutamatergic-GABAergic phenotype of the mossy fibers

As already mentioned, the expression of the neurotransmitter phenotype in the granule cells is initially GABAergic, next dual glutamatergic-GABAergic, next glutamatergic-only but, after a period of enhanced excitability, they can transiently become glutamatergic-GABAergic (Gutiérrez, 2005) (Fig. 5A and B). Given

that GABA actions in the pyramidal cells of the hippocampus are depolarizing during development and they become hyperpolarizing from the second postnatal week, it is expected that corelease of glutamate and GABA serves different functions in each developmental and physiological condition.

Several groups have proposed that glutamate-GABA corelease mediates the refinement of the circuit, as has been proposed to happen in the olivary nucleus, where GABAergic cells corelease glycine and glutamate (Gillespie et al., 2005); thus, the initial release of GABA and glutamate exerts a depolarizing action before the AMPA receptors are originated (Ben-Ari et al., 1997). During development GABA depolarizes the neurons due to a higher concentration of Cl⁻ inside the cell. The opening of the GABA_A-mediated Cl⁻ channels produces a net efflux of this anion, depolarizing the cell. The high intracellular concentration of

Programmed plasticity of the phenotype of the granule cells in the developing rat

Plasticity of the phenotype of newborn granule cells in the adult rat

Fig. 5. Programmed plasticity of the phenotype of the developing GCs in young and adult rats. (A) During the first 5 days of life, MFs are GABAergic. Minimal stimulation provokes synaptic currents that are partially inhibited by the glutamatergic antagonist DNQX and completely blocked by the GABA_A-R antagonist, picrotoxin (modified from Safiulina et al., 2006). (B) From the 6–7 postnatal day, responses observed by stimulation of single, identified MF boutons showed that they can either release glutamate-only or GABA-only or co-release both amino acids (modified from Beltrán and Gutiérrez, 2012). (C) GAD₆₇-IR in the stratum lucidum (sl) of a 15 day old (1) and a 2 mo old (2) preparation. The series of images below depicts GAD₆₇-IR in CA3b at the indicated ages, whereby a clear down-regulation is apparent towards the third week of life, when

PLASTIC CO-EXPRESSION OF THE GLUTAMATE AND GABA PHENOTYPES IN THE MOSS OF THE MESSY FIBERS

Fig. 6. Plastic co-expression of the glutamatergic and GABAergic phenotypes in the moss of the messy fibers. (A) Contents of a giant MF bouton and its respective post-synaptic target cell. (B) Contents of terminals that impinge on to interneurons. (C) Relative expression of glutamate and GABA in MF terminals during development (C), in adult, healthy rats (D) and in rats that had undergone seizures (E). Notice the complexity of the terminals that express several presynaptic receptors and chemical contents.

Cl[−] is reverted when KCC2, which extrudes Cl[−] from the cells, is expressed, producing a gradient that permits Cl[−] to enter the cell with a concomitant hyperpolarizing effect (Rivera et al., 1999; Ludwig et al., 2003). It has been suggested that because AMPA-type glutamate receptors are expressed late during development, the depolarizing GABA provides the necessary depolarization for the NMDA-type glutamate receptors to be activated. So, corelease of glutamate and GABA can be thought of as a means to provide the refinement in the configuration of the connectivity (Gutiérrez et al., 2003). Indeed, it was shown that the GABAergic signaling adds and synergizes with giant depolarizing potentials (Kasyanov et al., 2004). But, what then is its function when AMPA-type receptors are expressed and GABA released from the MFs do

not seem to exert this depolarizing effect? The way by which the NKCC1 is down-regulated by the neurons follows a somato-dendritic gradient, whereby it first disappears in the soma and days later in the dendrites (Marty et al., 2002). This prompted us to suggest that MF-GABA, activating the proximal part of the apical dendrite, would have an effect different to that of GABA released from the interneurons on distal dendrites and axon in CA3. We found electrophysiological evidence that favors the hypothesis that the intracellular concentration of Cl[−] follows a gradient in the somato-dendritic direction, paralleling that of the KCC2 protein. GABA applied in the distal dendrites, as well as activation of stratum radiatum interneurons produces a depolarizing response in the distal dendrites, whereas GABA applied in the soma, as well as

GAD₆₇-IR is restricted to terminals around pyramidal cells (sp) and to a few in sl and stratum radiatum (sr). Calibration bars for panels 1 and 2 and lower array 25 μm; for 3 and 4, 50 μm. (D) Percentage of pyramidal cells that responded with a fast IPSP to DG activation in the presence of nbqx + apv at different ages (rhombuses). In adults, MF-GABAergic transmission can be induced in an activity-dependent manner (stim). GAD₆₇ immunoreactivity determined by densitometric analysis in CA3a (open squares) and in CA3b (filled squares) follows a similar trend. (E) Expression of VGAT gene transcripts in the isolated DGs at the depicted ages. The bar graph shows the VGAT/HPRT ratio obtained from the whole series of experiments (n = 3 by triplicate) evidencing the expression of VGAT mRNA when MF-GABAergic transmission can be detected, and its age-dependent down regulation. (Modified from Gutiérrez et al., 2003). (F) A1: Panoramic view of the MFs showing, on the left hand, two GFP⁺ GCs (arrow) and GAD₆₇⁺-MFs. The area shown at high magnification in A2 is signaled. A2: Confocal image of an axon of a GFP⁺ granule cell is shown along the stratum lucidum of CA3. (B) Confocal images (50 optical slices of 0.5 μm) of mossy fiber giant boutons expressing GAD₆₇ at 15 dpi. (C) MF boutons of GFP⁺ GCs at 30 dpi, did express GAD₆₇ enzyme. (D) Three-dimensional reconstructions through the Z-axis of GFP⁺ boutons (fifty optical slices at 0.5 μm intervals) were used to verify the co-localization of GFP and GAD₆₇. Orthogonal projections are shown on the right most panel (from Lara et al., 2012).

activation of interneurons that innervate the soma of the pyramidal cells, produce hyperpolarization in the soma. Thus, MF GABA, which is released in the proximal third of the apical dendrite has a reversal potential value in between those predicted by the distal dendritic and somatic values (Romo-Parra et al., 2008). We proposed that MF GABA is still serving a fine-tuning action on the synaptic connectivity in the dendrites and in the interneurons of *stratum lucidum*. The possible outcome is filtering the output of information from the MFs and, possibly too, turning synapses silent for brief periods, maybe in a region-specific or even cell target-specific manner (Galván and Gutiérrez, 2011). Interestingly, it has been shown that KCC2 is up-regulated by seizures and BDNF and in tissue from epileptic animals and humans, originating an intracellular accumulation of Cl^- , which renders GABA depolarizing (Blaesse et al., 2009).

In the adult rat, MF glutamate-only transmission excites both interneurons and pyramidal cells of CA3 but seizures or LTP up-regulate the GABAergic phenotype allowing the MF to release GABA together with glutamate probably to dampen the excitability provoked by the seizures (Gutiérrez and Heinemann, 2001, 2006) (Fig. 5C–E). These simultaneous subthreshold currents, followed by a disynaptic GABAergic IPSP, produce a long-lasting shunt of the postsynaptic potential. Obtaining the underlying synaptic currents from the voltage recordings during subthreshold stimulation (i.e., no firing of APs) allowed us to unravel a higher evoked net conductance in post-seizure hippocampi than in controls (Treviño et al., 2011). On the other hand, interneurons receiving such a dual signal can still be excited, despite the opposing signs of the currents, probably due to a higher release of glutamate than that of GABA (Romo-Parra et al., 2008; Treviño et al., 2011). Thus, the output of CA3 is tonically modulated by the continuous input from the MF plus GABA coming from the interneurons (Treviño et al., 2007). Moreover, the EPSP-spike coupling is less effective when the glutamatergic-GABAergic signals are time locked, which is the case when they come from the same releasing fibers, the MFs. Our data also suggested that a frequency-dependent summation of these currents occurred at preferred frequencies in epileptic or post-seizure preparations (Treviño et al., 2011). We explored whether the preference to fire at a given frequency depended on the transmission of information of the MFs or on the proneness of the pyramidal cells to follow such a frequency. We found that it was the axon (MF) conduction properties that were involved in this phenomenon and revealed a resonance phenomenon of axonal action potential conduction, which was strongly affected by activation of presynaptic and axonal ionotropic glutamate and GABA_A receptors (Franco et al., 2015).

7. Plasticity of the neurotransmitter phenotype of the granule cells born in the adult rodent

Let us consider a particular characteristic of the DG: its ability to continuously generate and incorporate granule cells throughout life. Newborn granule cells in the adult DG have distinctive characteristics, making them more excitable as they develop until they are integrated to the circuit (Schmidt-Hieber et al., 2004; Toni et al., 2008; Vivar and van Praag, 2013). So if granule cells have a plastic phenotype during postnatal development, would it be that they also go through the same changes when they are born and develop in an adult DG? We have provided evidence supporting this hypothesis. Developing granule cells born in the adult hippocampus transiently express the markers of both, glutamatergic and GABAergic phenotypes (Lara et al., 2012). The transient nature of the coexpression of both phenotypes can be reflected in the results presented by Toni et al. (2008), who were unable to observe GABA mediated transmission from granule cells born in the adult rodent, probably because they were recorded when they

were already integrated in the circuit as adult neurons, when they no longer express GABAergic markers. Interestingly, GCs proliferation in the adult rodent increase after seizures (Parent et al., 1997) and it has been shown that they overexpress GAD₆₇ (Jiang et al., 2004). We have proposed that these GCs that can be recruited into dynamic, existing circuits and can transiently exert inhibitory actions (Lara et al., 2012). Additionally, newborn GCs in culture conditions can express a GABAergic phenotype in a manner dependent on activity, and by kainate or BDNF exposure (Babu et al., 2007), as previously described to occur in cultured adult GCs (Gómez-Lira et al., 2005). Thus, the expression of the neurotransmitter phenotypes in the GCs born in the adult rodent recapitulate postnatal development, however, whether they release GABA as they do in the developing rodent (Beltrán and Gutiérrez, 2012) is still to be confirmed. An interesting suggestion, that can apply to the GCs in the hippocampus, is the possible existence of reserve pools of neurons that can respectify their neurotransmitter phenotype (Dulcis and Spitzer, 2011) on demand (Gómez-Lira et al., 2005).

8. Future avenues

The GCs must have a programmed and an activity-dependent molecular switch, which regulate the turning on and off of genetic (or epigenetic?) programs. Thus, one or several key players that control the phenotypic plasticity of the GCs are still to be identified. In other words, the big open question is: what is the molecular switch that turns on and off the GABAergic and/or glutamatergic phenotypes in these hippocampal granule cells?

For example, in the spinal cord, *Tlx3* and *Tlx1* function as the selector genes for the expression of the glutamatergic over a GABAergic phenotype (Cheng et al., 2004) and *Lbx1* actively promotes a GABAergic over a glutamatergic phenotype (Cheng et al., 2005). Likewise, *Otx2* was determined to depress GABAergic differentiation and thus controls the fate of glutamatergic progenitors in the thalamus (Puelles et al., 2006). However, all these molecular switches were determined to act during the embryonic state. Interestingly, the transcription factor *Prox1* specifies GC identity in the hippocampus, whereby its expression can direct otherwise pyramidal cells to granule cells phenotype (Iwano et al., 2012). Because *Prox1*, as well as *Tbr1*, are selectively expressed in glutamatergic neocortical and granule cells (Hevner et al., 2001; Bedogni et al., 2010; Hodge et al., 2012), it is tempting to speculate that they may be related to the cascade of molecular events that may direct the plastic expression of their dual phenotype.

Several other questions are open and need to be addressed to fully understand the functional significance of glutamate-GABA co-release. Are there epigenetic mechanisms that control the plasticity of the phenotype of the GCs? Are there, besides pathological conditions (seizures, epilepsy), “physiological” processes that regulate the expression of the complementary GABAergic phenotype in the adult, glutamatergic granule cells? Are there distinct populations of vesicles at an active zone? Are these vesicles directed to distinct release sites? Do biphasic miniature events occur? Is there a selective control of release of each neurotransmitter? What is the mechanism responsible for that?

Finally, surprising new evidence appeared adding to the described contents and function of the moss in these messy fibers. Indeed, MFs were shown to form electrical synapses with pyramidal cells, confirmed by the following concurring findings: they express connexin36 in the vicinity of neurotransmitter vesicles and of the post-synaptic glutamate receptors, injection of pyramidal cells with a dye that crosses through gap junctions results in labeling of MFs, their activation produces fast spikelets

followed by excitatory postsynaptic potentials in a number of pyramidal cells, the spikelets persist during blockade of chemical transmission and are suppressed by the gap junction blocker carbenoxolone (Vivar et al., 2012). Therefore, MFs can also form mixed electrical-chemical synapses (for a review see Münster-Wandowski et al., 2013). Can they become more exciting? ... or “inhibiting”?, or both?. In any case, they continue to be electrifying!

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Environment- and activity-dependent dopamine neurotransmitter plasticity in the adult substantia nigra

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ABSTRACT

The ability of neurons to change the amount or type of neurotransmitter they use, or 'neurotransmitter plasticity', is an emerging new form of adult brain plasticity. For example, it has recently been shown that neurons in the adult rat hypothalamus up- or down-regulate dopamine (DA) neurotransmission in response to the amount of light the animal receives (photoperiod), and that this in turn affects anxiety- and depressive-like behaviors (Dulcis et al., 2013). In this Chapter I consolidate recent evidence from my laboratory suggesting neurons in the adult mouse substantia nigra pars compacta (SNc) also undergo DA neurotransmitter plasticity in response to persistent changes in their electrical activity, including that driven by the mouse's environment or behavior. Specifically, we have shown that the amounts of tyrosine hydroxylase (TH, the rate-limiting enzyme in DA synthesis) gene promoter activity, TH mRNA and TH protein in SNc neurons increases or decreases after ~20 h of altered electrical activity. Also, infusion of ion-channel agonists or antagonists into the midbrain for 2 weeks results in ~10% (~500 neurons) more or fewer TH immunoreactive (TH+) SNc neurons, with no change in the total number of SNc neurons (TH+ and TH-). Targeting ion-channels mediating cell-autonomous pacemaker activity in, or synaptic input and afferent pathways to, SNc neurons are equally effective in this regard. In addition, exposing mice to different environments (sex pairing or environment enrichment) for 1–2 weeks induces ~10% more or fewer TH+ SNc (and ventral tegmental area or VTA) neurons and this is abolished by concurrent blockade of synaptic transmission in midbrain. Although further research is required to establish SNc (and VTA) DA neurotransmitter plasticity, and to determine whether it alters brain function and behavior, it is an exciting prospect because: (1) It may play important roles in movement, motor learning, reward, motivation, memory and cognition; and (2) Imbalances in midbrain DA cause symptoms associated with several prominent brain and behavioral disorders such as schizophrenia, addiction, obsessive-compulsive disorder, depression, Parkinson's disease and attention-deficit and hyperactivity disorder. Midbrain DA neurotransmitter plasticity may therefore play a role in the etiology of these symptoms, and might also offer new treatment options.

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1. Introduction

Long-lasting changes in the brain or 'brain plasticity' underlie adaptive behavior or learning and memory in healthy individuals, and symptom etiology and brain repair in neurological disease or injury. Established forms of brain plasticity such as neurogenesis, neurodegeneration and synaptic plasticity arise following stimuli that trigger biochemical signaling leading to altered gene and protein expression inside cells, altered cell phenotype, and ultimately brain function and behavioral changes. Notionally, these generic mechanisms could encompass any gene or protein,

so it is likely that undiscovered or less-established forms of brain plasticity exist.

Neurotransmitter plasticity, the topic of this Special Issue, is a less-established form of brain plasticity that at its core comprises altered expression of genes and/or their protein products necessary for neurons to communicate using a particular neurotransmitter. This has the potential to alter neurotransmission in the brain and thereby brain function and behavior. Indeed, Dulcis et al. (2013) recently showed that neurons in the adult rat hypothalamus up- or down-regulate dopamine (DA) synthesis and neurotransmission in response to the amount of light exposure the animal receives (photoperiod), and that this in turn affects anxiety- and depressive-like behaviors. Thus, present indications are that neurotransmitter plasticity is a legitimate new form of adult brain plasticity with significant implications for brain function and behavior.

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In this Chapter I consolidate recent evidence from my laboratory suggesting environment- and activity-dependent DA neurotransmitter plasticity occurs also in the adult substantia nigra pars compacta (SNc) and ventral tegmental area (VTA) of the midbrain. Midbrain DA plays important roles in movement, motor learning, reward, motivation, memory and cognition; therefore midbrain DA neurotransmitter plasticity could potentially affect these behaviors. Furthermore, imbalances in midbrain DA cause symptoms associated with several prominent brain and behavioral disorders such as schizophrenia, addiction, obsessive-compulsive disorder (OCD), depression, Parkinson's disease (PD) and attention-deficit and hyperactivity disorder (ADHD). Therefore midbrain DA neurotransmitter plasticity may play a previously unrecognized role in the etiology of these symptoms and, more significantly, may offer new treatment options. In these lights I also review the cytoarchitecture and neurotransmitter phenotypes of SNc neurons with a view to helping identify those cells that are capable of DA neurotransmitter plasticity. This is crucial for verifying SNc DA neurotransmitter plasticity, as well as determining its cellular mechanisms. I conclude with a catalog of outstanding questions to help guide future research on the subject, including a summary of how I believe SNc DA neurotransmitter plasticity occurs and what its neurobiological and behavioral consequences are likely to be. This is helpful for designing future experiments to test whether SNc DA neurotransmitter plasticity is neurobiologically and behaviorally relevant.

2. Terminology

Before proceeding, it is helpful to define some terminology.

I define 'DA phenotype' as cellular expression of genes and their protein products that are necessary to synthesize and handle DA as a neurotransmitter [e.g. tyrosine hydroxylase (TH), aromatic L-amino acid decarboxylase (AADC), guanosine triphosphate cyclohydrolase (GTPCH), vesicular monoamine transporter (vMAT), DA transporter (DAT)]. I sometimes refer to these molecules as 'DA genes' or 'DA proteins'.

Also, in literature on the subject of neurotransmitter plasticity generally, the term 'neurotransmitter switching' is sometimes used. I view neurotransmitter switching as an extreme form of neurotransmitter plasticity, wherein expression of neurotransmitter genes or proteins is up-regulated from zero, i.e. 'switched on', or down-regulated to zero, i.e. 'switched off'. Nonetheless, any distinction between plasticity and switching is incidental because: (1) Current technologies are not sufficiently sensitive to distinguish whether a gene or protein has been switched completely on or off *versus* regulated above or below detection threshold; and (2) Either way there are large changes in the amounts of neurotransmitter genes or proteins in cells, which are likely to lead to large changes in neurotransmission and therefore brain function and behavior. In the interests of accuracy, clarity and consistency I will use 'neurotransmitter plasticity' rather than 'neurotransmitter switching' throughout most of this Chapter, although the two are sometimes interchangeable.

Another important point is 'neurotransmitter switching' can mean switching one (or more) neurotransmitter(s) on or off in a cell independent of changes in a different neurotransmitter(s) in the same cell, or it can mean a cell switching between different neurotransmitters, e.g. switching one neurotransmitter off and another neurotransmitter on. In our work so far we have evidence only of DA neurotransmitter plasticity in midbrain neurons, so it is presently unclear which of these two possibilities pertain.

3. Possible dopamine neurotransmitter plasticity following 6-OHDA substantia nigra lesions in rodents and in Parkinson's disease

I begin here because my laboratory's interest in DA neurotransmitter plasticity developed out of my colleagues' studies of axon sprouting by SNc neurons that survive partial 6-hydroxy-DA (6-OHDA; a neurotoxin that specifically kills DA neurons and is often used to model PD) SNc lesions in adult rats (Stanic et al., 2003). In addition to sprouting, they reported an early-phase loss of ~2500 TH immuno-positive (TH+) and concurrent gain of ~2500 TH immuno-negative (TH-) SNc neurons [2–16 weeks after lesion in Fig. 4b of (Stanic et al., 2003)], as well as a later-phase gain of ~2500 TH+ and concurrent loss of ~2500 TH- neurons [16–32 weeks after lesion in Fig. 4b of (Stanic et al., 2003)]. Both phases are consistent with changes or plasticity in the amount of TH protein in cells, specifically loss then re-acquisition of TH by the same SNc neurons. Transient loss of TH immunoreactivity in SNc neurons has also been reported following striatal infarction subsequent to transient focal cerebral ischemia (Soriano et al., 1997). Combinations of neurodegeneration and neurogenesis are unlikely to account for these changes because the weight of evidence indicates neurogenesis does not occur in the adult rodent SNc [(Aponso et al., 2008; Chen et al., 2005; Frielingsdorf et al., 2004; Lie et al., 2002; Peng et al., 2008; Yoshimi et al., 2005) but see (Zhao et al., 2003)].

In adult humans, and particularly those with PD, there is also evidence of SNc DA neurotransmitter plasticity. TH mRNA levels vary in different SNc neurons in both normal and PD brains (Javoy-Agid et al., 1990; Kingsbury et al., 1999). While low TH expression could be a pre-morbid state (Javoy-Agid et al., 1990), the similar number of cells with low TH in normal and PD brains (Kingsbury et al., 1999) suggests low TH has physiological significance. In normal brains the number of SNc cells expressing DA markers (TH, DAT and GTPCH) progressively decreases with age (Chen et al., 2000; Chu et al., 2002; Emborg et al., 1998; Ma et al., 1999) whereas the number of SNc cells containing neuromelanin (another marker of DA cells) does not change (Chu et al., 2002), indicating TH expression can be lost independent of neurodegeneration. With respect to up-regulation of DA genes and proteins, neuromelanin-negative cells have more TH mRNA in PD than control brains (Kingsbury et al., 1999), and surviving SNc neurons have increased TH but decreased DAT mRNA in PD compared with control brains (Joyce et al., 1997). Note that increased TH and decreased DAT appear also in SNc neurons following 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP, another neurotoxin that specifically kills DA neurons and is often used to model PD) administration in cats, and is associated with recovery of motor function in these animals [(Rothblat et al., 2001), see also (Blanchard et al., 1995)]. Thus, plasticity of DA genes may reflect compensatory upregulation of DA neurotransmission by fewer SNc neurons.

4. Substantia nigra dopamine neurotransmitter plasticity is activity-dependent

Prompted by the above suggestions of plasticity of TH expression in SNc neurons, we documented the functional properties, i.e. electrophysiology, of SNc neurons undergoing putative DA neurotransmitter plasticity following 6-OHDA lesions in mice (Aumann et al., 2008). We performed whole-cell patch clamp recordings of individual SNc cells in *ex vivo* midbrain slices that were acutely prepared at different times after 6-OHDA. Their TH expression status was determined by single-cell reverse transcriptase polymerase chain reaction (RT-PCR) of a sample of cytoplasm that was aspirated into the tip of the recording

micropipette immediately following electrophysiological characterization. We found a very strong correlation between the electrophysiology of SNc neurons and the presence and absence of TH mRNA. Cells with TH mRNA (TH+) exhibited spontaneous pacemaker activity in the form of slow oscillatory membrane potential fluctuations with or without an action potential on each depolarizing phase. At resting membrane potential these oscillations occurred at ~5 Hz, and the rate of action potential discharge in these cells was only slightly increased upon membrane depolarization. TH+ neurons also had relatively long duration action potentials, relatively prominent action potential after-hyperpolarizations that were mediated in part by apamin-sensitive small conductance calcium-activated potassium (SK) channels, and hyperpolarization-activated cyclic nucleotide-gated non-specific cation currents (HCN or Ih). In contrast, cells without TH mRNA (TH–) had no pacemaker activity at any level of membrane polarization, and they discharged action potentials at very high rates upon membrane depolarization. TH– neurons also had shorter duration action potentials, smaller spike after-hyperpolarizations with no SK component, and little or no HCN [see Fig. 4C in (Aumann et al., 2008)]. These two different electrophysiological phenotypes are consistent with those described for DA and non-DA SNc neurons in other species also [e.g. birds (Gale and Perkel, 2006), guinea pigs (Yung et al., 1991) and rats (Grace and Onn, 1989; Lacey et al., 1989)].

A crucial question arising from the above findings was whether these different electrophysiologies represent different cell-types with stationary phenotypes, or the same cell in different phenotypic states. As described later in this Chapter SNc contains DA nigrostriatal projection neurons as well non-DA and probably GABA interneurons and the two electrophysiologies could simply correspond to these. However, I propose they correspond to, and indeed cause, the same cell-type to have different neurotransmitter phenotypes, i.e. DA and non-DA. Support for this comes from Rodriguez and Gonzalez-Hernandez (1999) who reported evidence that nigrostriatal projection neurons indeed have different electrophysiological and neurotransmitter phenotypes. Specifically, they found 87% of antidromically identified nigrostriatal projection neurons display long duration action potentials (~2–6 ms, extracellular), slow conduction velocities (~0.5 m s⁻¹), and low (~3.5 Hz), stable rates of discharge, whereas 13% have short duration action potentials (<1 ms), faster conduction velocities (~3.5 m s⁻¹), and higher (~12.5 Hz) and more variable rates of discharge. They also reported indirect evidence that the larger population was DA and the smaller population non-DA (Rodriguez and Gonzalez-Hernandez, 1999). Others have also reported evidence that 5–15% of SNc projection neurons are non-DAergic (detailed later in the Chapter). Thus, it could be that nigrostriatal neurons exhibiting the DA phenotype have low rates of spontaneous pacemaker discharge, longer duration action potentials, SK mediated after-hyperpolarizations and HCN currents, whereas those without the DA phenotype have higher rates of sporadic or intermittent discharge, shorter duration action potentials, smaller after-hyperpolarizations with no SK component, and little or no HCN current. Indeed it may be that these different electrophysiologies play causal roles in the amounts of DA gene expression and DA proteins in nigrostriatal neurons *via* activity- and calcium-dependent mechanisms.

A causal relationship between SNc neuronal activity and putative DA neurotransmitter plasticity would necessitate altered DA gene expression in response to changes in neuronal activity. Indeed there are a large number of reports of causal relationships between neuronal activity, intracellular calcium, and TH gene and protein expression in other catecholaminergic cells and neurons [reviewed in (Aumann and Horne, 2012)]. Furthermore, pacemaker activity in SNc DA neurons is unusual in that it is driven largely by

calcium fluxes through voltage-activated calcium channels (Chan et al., 2007; Gale and Perkel, 2006; Guzman et al., 2009; Mercuri et al., 1994; Nedergaard et al., 1993; Ping and Shepard, 1996; Puopolo et al., 2007; Shi, 2009; Takada et al., 2001; Wolfart et al., 2001; Wolfart and Roeper, 2002) as opposed to sodium fluxes through voltage-activated sodium channels, which is more commonly observed pacemaker mechanism. In these lights it is reasonable to hypothesize that the rate of pacemaker activity in SNc neurons regulates their amount of DA gene expression *via* calcium-dependent cell signaling [see (Aumann and Horne, 2012)].

To test this hypothesis we incubated mouse midbrain slices in the specific SK channel antagonist apamin (or vehicle) chronically for up to 24 h then measured the amount of TH mRNA using reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) or the amount of TH protein using western blot (Aumann et al., 2011). We had shown in our electrophysiology experiments using this same preparation that apamin increases the rate of pacemaker activity in SNc neurons acutely, so we predicted midbrain slices chronically incubated in apamin would have different amounts of TH mRNA and protein compared with vehicle-treated slices at some time-point. Presumably it would take time for altered activity to effect differences in TH gene expression and TH protein levels. Indeed the amount of TH mRNA was significantly higher in apamin-treated slices sometime between 12 and 24 h later, and TH protein was significantly higher in apamin-treated slices sometime between 3 and 24 h later (Aumann et al., 2011). This supports the hypothesis that DA gene and protein expression in SNc (and VTA) neurons is regulated by their electrical activity. However the direction of these changes, i.e. increase, was puzzling because we had also shown in our electrophysiology experiments that cells with lower rates of pacemaker activity are TH+ whereas cells with no pacemaker activity but higher rates of activity upon depolarization are TH– (Aumann et al., 2008). This predicts apamin-treated slices should have less TH mRNA and TH protein than vehicle-treated slices, a point of discussion I return to later in the Chapter. In another experiment designed to interrogate activity-dependent regulation of TH gene expression, we applied the voltage-activated sodium channel blocker tetrodotoxin (TTX) (or vehicle) to primary cultures of midbrain neurons that had been genetically modified to express green fluorescent protein (GFP) in concert with TH gene promoter activity. TTX abolished all action potential activity in these neurons and also caused decreased GFP expression beginning 21 h later (Aumann et al., 2011). Overall, these experiments indicate that sustained increases and decreases in the rate of action potential discharge in SNc neurons lead, respectively, to increased and decreased TH gene and protein expression beginning ~20 h later.

To further test whether neuronal activity regulates DA neurotransmitter plasticity we infused apamin (or vehicle) directly into the midbrain of adult mice for 2 weeks using an osmotic minipump and brain infusion cannula whose tip was implanted ~1 mm above SNc on one side of the brain [Fig. 1A and (Aumann et al., 2011, 2008)]. The mice were then killed and midbrain sections were processed for TH immunohistochemistry and counterstained for Nissl substance [Fig. 1A and B, (Aumann et al., 2011, 2008)]. The numbers of SNc neurons that were immunopositive for TH (TH+) and immunonegative for TH but positive for Nissl substance (TH–) were then estimated using unbiased stereological methods by an observer blind to the treatment group [Fig. 1B and (Aumann et al., 2011, 2008)]. Glial cells were identified as positive for Nissl substance but with soma diameter <5 µm, and were excluded from the counts [Fig. 1B and (Aumann et al., 2011, 2008)]. Apamin resulted in ~500 fewer TH+ SNc neurons and ~500 more TH– SNc neurons with no change in total number of SNc neurons (TH+ and TH– combined) [Fig. 1C and (Aumann et al., 2011, 2008)]. This is consistent with reduction of TH

Fig. 1. Changes in numbers of tyrosine hydroxylase immunopositive (TH+, black bars) and immunonegative (TH-, white bars) SNc cells following 2-weeks of continuous infusions of ion-channel agonists or antagonists from osmotic minipumps attached to implanted cannulae into the midbrain in adult mice. **A** Low-power photomicrograph of ventral midbrain following infusion of the SK agonist 1-EBIO (100 μM, SK↑), TH immunohistochemistry and Nissl counterstain. Note the location of the tip of the infusion cannula immediately above the LHS SNc, and the increased number of TH+ SNc cells in the LHS SNc compared with the RHS (uninfused) in this section. **B** High-power photomicrograph showing examples of TH+, TH- and glial (gl) SNc cells. **C** Mean (±SE) change in number of SNc cells (infused or LHS SNc minus control or RHS SNc) in mice receiving the different treatments. Treatments were as follows from left to right: vehicle ($n=21$); SK agonist 1-EBIO (100 μM, SK↑, $n=5$); SK agonist riluzole (30 μM, SK↑, $n=5$); SK antagonist apamin (300 nM, SK↓, $n=5$); L-type voltage-activated Ca^{2+} channel agonist FPL64176 (100 nM, L-type↑, $n=5$); L-type Ca^{2+} channel antagonist nimodipine (10 μM, L-type↓, $n=4$); high extracellular K^+ (30 mM, K^+ ↑, $n=4$); high extracellular K^+ plus apamin (30 mM, K^+ ↑ and 300 nM SK↓, $n=3$); GABA_A receptor agonist muscimol (20 μM, GABA_A↑, $n=4$); GABA_A antagonist picrotoxin (100 μM, GABA_A↓, $n=5$). Notes: (1) the drug concentrations are those in the minipumps and would be lower in the midbrain; and (2) separate groups of vehicle infused mice were run concurrently with each drug (SK drugs, L-type Ca^{2+} drugs, high K^+ etc.) throughout this study and no statistically significant differences between left and right SNc ($p < 0.01$, z-test) were detected in numbers of TH+, TH- or total (TH+ and TH-) cells in any vehicle-treated group; vehicle groups have therefore been combined in this figure. Statistically significant changes ($p < 0.01$, z-test) are indicated with an asterisk. There was a trend for K^+ ↑ and SK↓ combined infusions to have greater effects than K^+ ↑ alone but these were not significantly different ($ns = p > 0.05$, t-tests). Modified from (Aumann et al., 2011) with permission.

protein levels to below immunohistochemically detectable levels in ~500 SNc neurons, which is ~10% of the normal number of SNc neurons on each side of the brain in this species. It is unlikely to reflect degeneration of ~500 TH+ and genesis of ~500 TH- neurons due to the lack of midbrain neurogenesis detailed earlier. Note that this putative change in level of TH protein, i.e. decrease, is in the opposite direction to what we observed in our *ex vivo* midbrain slice experiments, i.e. increase (Aumann et al., 2011), but is in the same direction predicted by our electrophysiology combined with single-cell RT-PCR experiments (Aumann et al., 2008), a point of discussion I return to later in the Chapter.

This ability to reduce the number of TH+ SNc neurons by infusing apamin led us to next test whether we could increase the number of these neurons. Our earlier work had hinted that drugs that produced opposite changes in SNc neuronal activity could also produce opposite changes in DA gene expression. Therefore we infused an SK channel agonist [1-Ethylbenzimidazolinone (1-EBIO) or riluzole or vehicle] into the midbrain continuously for 2 weeks and counted the number of TH+ and TH- SNc neurons. Indeed both drugs produced ~500 more TH+ and ~500 fewer TH- SNc neurons [Fig. 1C and (Aumann et al., 2011, 2008)], which is consistent with gain of TH protein expression by ~500 SNc neurons. These data add further support to the hypotheses that DA neurotransmitter plasticity occurs in the adult SNc, that it is activity-dependent, and that it is bi-directional. Note that riluzole also antagonizes voltage-activated sodium channels and glutamatergic neurotransmission, so its effects on the number of TH+ SNc neurons could be *via* these alternate, albeit still activity-dependent mechanisms.

We next asked whether other ion-channels could also be targeted to regulate the number of TH+ SNc neurons *in vivo*. We first targeted L-type voltage-activated calcium channels, which have been proposed as a major source of calcium entering SNc neurons during pacemaking (Chan et al., 2007; Mercuri et al., 1994; Nedergaard et al., 1993; Puopolo et al., 2007). Infusing the L-type calcium agonist FPL64176 resulted in 500–1000 fewer TH+ and 500–1000 more TH- SNc neurons [Fig. 1C and (Aumann et al., 2011)]. Conversely infusing the L-type antagonist nifedipine produced ~150 more TH+ neurons, although this was not statistically significant, as well as ~100 fewer TH- neurons, which was significant (unpublished data). Interestingly another L-type antagonist nimodipine reduced the number of TH+ neurons to ~50% of normal, i.e. ~2500 fewer cells, and also reduced the number of TH- neurons [Fig. 1C and (Aumann et al., 2011)]. Thus the effects of nimodipine are less consistent with putative DA neurotransmitter plasticity and more consistent with cell death, although the result might reflect an artifact of our stereological methods, which I discuss later in the Chapter. Thus, targeting either SK or L-type calcium channels, both of which are involved in generating SNc pacemaker activity, change the number of SNc TH+ neurons in a way consistent with DA neurotransmitter plasticity.

Another way to increase neuronal activity is by applying a higher than normal concentration of potassium (K^+) ions extracellularly. Two weeks' infusion of high K^+ resulted in ~500 fewer TH+ and ~500 more TH- SNc neurons, the same sign and magnitude of effect as apamin [Fig. 1C and (Aumann et al., 2011)]. Also, apamin and high K^+ infused together produced the same sign

and magnitude of effect as either agent alone, indicating both share downstream mechanisms [Fig. 1C and (Aumann et al., 2011)].

In addition to pacemaker activity, SNc DA neurons exhibit phasic increases (bursts) or decreases (pauses) in activity that are driven by synaptic inputs and environmental or behaviorally salient cues [e.g. (Kitai et al., 1999; Schultz, 2007; Tepper and Lee, 2007)]. We next tested whether synaptic inputs regulate putative DA neurotransmitter plasticity in SNc neurons by infusing drugs targeting GABA_A receptors, which mediate the majority (~70%) of fast synaptic inputs to these cells (Henny et al., 2012; Lee and Tepper, 2009). Infusing the GABA_A receptor agonist muscimol into midbrain continuously for 2 weeks resulted in ~500 fewer TH+ and ~500 more TH– SNc neurons, again the same sign and magnitude of effect produced when pacemaker activity was targeted via SK or L-type calcium channels [Fig. 1C and (Aumann et al., 2011)]. These data indicate that synaptic input, and therefore afferent pathways to SNc neurons, are at least as potent as pacemaker activity in regulating putative DA neurotransmitter plasticity and the number of SNc DA neurons.

The apparent involvement of GABAergic synaptic input in SNc DA neurotransmitter plasticity raised the question of whether and which afferent pathways are involved. Most GABAergic synapses on SNc neurons are derived from the direct and indirect striatonigral pathways (Lee and Tepper, 2009; Tepper and Lee, 2007), which originate from D1 DA receptor (D1R) expressing and D2 DA receptor (D2R) expressing striatal medium spiny neurons (MSNs), respectively. We therefore tested the influence of these pathways by infusing D1R and D2R agonists and antagonists into the striatum for 2 weeks. Only the D2R agonist quinpirole produced a pattern consistent with putative DA neurotransmitter plasticity in SNc, i.e. equal magnitude but opposite direction changes in TH+ and TH– neurons (Aumann et al., 2011). Indeed, striatal quinpirole produced the largest increase in TH+ SNc neurons we have observed so far, an average ~3000 or ~60% more neurons than normal. By contrast, striatal infusions of the D2R antagonist amisulpride had no effect on the number of TH+ or TH– SNc neurons, and striatal infusions of the D1R agonist dihydrexidine or the D1R antagonist SCH39166 had very small or no effects on the number of TH+ SNc neurons, or produced decreases in the number of SNc TH– neurons only (Aumann et al., 2011).

The apparent change in number of SNc DA neurons produced by striatal quinpirole infusion could have been mediated through post-synaptic D2Rs expressed on MSNs and altered activity in the indirect striatonigral pathway, or through D2 autoreceptors expressed pre-synaptically on nigrostriatal synapses leading to a retrograde influence on TH gene or protein expression in nigrostriatal cell bodies. There is evidence that the amount of extracellular DA binding to nigrostriatal D2 autoreceptors regulates the size of nigrostriatal axonal arbors and the number of synapses they form (Parish et al., 2002), which probably requires large-scale gene expression changes including DA genes such as TH. However, if D2 autoreceptors are involved in SNc DA neurotransmitter plasticity, quinpirole infusion in midbrain and striatum should have produced similar differences in numbers of TH+ and TH– SNc neurons because D2 autoreceptors are also expressed on nigrostriatal cell bodies. This was not the case however with midbrain quinpirole infusion producing no differences in numbers of TH+ or TH– SNc neurons (Aumann et al., 2011), arguing against involvement of D2 autoreceptors and for a role for post-synaptic D2Rs and the indirect striatonigral pathway in regulating SNc DA neurotransmitter plasticity.

To summarize, at this juncture our findings support a model whereby TH gene and protein expression in adult SNc neurons is regulated by the rate of cell-autonomous pacemaker activity, as well as by burst and pause activity driven by afferent pathways and synaptic inputs to SNc neurons, particularly the indirect

striatonigral pathway. The involvement of afferent pathways is particularly interesting because it implicates cues in our environment or aspects of our behavior that are relayed via afferent pathways to SNc neurons in regulating SNc DA neurotransmitter plasticity.

Before turning to evidence that environment and/or behavior do indeed regulate SNc neurotransmitter plasticity, I will discuss in more detail what we understand so far about the precise relationship between SNc neuronal activity and TH expression. If activity-dependent SNc DA neurotransmitter plasticity does play a role in the etiology of symptoms of midbrain DA imbalances, and especially if it is ever to be harnessed therapeutically, it will be important to know precisely which patterns of activity increase, and which patterns of activity decrease, the amount of nigrostriatal DA.

5. Relationship between substantia nigra neuronal activity and tyrosine hydroxylase expression

The relationship between neuronal activity and TH expression in SNc neurons is an open question that is important for elucidating mechanisms of putative SNc DA neurotransmitter plasticity as well as its potential involvement in symptom etiology and treatment. As detailed above, we have data showing TH+ SNc neurons exhibit ~5 Hz pacemaker activity, longer duration action potentials, SK-mediated after-hyper-polarizations and HCN (or Ih) currents, whereas TH– neurons do not have pacemaker activity, discharge at much higher rates when depolarized, have shorter duration action potentials, smaller after-hyper-polarizations with no SK component, and little or no HCN current [(Aumann et al., 2008); see also (Gale and Perkel, 2006; Grace and Onn, 1989; Lacey et al., 1989; Yung et al., 1991)]. This may indicate an inverse or negative relationship wherein low rates of activity are associated with high TH expression whereas high rates of activity are associated with low or no TH expression.

Other findings of ours also lend support to an inverse or negative relationship. Administration of drugs that ostensibly ought to increase the rate of SNc neuronal discharge, i.e. apamin, FPL64176, high K⁺, resulted in fewer TH+ neurons and more TH– neurons [Fig. 1C and (Aumann et al., 2011)], consistent with down-regulation of TH expression to below detectable levels in some cells. Conversely, infusions of drugs that ostensibly ought to decrease SNc neuronal discharge, i.e. 1-EBIO and riluzole, produced more TH+ neurons and fewer TH– neurons [Fig. 1C and (Aumann et al., 2011)], consistent with up-regulation of TH expression above detectable levels in some cells. However, recall that the mode of drug administration in these experiments was infusion into midbrain where the drugs could influence SNc neuronal activity directly via ion-channels or receptors expressed on SNc neurons, as well as indirectly via ion-channels or receptors expressed on neurons pre-synaptic to SNc neurons. For example, our apamin infusions probably increased the activity of SNc DA neurons directly, but probably also increased the activity of substantia nigra pars reticulata (SNr) neurons which provide inhibitory GABAergic synaptic input to SNc neurons, and thereby also decreased the activity of SNc neurons indirectly [see (Tepper and Lee, 2007)]. Likewise, our muscimol infusions probably decreased the activity of SNc neurons directly, but probably also decreased the activity of SNr neurons thereby dis-inhibiting, i.e. increasing, the activity of SNc neurons indirectly. Thus the net effects of our apamin, muscimol, and other drug infusions could be increases, decreases or no change in SNc neuronal activity, and only direct recordings of such activity during the infusions can resolve this.

On the other hand there are good reasons to posit that neuronal activity and TH expression in SNc neurons are positively correlated. There are many reports of positive correlations between rates of

activity, amounts of intracellular calcium, and amounts of TH expression in catecholaminergic cells generally [reviewed in (Aumann and Horne, 2012)], so the same relationship seems most likely in SNc neurons. Also, some of our own data support a positive relationship in SNc neurons. As detailed earlier in the Chapter we have shown that TH mRNA and protein are levels are increased in chronically apamin-treated *ex-vivo* midbrain slices (Aumann et al., 2011), which we know increases SNc neuronal activity in this preparation (Aumann et al., 2008). Also, the amount of GFP in cultured TH– GFP midbrain neurons, which is an indicator of TH gene promoter activity, decreases following addition of TTX (Aumann et al., 2011), a treatment we know abolishes action potential discharge in these cells (unpublished data). In addition, following our *in vivo* drug infusions we have shown that fewer TH+ SNc neurons are associated with higher TH immunoreactivity of remaining TH+ SNc neurons, and conversely, more TH+ SNc neurons are associated with lower TH immunoreactivity of TH+ SNc neurons (Aumann et al., 2011). In other words, infusing any one of a number of a number of different ion-channel agonists/antagonists has two effects on SNc neurons, each of which indicates opposing changes in TH expression. It is presently unclear which effect, *i.e.* difference in number of TH+ cells or difference in TH immunoreactivity, occurs first and is therefore most likely the direct effect of altered neuronal activity.

In summary, the most parsimonious explanation is that activity-dependent regulation of TH expression in SNc neurons

follows the same or similar rules as that in other catecholaminergic cells, namely that increases in neuronal activity and intracellular calcium cause increased TH expression, and decreases in neuronal activity and intracellular calcium cause decreased TH expression. This is then followed respectively by down- or up-regulation of TH expression in some SNc cells, evidenced by fewer or more TH+ SNc neurons, to maintain DA levels constant overall, *i.e.* homeostasis. Regardless, both effects qualify as DA neurotransmitter plasticity, albeit with probably different underlying mechanisms. It will be important to verify this and to ascertain the mechanisms of both because if either is to be harnessed therapeutically, any accompanying homeostatic response may need to be blocked to enable changes in nigrostriatal DA neurotransmission overall.

6. Midbrain dopamine neurotransmitter plasticity is environment- dependent

The dependence of the number of SNc TH+ and TH– neurons on GABA_A receptor function and afferent pathways indicated that aspects of our environment or behavior might influence SNc DA neurotransmitter plasticity. To test this we next exposed adult mice to different environments continuously for 1–2 weeks and counted the number of SNc (and VTA) TH+ and TH– neurons (immunohistochemistry). SNc neurons discharge bursts of action potentials in response to salient environmental cues such as rewards or stimuli that associate with rewards [e.g. (Schultz, 1998)]. Therefore, we

Fig. 2. Environmentally/behaviorally-induced differences in the number of tyrosine hydroxylase immunopositive (TH+) neurons in the adult mouse SNc. **A** Sex-pairing for 1 week. There was significantly (two-way ANOVA with factors sex and mating, $*p < 0.05$ Tukey multiple comparisons) more (Mean $\pm$ SE) TH+ SNc neurons in males paired with females (mated male, black bar) compared with male–male controls (white bar), and significantly fewer TH+ SNc neurons in females paired with males (mated female, black bar) compared with female–female controls (white bar). Note, the same differences were found in the ventral tegmental area (VTA) but not in the locus coeruleus (LC), where TH is used to synthesize noradrenaline. **B** Environment enrichment for 2 weeks. There was significantly (one-way ANOVA, $*p < 0.05$ Tukey multiple comparisons) more (Mean $\pm$ SE) TH+ SNc neurons in environmentally enriched (EE, black bar) male mice than in standard housed (SH, white bar) and running wheel only (RW, gray bar) male mice. Note, the same differences were also found in the VTA but not in the LC. **C** GABA_A receptor blockade completely abolishes the EE-induced increase in number of TH+ SNc neurons. Left: Mean $\pm$ SE number of TH+ SNc neurons in SH (white bars) and EE (black bars) male mice subjected to concurrent vehicle, picrotoxin (10 μ M) or bicuculline (5 μ M) midbrain infusion. As in B, EE significantly (two-way ANOVA with factors environment and drug, $*p < 0.05$ Tukey multiple comparisons) increased the number of TH+ SNc neurons in vehicle-infused mice. However, GABA_A receptor blockade with either antagonist completely abolished this increase. Right: Photomicrographs of sections through the ventral midbrain at approximately the same rostrocaudal level showing TH immunoreactive neurons (black reaction product) in an SH and EE mouse receiving vehicle infusions (top row), and an SH and EE mouse receiving bicuculline infusions (5 μ M, bottom row). * = damage caused by the cannula tip (which is not seen in two of the sections because the cannula was implanted outside of the plane of section). SNr = substantia nigra pars reticulata.

Modified from (Aumann et al., 2013) with permission.

exposed mice to what we considered would be rewarding environments. The first environment (termed ‘sex-pairing’) comprised pairing males and females together (2 mice/cage) continuously for 1 week in otherwise standard housing conditions. Comparison groups (controls) were male–male pairs and female–female pairs. We found ~350 more TH+ SNc neurons in males paired with females compared with male–male controls, and ~900 fewer TH+ SNc neurons in females paired with males compared with female–female controls [Fig. 2A and (Aumann et al., 2013)]. Furthermore, in the adjacent VTA we also found more TH+ neurons in males paired with females compared with male–male controls, and fewer TH+ neurons in females paired with males compared with female–female controls (Aumann et al., 2013). Thus, the phenomenon extended to VTA, which was not surprising given VTA DA plays a key role in higher-level aspects of behavior such as reward, motivation and decision-making, all of which are expected to vary across the different sex-pairings. By contrast, there were no differences in the numbers of TH+ neurons in the locus coeruleus (LC) (Aumann et al., 2013), where TH is used to synthesize noradrenaline instead of DA. Thus, sex-pairing regulation of the number of TH+ cells is specific to midbrain, at least to this extent.

While the sex-pairing result demonstrated environmental influence over the number of midbrain TH+ neurons, it shed little light on the identity of the salient environmental cue(s). They could have been, for example, sensory (e.g. sight, sound, touch, smell, taste), hormonal (e.g. pheromones, sex hormones, stress) and/or behavioral (e.g. motor, cognitive, emotive). SNc DA plays a key role in motor behavior and motor learning so we next designed environments that promoted motor behavior and motor learning over some of these other environmental cues. Specifically, adult male mice ($n=6$) were housed together in one of 3 different environments:

1. Standard housed (SH), comprising a large cage with only bedding, food and water;
2. Running wheel (RW), comprising SH plus running wheels to stimulate habitual motor activity;
3. Environment-enriched (EE), comprising RW plus toys, ladders, ropes, etc. In addition, these EE mice received ‘super enrichment’ comprising placement into a larger cage containing novel toys for 1 h/day, 5 days/week to stimulate novel motor activity (motor learning).

Immediately following continuous exposure to these environments for 2 weeks, EE mice had ~800 more TH+ SNc neurons than SH mice. RW mice also had more (~300) TH+ SNc neurons than SH mice, but this was not significantly different [Fig. 2B and (Aumann et al., 2013)]. As was seen in the sex-pairing experiments, this difference in number of TH+ cells occurred also in VTA, but not in LC, demonstrating a degree of midbrain specificity. These findings corroborate those in the sex-pairing experiment with respect to environmental influence over the number of midbrain TH+ neurons. They also highlight motor activity, and in particular motor learning, as providing salient environmental cue(s) regulating the number of midbrain TH+ neurons.

To test the potential role of afferent pathways and synaptic input in relaying salient environmental cues to SNc neurons to alter the number of TH+ neurons, we next exposed groups of adult male mice to 2 weeks of continuous SH or EE while they also received midbrain infusion of a GABA_A receptor antagonist (bicuculline or picrotoxin or vehicle). In vehicle-infused mice, EE again produced ~850 more TH+ SNc neurons, confirming the EE influence [Fig. 2C and (Aumann et al., 2013)]. However, this increase was completely abolished in EE mice receiving either of the two GABA_A antagonists [Fig. 2C and (Aumann et al., 2013)]. Thus normally functioning midbrain GABA_A receptors are essential for EE-induction of more SNc TH+ neurons, which implicates afferent pathways in environmental-induction of putative SNc DA neurotransmitter plasticity.

Notably, in many of the above sex-pairing and EE experiments, the differences in number of TH+ neurons were not accompanied by equal but opposite differences in number of TH– neurons (Aumann et al., 2013). While this could indicate mechanisms other than DA neurotransmitter plasticity, I think it may be an artifact of our analysis method. Specifically, during stereology we define SNc (and VTA) as the volume of tissue containing TH+ neurons. Therefore if cells undergoing DA neurotransmitter plasticity are located on or near the borders of SNc (or VTA), as is known to be the case in the ventral supra-chiasmatic nucleus of *Xenopus laevis* (Dulcis and Spitzer, 2008), and appears to be the case in midbrain also (e.g. Fig. 3), the borders of SNc (and VTA) will be different in control and environmentally manipulated mice. While this will not affect estimates and comparisons of the true number of TH+ neurons, it may cause artifactual differences in the number of TH–

Fig. 3. SNc cells that have down-regulated expression of TH protein. To detect these cells we use transgenic TH–Cre/GtROSA mice, in which any cell that has ever expressed TH will permanently express β -galactosidase (β -gal). By performing double immunohistochemistry against β -gal and TH, we can visualize cells that were TH+ at the time of death (TH+ and β -gal+, yellow cells), and cells that were TH– at the time of death but were TH+ at some earlier time (TH– and β -gal+, green cells). Note that there are a substantial number of SNc cells that have down-regulated TH protein in a normal, vehicle-treated SNc (A, green cells), and there are more of these cells following infusion of the SK antagonist apamin (300 nM) into the midbrain for 2 weeks (B, green cells). Apamin is a specific SK ion-channel antagonist that should alter the activity of SNc neurons, and produces significantly fewer TH+ SNc neurons when infused into midbrain for 2 weeks (see text for further details). Note also that many of these green cells are located on or near the borders of SNc, which may lead to artifactual differences in the number of TH– SNc neurons in some of our experiments (see text for further details). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

neurons. For example, if TH[−] neurons located just outside the borders of SNc in SH mice acquire TH expression following EE, the boundaries of SNc will expand in EE relative to SH (control) mice, which will cause more TH[−] neurons to be counted in EE relative to SH mice (assuming the expanded border also encompasses some 'new' TH[−] neurons). This artifactual increase in TH[−] neurons would counteract any decrease in TH[−] neurons caused by their acquisition of TH expression. Likewise if TH⁺ neurons on and near the border of female mice lose TH expression following pairing with males, the boundaries of SNc will retract relative to those paired with females (controls), which will cause fewer TH[−] neurons to be counted in female mice paired with males relative to those paired with females. This artifactual decrease in TH[−] neurons would counteract any increase in TH[−] neurons brought about by loss of TH expression. A better way to measure differences in number of TH[−] neurons caused by gain or loss of the DA phenotype awaits identification of a positive phenotype marker of cells capable of putative DA neurotransmitter plasticity.

In summary thus far, sustained (1–2 weeks) alterations in midbrain neuronal activity, including those driven by afferent pathways and environmental cues, cause $\pm 10\%$ differences in the number of TH⁺ SNc (and VTA) neurons in adult mice. Our evidence that this is indicative of underlying SNc DA neurotransmitter plasticity is: (1) The amounts of TH gene promoter activity, TH mRNA and TH protein in *in vitro* midbrain preparations begin to increase or decrease (depending on the drug) ~ 20 h after addition of drugs that alter the electrical activity of constituent neurons; (2) Infusion of these same or similar drugs into midbrain *in vivo* for 2 weeks increases or decreases the number of SNc TH⁺ neurons, and increase or decrease the immunoreactivity of SNc TH⁺ neurons; and (3) Differences in the number of SNc TH⁺ neurons are often accompanied by equal but opposite differences in the number of TH[−] neurons. The alternative explanation of neurogenesis combined with neurodegeneration is not supported by the literature [(Aponso et al., 2008; Chen et al., 2005; Frielingsdorf et al., 2004; Lie et al., 2002; Peng et al., 2008; Yoshimi et al., 2005) but see (Zhao et al., 2003)]. In addition (4) our findings resemble those of Dulcis et al. (Dulcis et al., 2013; Dulcis and Spitzer, 2008), who showed that manipulating the environment of *Xenopus laevis* or adult rats, or manipulating the activity of hypothalamic neurons, causes increases or decreases in the number of hypothalamic TH⁺ neurons and equal but opposite differences in the number of hypothalamic TH[−] neurons.

Note that Dulcis et al. have gone further to establish hypothalamic DA neurotransmitter plasticity, as well as to show that it is neurobiologically and behaviorally relevant. Specifically, they have shown differences in the amounts of TH, DA and DA receptors at the terminal end of hypothalamic neurons that concord with differences in the parent cell bodies, and they have shown that environmental-induction of hypothalamic DA neurotransmitter plasticity causes behavioral changes (Dulcis et al., 2013; Dulcis and Spitzer, 2008). These experiments by Dulcis et al. in hypothalamus serve as a useful template for the kinds of experiments needed to establish midbrain DA neurotransmitter plasticity, and to determine whether it is also neurobiologically and behaviorally relevant. An important step in this process is identifying cells that are capable of DA neurotransmitter plasticity. For this reason I now turn to reviewing literature on the cytoarchitecture and neurotransmitter phenotypes of SNc neurons with a view to helping achieve this.

7. Substantia nigra pars compacta cytoarchitecture

Studies of substantia nigra cytoarchitecture [including SNc, substantia nigra pars lateralis (SNl) and SNr] describe continuous distributions of neuronal somata sizes that are arbitrarily divided

into small, medium and large (Gulley and Wood, 1971; Hanaway et al., 1970; Juraska et al., 1977). Most (~ 82 – 90%) SNc neurons are medium-sized, with the remainder small-sized. Medium-sized SNc neurons have various morphologies (ovoid, fusiform, pyramidal, polygonal) with some tendency towards pyramidal. They are best distinguished from other SNc neuron types by their 'inverted pyramidal cell' dendrites comprising 1–2 large and long 'apical' dendrites extending ventrally through the underlying SNr, and 2–5 'basal' dendrites extending shorter distances in all directions but confined within SNc (Gulley and Wood, 1971; Hanaway et al., 1970; Juraska et al., 1977). The axons of medium-sized SNc neurons form synapses in efferent telencephalic target nuclei, primarily the striatum. Thus, most if not all medium-sized SNc neurons are projection neurons. In contrast, small-sized SNc neurons have stellate dendritic fields comprising 3–5 thin dendrites confined within SNc [(Gulley and Wood, 1971; Juraska et al., 1977), but see (Hanaway et al., 1970) who did not find this population]. The axons of some of these neurons were able to be traced and terminated locally along the dendrites of SNc projection neurons (Gulley and Wood, 1971; Juraska et al., 1977). To this extent small-sized SNc neurons are probably local interneurons.

An unresolved question is the identity of SNc neurons that are capable of putative DA neurotransmitter plasticity. It is important to know this because these cells need to be targeted in future studies aimed at elucidating cellular mechanisms. For example, Dulcis and Spitzer (2008) identified neuropeptide Y (NPY) containing neurons located in an annular region surrounding a core of permanently DA neurons in the hypothalamus of *Xenopus laevis* as those capable of DA neurotransmitter plasticity. This then enabled Dulcis and Spitzer to further characterize these NPY cells as expressing LIM1/2 but not PAX6 transcription factors, as maintaining the same efferent synaptic connectivity regardless of their DA neurotransmitter status, and as undergoing concordant changes in DA protein expression in both their cell body and efferent synaptic terminals (Dulcis and Spitzer, 2008). Also, Dulcis et al. (2013) identified somatostatin-containing neurons in the hypothalamus of adult rats as those capable of DA neurotransmitter plasticity. This enabled them to further reveal these neurons similarly maintain the same efferent synaptic connectivity regardless of their neurotransmitter phenotype, and undergo concordant changes in DA protein expression in both their cell body and efferent synaptic terminals. These insights significantly strengthen the case that DA neurotransmitter plasticity affects DA neurotransmission and thereby brain function and behavior.

Given hypothalamic neurons maintain their efferent connectivity regardless of their DA neurotransmitter status, it seems likely SNc neurons do the same. Thus, SNc neurons that are capable of DA neurotransmitter switching are more likely to be medium-sized SNc neurons with extant axon projections to, and synapses in, the dorsal striatum than small-sized SNc interneurons. Teleologically this also makes more sense because if the purpose of SNc DA neurotransmitter plasticity is to provide medium to long-term alterations in SNc DA neurotransmission affecting brain function and behavior, why expend additional energy growing and retracting axons and synapses to and from the requisite efferent target? I will therefore concentrate the remainder of this Chapter on medium-sized SNc projection neurons.

8. Neurotransmitter phenotypes of substantia nigra projection neurons

While it is true most medium-sized SNc projection neurons are DA, anywhere between 5 and 15% are non-DA, as defined by lack of glyoxylic acid-induced catecholamine histofluorescence [a more direct measure of DA itself (van der Kooy et al., 1981)], or lack of TH

immunoreactivity (Gerfen et al., 1987; Rodriguez and Gonzalez-Hernandez, 1999; Swanson, 1982; Villar-Cervino et al., 2009). However, care must be taken when synthesizing the literature on this because several studies report most non-DA nigrostriatal neurons are located in SNr and VTA, not in SNc proper [e.g. (Rodriguez and Gonzalez-Hernandez, 1999; van der Kooy et al., 1981)].

Non-DA nigrostriatal neurons have been described as polymorphic elongated and round cells (largest diameter 15–23 μm) (Rodriguez and Gonzalez-Hernandez, 1999) with larger diameter axons and larger, more bulbous varicosities than their DA counterparts (Gerfen et al., 1987). The identity of their neurotransmitter(s) is not known, but is likely to be GABA. Rodriguez and Gonzalez-Hernandez (1999) reported data suggesting 5–8% of nigrostriatal non-DA neurons are immunoreactive against GABA, glutamic acid decarboxylase (GAD) or parvalbumin, all markers of GABAergic neurons. Glutamate is another possibility (although more likely for VTA than SNc projection neurons, see below) because Yamaguchi et al. (2013) found ~15% of SNc neurons contain mRNA for the vesicular glutamate transporter (vGlut2, but not vGlut1 or vGlut3) and are not TH immunoreactive [but see (Nair-Roberts et al., 2008)]. Although Yamaguchi et al. (2013) did not determine whether these were projection or interneurons, examination of their Figures clearly shows vGlut2+ cells are the same size as TH+ cells, and are therefore projection neurons on cytoarchitectonic grounds. To my knowledge these are the only studies that have thoroughly examined for the neurotransmitter identity of non-DA SNc projection neurons.

Thus, current evidence suggests that, in rats at least, ~85–95% of nigrostriatal neurons are DA and ~5–15% of nigrostriatal neurons are non-DA and GABAergic or glutamatergic [see also (Yetnikoff et al., 2014)]. If some or all nigrostriatal neurons switch between these neurotransmitters, one would expect to see evidence of transitional phenotypes such as co-labelling of nigrostriatal neurons for markers of DA and GABA or glutamate. However, TH+ SNc neurons are not convincingly co-immunoreactive against GAD [e.g. (Gonzalez-Hernandez et al., 2001)], and this is our experience also (unpublished data). The uncertainty stems largely from the high density of GAD immunoreactive synapses circumscribing SNc cell bodies, which makes it difficult to assess whether the cell bodies themselves are weakly GAD immunopositive or GAD immunonegative. To overcome this Gonzalez-Hernandez et al. (2001) performed *in situ* hybridization against GAD mRNA and found ~10% of TH immunoreactive SNc neurons (including nigrostriatal neurons) contained GAD mRNA ('DA/GABA' cells) in normal rats. Moreover, following 6-OHDA degeneration of DA nigrostriatal neurons, these DA/GABA cells become more immunoreactive for GAD (and GABA) (Gonzalez-Hernandez et al., 2004), indicating up-regulation or plasticity of GAD protein and GABA synthesis is possible in these neurons.

More recent studies using optogenetic approaches to stimulate neurotransmitter release specifically from midbrain projection neurons indicate functional, fast, *i.e.* ionotropic *via* post-synaptic GABA_A receptors, GABA co-transmission occurs from all (or most) nigrostriatal DA neurons (Tritsch et al., 2012, 2014). Furthermore, they show nigrostriatal DA synapses can derive their GABA locally from extracellular sources *via* the membrane GABA transporter (mGAT) (Tritsch et al., 2014), followed by transport into synaptic vesicles *via* vMAT, the same transporter used for vesicular DA loading. (Tritsch et al., 2012), see also (Stensrud et al., 2014)]. Thus, GABA synthesis by midbrain neurons is not necessary for nigrostriatal DA/GABA co-transmission. Also, a recent report raises the possibility that GABA synthesis by midbrain DA neurons does not use the conventional GAD enzymes, but rather an evolutionarily conserved pathway mediated by aldehyde dehydrogenase enzymes (ALDH) (Kim et al., 2015). This might explain

why we and others have had difficulty detecting GAD in SNc neurons. Any future attempts to identify GABA as a switching partner for DA should therefore include mGAT and ALDH as markers.

Notes: (1) In other CNS locations there is evidence of a close ontogenetic relationship between GABA neurons and DA neurons [e.g. (Abbott et al., 1996; Barreiro-Iglesias et al., 2009; Gall et al., 1987; Kiyokage et al., 2010; Velazquez-Ulloa et al., 2011)]; (2) Similar optogenetic approaches have also yielded evidence of fast glutamatergic co-transmission from nigrostriatal DA neurons (Tritsch et al., 2012), although others report this is limited to VTA neurons (Hnasko and Edwards, 2012; Stuber et al., 2010; Tecuapetla et al., 2010); and (3) Neuropeptides, in particular cholecystokinin (CCK), are also potential DA switching partners in SNc neurons [(Artaud et al., 1989; Gonzalez-Hernandez and Rodriguez, 2000; Lanca et al., 1998; Savasta et al., 1989; Schuligoi et al., 1993; Seroogy et al., 1988a,b, 1989; Sirinathsinghi et al., 1992; Wotherspoon et al., 1994), but see (Palacios et al., 1989)].

In relation to DA and all of the above potential co-transmitters, and particularly their putative plasticity in SNc neurons, a role for neurotrophins must be considered. Neurotrophins (BDNF and NT-3 and NT-4/5) and their receptors (TrkB and TrkC) are expressed by all SNc (and VTA) DA and non-DA/GABAergic neurons (Numan and Seroogy, 1999). Neurotrophin infusion into SNc either promotes neuronal survival following axotomy but suppresses their TH phenotype (BDNF or NT-4/5), or has little effect on survival but maintains the TH phenotype in surviving cells (NT-3) (Hagg, 1998). Infusion of either BDNF or NT-4/5 into midbrain also increases GAD expression and the size of GABAergic neurons in the rat SNc (Arenas et al., 1996). With respect to neuropeptides, neurotrophin administration enhances the neuropeptide phenotype of cortical and striatal neurons (Croll et al., 1994; Mizuno et al., 1994; Nawa et al., 1993, 1994; Sauer et al., 1994, 1995) so might alter the neuropeptide phenotype of SNc neurons also.

In addition, it is well-known neurotrophin synthesis and release are regulated by environmental stimulation and neuronal activity. Thus, neurotrophins may mediate putative environment- and activity-dependent midbrain DA neurotransmitter plasticity. This idea is illustrated in Fig. 4 as an alternative to intracellular calcium regulating expression of DA genes directly.

9. Outstanding questions

To further establish midbrain DA neurotransmitter plasticity and to ascertain its neurobiological, etiological, and therapeutic relevance, I propose the following questions to help guide further research in this area.

- 1) What are the mechanisms of putative midbrain DA neurotransmitter plasticity? See Fig. 4A.
 - a) Is it regulated at the gene expression level and how (classical transcriptional regulation and/or epigenetics) ?
 - b) What are the signaling pathways? Is it relatively direct between neuronal activity, intracellular calcium, and calcium-dependent gene and protein expression, or does it involve activity-dependent regulation of neurotrophins (e.g. GDNF, BDNF, NT-3, NT-4/5) and their signaling pathways to altered DA gene and protein expression?
 - c) Is expression of the panoply of different DA genes and proteins co-regulated by a common upstream signaling pathway, or is each DA gene/protein regulated independently?

Fig. 4. Models of how dopamine (DA) neurotransmitter plasticity in the adult substantia nigra pars compacta (SNc) might be regulated by neuronal activity (A), and how this might in turn be regulated by environmental cues to affect brain function and behavior (B). **A** We know putative SNc DA neurotransmitter plasticity is ion-channel- and activity-dependent, but the cellular signaling mediating this is unclear. It is probably *via* changes in intracellular Ca²⁺ concentration (transients) and altered Ca²⁺-dependent intracellular signaling. This is known to affect gene expression generally, and it is known to affect expression of DA genes in other catecholaminergic cells [see (Aumann and Horne, 2012)]. The question is whether altered Ca²⁺-dependent signaling has a direct effect on the expression of DA genes (shown here as the red dashed arrow from Ca²⁺ to the red DA genes TH, VMAT, DAT and D2R), or an indirect effect *via* altered neurotrophin signaling (shown here as the black dashed arrows from Ca²⁺ to BDNF and NT-3 genes, and then to neurotrophin receptors expressed by SNc neurons). Note, the neurotrophin pathway could also be altered neurotrophin release independent of altered neurotrophin gene expression. Another question is whether there is a neurotransmitter plasticity partner for DA in SNc neurons, as has been shown for somatostatin in rat hypothalamic DA neurons (Dulcis et al., 2013). Data suggest this might be GABA and expression of 'GABA genes' might be co-regulated with expression of DA genes in SNc neurons (shown here as the blue dashed arrows). **B** I propose SNc neurons that undergo DA neurotransmitter plasticity are pre-existing nigrostriatal neurons with the same afferent and efferent connectivity as other DA nigrostriatal neurons. Our data suggest that ~10% (~500 neurons) of mouse nigrostriatal neurons exhibit DA neurotransmitter plasticity to an extent where tyrosine hydroxylase (TH, the rate limiting enzyme in DA synthesis) expression is 'switched' on or completely off. One such neuron is illustrated here in three different DA phenotypic states: DA (red); non-DA (blue); and in transition between DA and non-DA (purple). We also have evidence that most or all nigrostriatal neurons undergo DA neurotransmitter plasticity to some degree (see text). I further propose that when our environment contains cues that are salient with respect to requiring more nigrostriatal DA (e.g. rewards or a requirement to learn a new motor skill), those cues are relayed to SNc neurons in the form of altered afferent activity leading to increased burst activity in SNc neurons (illustrated here by an increased number of yellow lightning bolts). This increased burst activity has two effects: (1) acutely it increases DA exocytosis from nigrostriatal neurons; and (2) over the longer term it up-regulates expression of DA genes and proteins in nigrostriatal neurons (including an apparent increase in the number of DA neurons). This is illustrated here as the blue non-DA neuron becoming DA (purple then red). The net result is increased → nigrostriatal DA neurotransmission in the longer-term (days-weeks), leading to altered brain function and behavior. A tangible example might be learning to drive a car. In the beginning the environment of the car and its interactions with the road and other traffic are novel and we are required to learn or adapt our behavior to survive, drive and thrive. This requires adaptation, learning and memory in various faculties including sensory, cognitive and motor. Since SNc and dorsal striatum are generally associated with motor function I will use motor learning as an example, but the model could equally apply to neurons in the ventral tegmental area and cognitive function for example. A motor sequence that might be learned in this scenario is shifting gears' i.e. left foot on clutch → right hand gear-shift → left foot off clutch → right foot on accelerator. I propose that salient cues in this novel environment are relayed to SNc neurons in the form of altered afferent activity, which causes increased burst activity in SNc neurons leading longer-term to increased expression of DA genes and proteins in these neurons. This then leads to increased DA neurotransmission in the dorsal striatum, altered brain function and behavior. Altered brain function and behavior could be in the forms of increased cortico-striatal synaptic plasticity underlying reorganization of cortical-basal ganglia-thalamo-cortical circuitry driving gear-shift motor sequences, and learning or refinement of gear-shift motor sequences. Once the motor sequences are learned, the movements become habitual and can be performed almost subconsciously whilst our attention is directed towards other things. This habitualization of the task may correlate with elimination of the salient environmental cues (represented as the dotted arrow between behavior and environment) leading to normalization of afferent activity and SNc neuronal activity, and reversion of DA neurotransmitter plasticity in SNc neurons (represented here as the red DA neuron becoming blue and non-DA). Nigrostriatal DA neurotransmission then returns to baseline, however the learned behavior persists because it is now driven independently of nigrostriatal DA by the newly organized cortico-basal ganglia-thalamo-cortical circuitry. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Important for future studies aimed at identifying and developing ways to manipulate midbrain DA neurotransmitter plasticity for therapeutic purposes.

- 2) Is putative midbrain DA neurotransmitter plasticity functionally and behaviorally relevant? See Fig. 4B.
 - a) Do differences in the number of midbrain TH+ neurons translate to differences in the amounts of midbrain DA and midbrain DA signaling?
 - b) Does this, in turn, translate to differences in brain function (e.g. cortico-striatal synaptic plasticity) and behavior (e.g. motor behavior or motor learning)?

Important for establishing midbrain DA neurotransmitter plasticity as: (1) a new facet of adult brain plasticity; and (2) neurobiologically and behaviorally relevant.

- 3) Which midbrain cells are capable of putative DA neurotransmitter plasticity?

- a) What is their co-transmitter(s)?
- b) Is their co-transmitter regulated in parallel with DA?
- c) Other biochemical characteristics?

Important for identifying midbrain cells that undergo DA neurotransmitter plasticity for future mechanistic studies.

- 4) Does putative midbrain DA neurotransmitter plasticity occur in humans?

Important for ascertaining its relevance as: (1) a potential contributor to the etiology of symptoms of brain and behavioral disorders associated with midbrain DA imbalances (e.g. schizophrenia,

addiction, OCD, depression, PD and ADHD); and (2) a potential new approach to treating these symptoms.

10. Summary

In summary, evidence for environment- and activity-dependent DA neurotransmitter plasticity in the adult SNc and VTA includes: (1) The amounts of TH gene promoter activity, TH mRNA and TH protein in midbrain neurons begins to increase or decrease ~20 h after sustained changes in their electrical activity *in vitro*. (2) There are ~10% (~500 neurons) more or fewer TH immunoreactive (TH+) SNc neurons following 2 weeks' continuous infusion of ion-channel agonists or antagonists directly into midbrain, and this occurs without any change in the total number (TH+ and TH-) of SNc neurons. (3) Exposing mice to different environments (sex pairing or environment enrichment) for 1–2 weeks induces ~10% more or fewer TH+ SNc and VTA neurons, and this is abolished by concurrent blockade of synaptic input in midbrain. These data also share similarities with DA neurotransmitter plasticity described in the hypothalamus of *Xenopus laevis* (Dulcis and Spitzer, 2008) and adult rats (Dulcis et al., 2013). Specifically they are all bi-directional, appear to occur in extant neurons rather than *via* neurogenesis and neurodegeneration, and are dependent on neuronal activity- and/or environmental stimuli. In hypothalamus the case for DA neurotransmitter plasticity has made stronger by showing concurrent changes in other DA genes or proteins, concordant changes in DA genes or proteins in the cell bodies and synapses of these neurons including neurotransmitter receptors post-synaptically, and that environment-induced DA neurotransmitter plasticity affects the behavior of these animals. Experiments addressing these questions in midbrain neurons are necessary to further establish adult midbrain DA neurotransmitter plasticity and its neurobiological and behavioral relevance; a worthwhile pursuit in my opinion because, apart from being another example of a novel form of adult brain plasticity, it could herald new therapeutic options for symptoms of midbrain DA imbalances associated with a host of prominent brain and behavioral disorders (e.g. schizophrenia, addiction, OCD, depression, PD and ADHD).

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Multiplexed neurochemical signaling by neurons of the ventral tegmental area

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ABSTRACT

The ventral tegmental area (VTA) is an evolutionarily conserved structure that has roles in reward-seeking, safety-seeking, learning, motivation, and neuropsychiatric disorders such as addiction and depression. The involvement of the VTA in these various behaviors and disorders is paralleled by its diverse signaling mechanisms. Here we review recent advances in our understanding of neuronal diversity in the VTA with a focus on cell phenotypes that participate in 'multiplexed' neurotransmission involving distinct signaling mechanisms. First, we describe the cellular diversity within the VTA, including neurons capable of transmitting dopamine, glutamate or GABA as well as neurons capable of multiplexing combinations of these neurotransmitters. Next, we describe the complex synaptic architecture used by VTA neurons in order to accommodate the transmission of multiple transmitters. We specifically cover recent findings showing that VTA multiplexed neurotransmission may be mediated by either the segregation of dopamine and glutamate into distinct microdomains within a single axon or by the integration of glutamate and GABA into a single axon terminal. In addition, we discuss our current understanding of the functional role that these multiplexed signaling pathways have in the lateral habenula and the nucleus accumbens. Finally, we consider the putative roles of VTA multiplexed neurotransmission in synaptic plasticity and discuss how changes in VTA multiplexed neurons may relate to various psychopathologies including drug addiction and depression.

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1. Introduction

Midbrain dopamine neurons (DA) are most often associated with reward processing of both natural rewards (e.g., food, water, etc.) and drugs of abuse (Schultz, 2002; Wise, 2004; Sulzer, 2011). Over fifty years of intense research has led to the proposal that neurons belonging to the ventral tegmental area (VTA), which includes but is not limited to DA neurons, are paramount to reward processing. Many hypotheses have been put forward regarding the specific function of VTA DA neurons in reward processing, such as decision making (Salamone and Correa, 2002a, 2002b; Saddoris et al., 2015), flexible approach behaviors (Nicola, 2010), incentive salience (Berridge and Robinson, 1998; Berridge, 2007), and learning or the facilitation of memory formation (Adcock et al., 2006; Steinberg et al., 2013). However, several studies have also shown that VTA DA neurons are involved in the processing of aversive outcomes (Laviolette et al., 2002; Young, 2004; Pezze and Feldon, 2004; Brischoux et al., 2009; Lammel et al., 2012; Twining

et al., 2014; Hennigan et al., 2015), fear (Abraham et al., 2014), aggression (Yu et al., 2014a, 2014b), depression (Tidey and Miczek, 1996; Tye et al., 2013), and drug withdrawal (Grieder et al., 2014). Other hypotheses have proposed that VTA DA neurons play a more general role in processes such as associative learning (Brown et al., 2012), arousal (Horvitz, 2000), or general motivational salience and cognition (Bromberg-Martin et al., 2010).

The functional diversity associated with the VTA may be mediated, in part, by different VTA subpopulations of neurons. A particular advancement that may subserve the functional diversity of the VTA is the recent discovery of neurons that are capable of signaling using one or more neurotransmitters. In the present review, we cover recent literature on the diversity of VTA neuronal phenotypes as they relate to 'multiplexed neurotransmission'. We refer the reader to recent comprehensive reviews detailing VTA cellular composition, VTA efferent and afferents, and VTA functions (Oades and Halliday, 1987; Fields et al., 2007; Ikemoto, 2007; Nair-Roberts et al., 2008; Morales and Pickel, 2012; Trudeau et al., 2013; Morales and Root 2014; Pignatelli and Bonci, 2015; Saunders et al., 2015b; Lüthi and Lüscher, 2014). Moreover, the present review does not cover co-transmission of

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neurotransmitters and neuropeptides, which has long been known and recently reviewed (Morales and Pickel, 2012). Here, we use the phrase “multiplexed neurotransmission” to describe neurons that are capable of signaling using two or more neurotransmitters. In many circuits, our understanding of the specific mechanisms by which neurons utilize multiple neurotransmitters is limited. Thus, we have chosen the term multiplexed neurotransmission to encompass known and unknown mechanisms of co-release and co-transmission (e.g., Nusbaum et al., 2001; El Mestikawy et al., 2011), while also allowing for the possibility of independent release of individual neurotransmitters either in time or space.

2. Cellular diversity in the ventral tegmental area

Following the discovery of DA as a chemical neurotransmitter in the brain (Montagu, 1957), the DAergic neurons in the “ventral tegmental area of Tsai” (Nauta, 1958) were identified by formaldehyde histofluorescence (Carlsson et al., 1962). These neurons, along with other catecholaminergic and serotonergic neurons throughout the brain were shown to comprise twelve discrete cell groups (labeled as A1–A12 groups; Dahlström and Fuxe, 1964). One feature of the A10 group, in particular, is the heterogeneous morphology among its neurons. Based on cytoarchitecture, the A10 region has been divided into two lateral nuclei [the Parabrachial Pigmented Nucleus (PBP) and Paranigral Nucleus (PN)], and three midline nuclei [the Rostral Linear Nucleus of the Raphe (RLi), Interfascicular Nucleus (IF), and Caudal Linear Nucleus (CLi)]. Traditionally, the VTA has been considered to include just the lateral nuclei (PBP, PN) (Swanson, 1982), however, modern conceptions of VTA function have often included the midline nuclei (RLi, IF, CLi) as subnuclei of the VTA (Ikemoto, 2007; Nair-Roberts et al., 2008; Morales and Root, 2014). Thus, in this review, we use the term VTA to define the midbrain A10 structure containing lateral (PBP, PN) and midline nuclei (RLi, IF, CLi). The cellular heterogeneity within the VTA subnuclei, together with findings showing that a single A10 neuron rarely innervates multiple structures (Swanson, 1982; Takada and Hattori, 1987; Lammel et al., 2008; Hosp et al., 2015), suggests that the VTA utilizes highly specific projections from different sets of neurons.

Dopamine neurons, defined by the expression of tyrosine hydroxylase (TH) protein (Fig. 1), are interspersed throughout all VTA nuclei, but are most prevalent in the lateral PBP and PN (Swanson, 1982; Ikemoto, 2007; Li et al., 2013). In addition to the co-expression of TH and aromatic decarboxylase (AADC), the majority of rat lateral PBP and lateral PN neurons co-express the dopamine transporter (DAT), D2 receptor (D2R), and vesicular monoamine transporter 2 (VMAT2) mRNA (Li et al., 2013). More medially within the rat PBP and PN, as well as within the RLi, CLi, and IF, subsets of TH-expressing neurons either express or lack different combinations of DAT, VMAT2, or D2 receptor (Li et al., 2013; reviewed in Morales and Root, 2014). Our understanding of diversity among DAergic neurons in other species than the rat is less understood. However, recent studies have shown that, while all VTA neurons in the rat VTA expressing TH mRNA co-express the TH protein, some mouse VTA neurons expressing TH mRNA lack TH protein (Yamaguchi et al., 2015). In addition, ventrally to the VTA within the interpeduncular nucleus, there is in the mouse, but not in the rat, a subpopulation of neurons expressing TH mRNA, but lacking TH protein (Yamaguchi et al., 2015; Lammel et al., 2015). So far, detailed molecular characterizations of VTA neurons in nonhuman primates or humans has not been reported.

Rat TH-expressing neurons within the lateral PBP and lateral PN have also been electrophysiologically characterized (so-called ‘primary’ neurons) based on their long-duration action potentials and hyperpolarization-activated cation currents (Grace and Onn, 1989). However, recent findings have shown that not all VTA TH-expressing neurons share these electrophysiological criteria (Margolis et al., 2006). In addition, although lack of direct electrophysiological responses to the μ -opioid receptor agonist DAMGO has been proposed as a property shared by VTA DAergic neurons (Johnson and North, 1992), the VTA has a subpopulation of TH-expressing neurons that are directly excited or inhibited by DAMGO (Margolis et al., 2014). So far, it seems that hyperpolarization-activated cation currents, spike duration, inhibition by D2R agonist and other electrophysiological properties are unreliable predictors for the identification of all VTA DAergic neurons (Margolis et al., 2006), further supporting the heterogeneity of VTA DAergic neurons.

Fig. 1. Neurons in the ventral tegmental area (VTA) are capable of multiplexed neurotransmission. Detection of tyrosine hydroxylase (TH) immunoreactivity within the VTA, (low magnification, left panel). VTA combined immunohistochemistry and *in situ* hybridization showing at high magnification (right panel) neurons expressing TH (dopamine neurons; green cells), glutamic acid decarboxylase mRNA (GABA neurons; GAD 65/67; purple cells), vesicular glutamate transporter 2 mRNA (glutamate neurons; VGLUT2; green or white grain aggregates) or combinations of these cell markers.

Abbreviations: Left: RLi, Rostral Linear Nucleus, IF, Interfascicular Nucleus, PBP, Parabrachial Pigmented Nucleus, PN, Paranigral Nucleus, Snc, Substantia Nigra Pars Compacta, fr, fasciculus retroflexus, mp, Mammillary Peduncle, Right: TH, tyrosine hydroxylase, GAD, glutamic acid decarboxylase, VGLUT2, vesicular glutamate transporter 2.

Along with DAergic neurons, γ -aminobutyric-acid (GABA) neurons are also present in the VTA (Nagai et al., 1983; Kosaka et al., 1987). These GABAergic neurons are relatively less prevalent than the DAergic neurons, and are identified by their expression of glutamic acid decarboxylase (GAD) 65 or 67 mRNA, isoforms of the enzyme involved in the synthesis of GABA. GABAergic VTA neurons are also identified by their expression of vesicular GABA transporter (VGAT) mRNA. Electrophysiologically, putative VTA GABAergic 'secondary' neurons have been characterized based on the observation that these cells are hyperpolarized by μ -opioid agonists (Johnson and North, 1992). As with VTA DAergic neurons, VTA GABAergic neurons are pharmacologically and electrophysiologically heterogeneous. For example, approximately 60% of VTA GABAergic neurons are inhibited by the μ -opioid receptor agonist DAMGO, but all seem to be unaffected by the GABA_B agonist baclofen (Margolis et al., 2012). GABAergic neurons in the VTA are known to establish local inhibitory connections on DAergic neurons (Johnson and North, 1992; Omelchenko and Sesack, 2009), but have also been shown to project outside of the VTA to the ventral striatum (nAcc; Van Bockstaele and Pickel, 1995) basal forebrain (Taylor et al., 2014), the prefrontal cortex (Steffensen et al., 1998; Carr and Sesack, 2000), the lateral habenula (LHb; Stamatakis et al., 2013; Root et al., 2014a; Taylor et al., 2014; Lammel et al., 2015), lateral hypothalamus, preoptic area, and amygdala, as well as to structures in the thalamus, midbrain, pons and medulla (Taylor et al., 2014). GABAergic neurons are scattered throughout the A10 region, and although a detailed subregional mapping of these neurons has not been yet reported, a dense group of GABAergic neurons has been identified in an area ventro-caudal to the VTA, referred as the 'tail of the VTA' (tVTA; Kaufling et al., 2009) or the rostromedial tegmental area (RMTg; Jhou, 2005; Jhou et al., 2009a, 2009b; Geisler et al., 2008; Lavezzi and Zahm, 2011). The GABAergic neurons of the tVTA/RMTg provide a major

inhibitory control to VTA DAergic neurons (Kaufling et al., 2010; Matsui and Williams, 2011).

In addition to VTA DAergic and GABAergic neurons, early electrophysiological studies of the midbrain suggested the possibility of glutamatergic signaling by some VTA neurons (Wilson et al., 1982; Mercuri et al., 1985; Sulzer et al., 1998; Joyce and Rapport, 2000; Chuhma et al., 2004; Ungless et al., 2004; Lavin et al., 2005; Chuhma et al., 2009). Anatomical identification of glutamatergic neurons has recently become possible due to the cloning of three distinct vesicular glutamate transporters (VGLUT1, VGLUT2, and VGLUT3; Bellocchio et al., 1998; Bai et al., 2001; Fremeau et al., 2001, 2002; Fujiyama et al., 2001; Hayashi et al., 2001; Herzog et al., 2001; Takamori et al., 2000; Varoqui et al., 2002; Gras et al., 2002). By *in situ* hybridization, it has been demonstrated that some neurons within the VTA (Kawano et al., 2006; Yamaguchi et al., 2007, 2011), substantia nigra and retrorubral field (Yamaguchi et al., 2013) express VGLUT2 mRNA, but not VGLUT1 or VGLUT3. The VTA-VGLUT2 neurons are present in all A10 nuclei, but are particularly prevalent within midline nuclei (Yamaguchi et al., 2007, 2011). In fact, glutamatergic neurons outnumber the DAergic neurons in the rostral and medial portions of the VTA (Yamaguchi et al., 2007, 2011). Thus, these neurons represent a major subpopulation in certain parts of the VTA. Similar to VTA GABAergic neurons, VGLUT2-glutamatergic neurons in the VTA establish local and extrinsic synapses (Dobi et al., 2010; Yamaguchi et al., 2011; Zhang et al., 2015; Wang et al., 2015). Specifically, glutamatergic VTA neurons establish local asymmetric synapses with both DAergic and non-DAergic neurons (Dobi et al., 2010; Wang et al., 2015). Additionally, glutamatergic VTA neurons project to other regions of the brain including the LHb (Root et al., 2014a, 2014b), nAcc (Zhang et al., 2015), amygdala, basal forebrain, and prefrontal cortex (Hnasko et al., 2012; Taylor et al., 2014).

Fig. 2. Ultrastructural immunolabeling reveals unpredicted mechanisms of neurotransmission within the mesohabenular and mesoaccumbal pathways. (a) Lateral habenula micrograph (left panel) showing a single mesohabenular axon terminal containing VGLUT2 (scattered dark material detected by immunoperoxidase labeling) and VGAT (gold particles detected by immunogold; blue arrowheads). This single axon terminal forms both an asymmetric synapse (green arrow) and symmetric synapses (blue arrows) with a common postsynaptic dendrite (De). Postsynaptic to a single axon terminal (middle panel), GluR1 receptors (green arrowhead) are found adjacent to asymmetric synapses (green arrows), while GABA_A receptors (blue arrowhead) are found adjacent to symmetric synapses (blue arrow). (b) Nucleus Accumbens micrograph showing a mesoaccumbal axon containing both VMAT2 (scattered dark material) and VGLUT2 (gold particles). VMAT2 and VGLUT2 are segregated within the same axon. Note that the VGLUT2 microdomain corresponds to an axon terminal establishing an asymmetric synapse (arrow) with a postsynaptic dendritic spine (sp). All scale bars represent 200 nm.

Moreover, increasing evidence indicates that subpopulations of VTA neurons are capable of releasing DA and GABA, or DA and glutamate (Kosaka et al., 1987; Sulzer et al., 1998; Rayport, 2001; Dal Bo et al., 2004; Trudeau, 2004; Seutin, 2005; Lapish et al., 2006; Yamaguchi et al., 2007, 2011; Hnasko et al., 2010; Tritsch et al., 2012; Li et al., 2013; Mingote et al., 2015). Recent work from our laboratory has shown that there is a subset of VTA neurons capable of co-releasing DA and glutamate or glutamate and GABA (Zhang et al., 2015; Root et al., 2014a).

Multiplexed neurotransmission by some VTA neurons and associated circuits is a property shared by other brain structures. For instance, glutamate and GABA co-neurotransmission has been reported in epilepsy models within mossy fiber terminals (Gutiérrez et al., 2003; Gutiérrez, 2003, 2005; Trudeau and Gutiérrez, 2007; Münster-Wandowski et al., 2013), developing medial trapezoid body terminals from the lateral superior olive (Gillespie et al., 2005; Noh et al., 2010), entopeduncular nucleus projection to the LHB (Shabel et al., 2014), and cortex (Fattorini et al., 2015). In addition, there is evidence for GABA and DA co-transmission in by substantia nigra pars compacta neurons as well as retinal amacrine neurons (Tritsch et al., 2012; Hirasawa et al., 2012). Other forms of neurotransmission include GABA and histamine by hypothalamic neurons (Yu et al., 2015), glutamate and acetylcholine co-transmission in striatal interneurons or medial habenula neurons (Gras et al., 2008; Ren et al., 2011; Higley et al., 2011; Nelson et al., 2014), and GABA and acetylcholine co-transmission in corticopetal globus pallidus neurons (Saunders et al., 2015a).

Based on the discovery that some VTA neurons exhibit multiple vesicular transporters, we have applied ultrastructural and electrophysiological approaches to determine the possible cellular mechanisms by which multiple neurotransmitters are released at the synaptic level. In the process of answering this question, we have revealed complex ultrastructural architectures suggesting that glutamate and GABA neuronal signaling by VTA neurons can be integrated into a single complex terminal with spatially distinct synaptic release sites for glutamate or GABA (Fig. 1) (Root et al., 2014a). We have also revealed that DA and glutamate neuronal signaling by VTA neurons can be segregated to distinct microdomains within the same axon (Fig. 2), allowing for the spatially distinct release of DA or glutamate (Zhang et al., 2015).

3. Multiplexed signaling by VTA-GluT2 neurons

Following the discovery of VTA-VGluT2 neurons, further characterization of these neurons demonstrated that they are very diverse in their molecular composition, signaling properties and neuronal connectivity. Whereas many VTA-VGluT2 neurons lack both DAergic and GABAergic markers, there are subpopulations of VTA-VGluT2 neurons that co-express molecules responsible for the synthesis or vesicular transport of either DA or GABA (Li et al., 2013; Root et al., 2014a). Although the distinct targets for VTA-VGluT2 neurons remains to be determined, emerging evidence suggests preferential target sites for specific subsets of VTA-VGluT2 neurons. For instance, by a combination of retrograde tract tracing and *in situ* hybridization, it has been demonstrated that VTA VGluT2⁽⁺⁾/GAD⁽⁺⁾ neurons provide the major mesohabenular input to the LHB (Root et al., 2014a), and by contrast, VTA VGluT2⁽⁺⁾/TH⁽⁺⁾ neurons largely target the nAcc shell (Yamaguchi et al., 2011). While the molecular characterization of mesohabenular and mesoaccumbens neurons provides support for multiplexed signaling by some VTA neurons, this characterization does not provide information on the cellular mechanisms by which these neurons release more than one neurotransmitter. As discussed below, recent findings obtained by a combination of cell-type specific anterograde tract tracing and immuno-electron

microscopy have demonstrated that the VTA-VGluT2 neurons establish unique synaptic architectures for multiplexed signaling in both the LHB (Root et al., 2014a) and the nAcc (Zhang et al., 2015).

3.1. Multiplexed signaling by mesohabenular VGluT2 neurons

Recently available viral vectors and transgenic mice have facilitated the elucidation of the cellular mechanisms by which some VTA neurons use two distinct signaling molecules. To determine the synaptic ultrastructural features of mesohabenular axons we have taken advantage of the cell-specific viral tagging of VTA-VGluT2 neurons through the Cre-dependent expression of mCherry tethered to channelrhodopsin (ChR2) under the regulation of the VGluT2 promoter in VGluT2:Cre mice. By applying cell-specific tagging we estimated that more than 70% of mesohabenular axon terminals within the LHB co-express VGluT2 and VGaT (Root et al., 2014a). Moreover, we estimated that within the LHB both VGluT2 and VGaT are present in half of the total population of axon terminals, some of which derive from brain structures others than the VTA (e.g., from the basal ganglia; Shabel et al., 2014). While the presence of VGluT2 and VGaT within the same axon terminal has been established by immuno-electron microscopy, it remains to be determined whether each vesicular transporter is integrated into the membrane of distinct vesicles or in the same vesicular membrane. However, ultrastructural findings of the synaptic composition of individual VGluT2⁽⁺⁾/VGaT⁽⁺⁾ axon terminals show that the plasma membrane of single VGluT2⁽⁺⁾/VGaT⁽⁺⁾ axon terminals participates in the formation of both asymmetric and symmetric synapses, suggesting that glutamate-signaling is segregated to the asymmetric synapse and GABA-signaling to the symmetric (Fig. 2).

In addition to the suggestion that asymmetric synapses participate in excitatory neurotransmission (Peters and Palay, 1996), GluR1-containing AMPA receptors are located in the membrane postsynaptic to the mesohabenular asymmetric synapses, but not to the symmetric synapses (Root et al., 2014a). In contrast, consistent with the suggestion that symmetric synapses participate in inhibitory neurotransmission (Peters and Palay, 1996), GABA_A receptors are located postsynaptically to the mesohabenular symmetric synapses, but not to the asymmetric synapses (Root et al., 2014a). The selective postsynaptic distribution of GluR1 to VGluT2⁽⁺⁾/VGaT⁽⁺⁾ mesohabenular terminals making asymmetric synapses and GABA_A to those making symmetric synapses indicates that VGluT2⁽⁺⁾/VGaT⁽⁺⁾ terminals release glutamate at the asymmetric synapse, and GABA at the symmetric synapses (Root et al., 2014a). Besides the formation of asymmetric and symmetric synapses by individual VGluT2⁽⁺⁾/VGaT⁽⁺⁾ axon terminals, both synapses may target separate postsynaptic dendritic spines or dendritic shafts or share a common postsynaptic dendrite. These ultrastructural findings underlie the multiplexed signaling and potential neuroplastic capacity endowed by dual VGluT2⁽⁺⁾/VGaT⁽⁺⁾ axon terminals from the VTA to the LHB.

3.2. Multiplexed Signaling by Mesoaccumbens VGluT2-DA neurons

Pioneering electrophysiological *in vitro* studies demonstrated that dopamine neurons in primary culture have the capability to release glutamate, which lead to the hypothesis that midbrain neurons co-transmit DA and glutamate (Sulzer et al., 1998; Joyce and Rayport 2000; Bourque and Trudeau, 2000). Since then, anatomical studies demonstrated that subsets of TH-positive neurons co-express VGluT2 mRNA throughout the brain (Stornetta et al., 2002; Kawano et al., 2006), including some TH-neurons within the midline nuclei of the VTA in rats (Yamaguchi et al., 2007,

2011) and mice (Yamaguchi et al., 2015). Moreover, findings from optogenetic electrophysiological *ex vivo* recordings have shown that the VGLuT2⁽⁺⁾/TH⁽⁺⁾ mesoaccumbens neurons use glutamate as signaling molecule (Stuber et al., 2010; Tecuapetla et al., 2010; Zhang et al., 2015; Mingote et al., 2015), and recent *in vitro* voltammetry measurements have shown that VGLuT2-TH co-expressing neurons that project to nAcc release DA (Zhang et al., 2015).

So far two opposing hypotheses have been proposed to mediate the dual glutamate and DA signaling by VGLuT2⁽⁺⁾/TH⁽⁺⁾ mesoaccumbens neurons. One of them proposes that glutamate and DA coexist (and are co-released) from the same pool of vesicles (Hnasko et al., 2010; Hnasko and Edwards, 2012). This hypothesis has been based on the co-immunoprecipitation of VMAT2 and VGLuT2 from nAcc preparations (Hnasko et al., 2010). In clear contrast, a recent study has shown lack of VMAT2 and VGLuT2 co-immunoprecipitation when ultrastructurally confirmed pure nAcc synaptic vesicles were used (Zhang et al., 2015). These recent findings have led to the hypothesis that dual VGLuT2⁽⁺⁾/TH⁽⁺⁾ mesoaccumbens neurons contain independent pools of vesicles for the accumulation of either DA or glutamate. Moreover, immuno-electron microscopy findings from intact brain tissue have shown that TH and VGLuT2 do not coexist in the same axon terminal in the nAcc of either adult rats (Bérubé-Carrière et al., 2009; Moss et al., 2011) or mice of any age (Bérubé-Carrière et al., 2012). These immuno-electron microscopy findings are consistent with nAcc structural studies published over the last 40 years showing that axonal compartments engaged in excitatory signaling do not overlap with axonal compartments engaged in DA signaling (reviewed in Morales and Pickel, 2012). The lack of overlap between DA-vesicles and glutamate-vesicles may result from their segregation into two different sets of axons or segregation into micro-domains within the same axon. Although the possibility of vesicular segregation to different axons has not been discarded, recent immuno-electron microscopy findings indicate that VGLuT2-vesicles from VGLuT2⁽⁺⁾/TH⁽⁺⁾ neurons are located in axon terminals that establish asymmetric synapses, and that axonal segments adjacent to these VGLuT2- axonal terminals contain TH, VMAT2 and DAT (Zhang et al., 2015). The segregation between glutamate-vesicles and DA-vesicles within the same axon appears to be highly regulated, as *in vivo* overexpression of VMAT2, in the rat, does not disrupt the segregation between these two different types of vesicles. Moreover, the vesicular segregation by VGLuT2⁽⁺⁾/TH⁽⁺⁾ mesoaccumbens neurons is maintained in the nAcc of transgenic mice expressing ChR2 (following their viral mediated expression in VTA neurons under the regulation of either the TH-promoter or VGLuT2-promoter; Zhang et al., 2015).

In summary, the characterization of mesoaccumbens and mesohabenular ultrastructural features together with the characterization of their electrophysiological and chemical properties have provided evidence for multiplexed signaling by VTA-VGLuT2 neurons. These findings have demonstrated that dual rodent mesoaccumbens VGLuT2⁽⁺⁾/TH⁽⁺⁾ neurons have adjacent cellular compartments that participate in independent glutamate-signaling and DA-signaling (Zhang et al., 2015). In contrast, the dual rodent mesohabenular VGLuT2⁽⁺⁾/GABA⁽⁺⁾ neurons concentrate both glutamate-vesicles and GABA-vesicles within a single axon terminal that establishes both excitatory and inhibitory synapses (Root et al., 2014a). Future studies are necessary to determine the molecular and signaling mechanisms involved in the sorting and retention of VGLuT2-vesicles, GABA-vesicles (VGaT) and DA-vesicles (VMAT2) to specific microdomains within the same axon. Additional studies are also necessary to determine the extent to which the multiplexed signaling by VTA neurons is affected in brain disorders, such as addiction and depression.

4. Functional diversity by VTA neurons

The functional diversity of VTA neurons has been constantly updated (Unlghess et al., 2004; Stamatakis et al., 2013; Root et al., 2014b; Mejias-Aponte et al., 2015; Eddine et al., 2015; Kotecki et al., 2015; Beier et al., 2015). As detailed above, the multiplexed neurotransmission of the VTA-VGLuT2 neurons is an emerging factor involved in the complexity of VTA function. Based on observations that different combinations of neurotransmitters are multiplexed throughout the brain (Trudeau, 2004; Gillespie et al., 2005; Zhou et al., 2005; Gras et al., 2008; Noh et al., 2010; Higley et al., 2011; Tritsch et al., 2012; Hnasko and Edwards, 2012; Münster-Wandowski et al., 2013; Nelson et al., 2014; Root et al., 2014a; Shabel et al., 2014; Qi et al., 2014; Zhang et al., 2015; Fattorini et al., 2015; Saunders et al., 2015a, 2015b), we suggest that multiplexed neurotransmission conveys distinct messages depending on the neurotransmitter content of each circuit, momentary singular or multiplexed signaling, and perhaps even the time scale of neurotransmitter function. Furthermore, we speculate that changes in the influence of one or more of the multiplexed neurotransmitters, by way of either presynaptic or postsynaptic changes, may result *from* and result *in* observable changes in behavior. Recent advances in the functional diversity within the VTA neurons targeting the LHB or nAcc will be presented in the following paragraphs.

4.1. Functional diversity by VGLuT2 mesohabenular neurons

An example of the circuit specific nature of multiplexed neurotransmission is found in the LHB. By combination of optogenetics and electrophysiology, we have shown that activation of the mesohabenular pathway evokes release of GABA and glutamate, and that the co-transmitted GABA is capable of shunting the co-transmitted glutamate-mediated currents (Root et al., 2014a). Therefore, the simultaneous release of glutamate and GABA may be a mechanism by which the glutamatergic excitation within the LHB is autoregulated by the co-transmitted GABA. *In vivo* recordings of LHB neurons following ChR2 activation of mesohabenular fibers have shown that this activation results in GABA-induced decreases in the firing rates of most recorded LHB neurons, and in glutamate-induced increases in firing rates in fewer neurons. In addition, secondary firing patterns are often observed in which initial increases in firing rates are followed by decreased firing rates or initial decreases in firing rates are followed by increased firing rates. The *in vivo* recordings of LHB neurons suggest that stimulation on mesohabenular fibers induces predominantly GABAergic neurotransmission. Nevertheless, the observed secondary firing patterns suggest that signaling might also occur over multiple time-scales or that the contribution of each neurotransmitter might be shifted in response to specific stimuli. For instance, rat depression models reduce GABA signaling from the multiplexed glutamate-GABA inputs to LHB from entopeduncular neurons (Shabel et al., 2014). In addition, findings from combinations of optogenetics and behavioral analysis have shown that mesohabenular stimulation of fibers from different pools of VTA neurons, including multiplexed signaling neurons, promotes different behaviors. For instance, a LHB GABA receptor-mediated reward is evoked by mesohabenular stimulation of fibers expressing ChR2 under the TH-promoter (Stamatakis et al., 2013), likely to include activation of fibers from VGLuT2⁽⁺⁾/GAD⁽⁺⁾/TH⁽⁺⁾, VGLuT2⁽⁺⁾/TH⁽⁺⁾/GAD⁽⁻⁾, and VGLuT2⁽⁻⁾/GAD⁽⁺⁾/TH⁽⁺⁾ mesohabenular neurons. However, a mild reward is evoked by mesohabenular stimulation of fibers expressing ChR2 under the GAD2-promoter (Lammel et al., 2015), likely to include activation of fiber from VGLuT2⁽⁺⁾/GAD⁽⁺⁾/TH⁽⁺⁾, VGLuT2⁽⁺⁾/GAD⁽⁺⁾/TH⁽⁻⁾, VGLuT2⁽⁻⁾/GAD⁽⁺⁾/TH⁽⁻⁾, and VGLuT2⁽⁻⁾/GAD⁽⁺⁾/TH⁽⁺⁾ mesohabenular neurons. In

contrast, a LHB glutamate receptor-mediated conditioned place aversion is evoked by mesohabenular stimulation of fibers expressing Chr2 under the VGluT2-promoter (Root et al., 2014b; Lammel et al., 2015), likely to include activation of fibers from VGluT2⁽⁺⁾/GAD⁽⁺⁾/TH⁽⁻⁾, VGluT2⁽⁺⁾/GAD⁽⁺⁾/TH⁽⁺⁾, and VGluT2⁽⁺⁾/GAD⁽⁻⁾ neurons. These behavioral findings underlie the need for targeted intersectional approaches to dissect the behavioral contributions of each mesohabenular neuronal phenotype.

Multiplexed neurotransmission may affect neuronal regulation over multiple time scales, for instance “prolonged slow-actions” by monoamines (*i.e.*, serotonin or dopamine) and “fast short actions” provided by the concomitant release of glutamate or GABA. This multiple time scale neurotransmission, by neurons endowed with the capacity for multiplexed signaling, may be found in a single DA-glutamate mesoaccumbens axon establishing segregated postsynaptic targets for DA- or glutamate-signaling (Zhang et al., 2015). Although the extent to which these mesoaccumbens DA-glutamate fibers participate in the neurobiology of drugs of abuse remains to be determined, we speculate that these axons may participate in the regulation of neuronal activity in cocaine self-administration. Specifically, electrophysiological recordings have shown that nAcc neurons exhibit rapid phasic firing patterns to related cues and actions to obtain the drug (Peoples et al., 1998; Ghitza et al., 2003, 2004, 2006; Fabbriatore et al., 2009, 2010; Coffey et al., 2015). The nAcc neurons also exhibit slow-phasic and tonic changes in firing rate that correlate with the pharmacological effects of cocaine, and do not correlate with the rapid phasic firing patterns (Fabbriatore et al., 2010). Furthermore, slow phasic pharmacologic and rapid phasic behavioral firing patterns are similarly processed in downstream accumbal targets (ventral pallidum and lateral preoptic area; Root et al., 2012, 2013; Barker et al., 2014). These dissociable fast and slow signaling patterns in the accumbens are consistent with findings suggesting that glutamate and dopamine each have specific roles in addiction-associated behaviors (Birgner et al., 2010; Alsiö et al., 2011).

4.2. Functional diversity by TH mesohabenular neurons

Phenotypic characterizations of VTA-TH neurons have revealed the heterogeneous expression of several transcripts, some of which may be expressed transiently during development or may be induced in the adult brain in response to insults (*e.g.*, drugs, stress, illness). In addition, some of these transcripts may not be translated into detectable protein levels under normal conditions, instead, this translation may depend on VTA circuit activity or be induced as a result of various brain insults (*e.g.*, Bayer and Pickel, 1990, 1991; García-Pérez et al., 2014). For instance, we have identified a subset of VTA neurons, in wild type mice, that express TH mRNA, but lack detectable levels of TH-protein in cell bodies, dendrites and axons. Some of these neurons send projections to the LHB (Yamaguchi et al., 2015). In agreement with these findings, revealing TH-mRNA⁽⁺⁾/TH-protein⁽⁻⁾ mesohabenular neurons in wild type mice, viral-induced expression of reporter genes (*i.e.*, green-fluorescent-protein under the regulation of the TH-promoter) within the VTA of TH:cre mice has shown expression of fluorescent fibers without detectable TH-protein in the LHB (Stamatakis et al., 2013; Lammel et al., 2015; Stuber et al., 2015). These findings underlie the need to better characterize the VTA cellular composition in wild type mice, and reveal that expression of reporter genes in the mouse under the control of the TH-promoter does not guarantee the selective manipulation or mapping of DA projections.

In contrast to the mouse TH-mRNA⁽⁺⁾/TH-protein⁽⁻⁾ mesohabenular neurons, subsets of rat mesohabenular neurons contain detectable levels of TH-protein in the cell bodies, dendrites and

axons (Root et al., 2015). However, these rat TH-protein⁽⁺⁾ mesohabenular neurons rarely co-express VMAT2-mRNA in their cell bodies or VMAT2-protein in their axon terminals in LHB (Root et al., 2015). The lack of VMAT2 within mesohabenular neurons has also been documented in the mouse (Stamatakis et al., 2013; Lammel et al., 2015). Overall, these findings provide crucial information when considering the functional properties of multi-neurotransmitter neurons, as they demonstrate that specific neuronal subsets have the capacity to synthesize DA but lack the capability to package DA into synaptic vesicles for traditional vesicular release. These findings are intriguing because DA has been detected in LHB homogenates (Phillipson and Pycck, 1982; Root et al., 2015), D2 receptors have been found in a subset of LHB neurons (Aizawa et al., 2012), and exogenous DA evokes currents in LHB neurons, currents that are eliminated by D2 or D4-receptor antagonists (Jhou et al., 2013; Good et al., 2013; Root et al., 2015). However, recordings of LHB neurons from rats treated with toxins for either the elimination of VTA-TH neurons or noradrenergic fibers have demonstrated that noradrenergic habenular afferents specifically activate D4-receptors in the LHB neurons and that VTA TH-expressing neurons are not necessary for this effect (Root et al., 2015). Thus, it seems that the LHB effects on DA-receptors previously ascribed to DA release from mesohabenular fibers may be instead mediated by noradrenergic fibers.

5. Multiplexed transmission: future directions and considerations for synaptic plasticity

Our ever-expanding knowledge of multiplexed signaling opens the door to new predictions about synaptic plasticity. For example, though activation of the mesohabenular projection results in glutamate and GABA release, firing patterns of LHB neurons indicate a predominant GABA-induced decrease in firing rate of LHB neurons in rodents (Root et al., 2014a). Drugs of abuse, depression, and stress alter LHB function to favor glutamatergic excitation and demote GABAergic inhibition (Meshul et al., 1998; Li et al., 2011; Shabel et al., 2014), suggesting the potential for mesohabenular plasticity in mediating part of these effects. The ability of “neurotransmitter-switching” depending on circadian and seasonal variations has also been documented (Dulcis et al., 2013; Farajnia et al., 2014), and further investigation is necessary to determine if these factors influence multiplexed signaling.

The postsynaptic signaling dominance by one neurotransmitter may also be used to balance signals from other neurotransmitters and maintain homeostasis. Indeed, it has been shown that the same neurotransmitter may elicit different responses depending on the overall extracellular environment (Laviolette et al., 2002; Twining et al., 2014). For example in the mesohabenular projection, depending on the membrane potential of the postsynaptic LHB neuron, mesohabenular stimulation results in GABA_A receptor or AMPA-receptor currents (Root et al., 2014b). With this in mind, we speculate that drugs of abuse, neurodegenerative diseases, or other circumstances that affect synapses capable of multiplexed neurotransmission may produce aberrant signaling and thus affect cognition and behavior. It is likely that the recent discoveries of unpredicted synaptic arrangements will lead to novel experimental approaches to have a better understanding of how neurotransmission shifts from homeostatic conditions, how certain neurotransmitters become amplified or silenced, and how multiplexed signals might be simultaneously sent and received.

Because many neurotransmitters have multiple postsynaptic and presynaptic effects, multiplexed neurotransmission expands the repertoire of synaptic capabilities of single neurons. In this regard, electrophysiological evidence indicates that DA neurons are capable of acting on GABA_A receptors (Tritsch et al., 2012; Kim et al., 2015; Hoerbelt et al., 2015). Thus, when glutamate and DA are

multiplexed together – as they are in some mesoaccumbal projections (Zhang et al., 2015) – DA may act to facilitate glutamatergic signaling, counter glutamatergic signaling, or might even behave differently depending on the postsynaptic cell (e.g., targeting of D1 receptor neurons or D2 receptor neurons). With this in mind, it is clear that novel technologies and intersectional genetic strategies will be necessary in order to decipher the unique contributions of each component of multiplexed signals (Pupe and Wallén-Mackenzie, 2015).

A better understanding of the presynaptic and postsynaptic elements will allow better understanding of how these specialized synapses, described above, manage multiplexed neurotransmission in the presynaptic terminal and are subsequently integrated by the postsynaptic neuron. For example, these elements may work together to facilitate spike-timing dependent mechanisms for plasticity (e.g., Watanabe et al., 2002), as it is known that neuromodulators can affect the temporal window necessary for spike timing dependent activation, or that neuromodulators can cause a switch from long-term potentiation to long term depression (Caporale and Dan 2008; Bissière et al., 2003). Thus, the spatiotemporal relationship of segregated DAergic and glutamatergic signaling in the nAcc may act to enhance the probability of signal transduction when both transmitters are released within a short time window. A similar mechanism might apply to mesohabenular signaling, although the precise mechanism by which glutamate and GABA might work to facilitate or shunt one another is still unclear. One possibility is that the integration for either a GABAergic or a glutamate response by the post-synaptic cell would depend on the timing of other habenular afferents.

Overall, it is becoming clear that the VTA is far more complex than was initially realized. Indeed, many studies have reported heterogeneous responses of specific VTA neurons (Brischoux et al., 2009; Borgkvist et al., 2011; Margolis et al., 2014; Eddine et al., 2015; Mrejeru et al., 2015; Mejias-Aponte et al., 2015), and it would seem likely that this diversity is due (at least in part) to cells that are capable of multiplexed neurotransmission. With this in mind, it is clear that the discovery of compound cell types has wide-reaching implications for our understanding of VTA circuit functions and that the use of intersectional strategies will become increasingly critical. Moreover, multiplexed signaling neurons are increasingly identified throughout the brain, suggesting that this unique type of signaling plays important roles in health and disease.

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