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Cholinergic modulation of the mesostriatal pathway

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By

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Abstract

Cholinergic neurotransmission exerts widespread modulation of brain function. The focus of my thesis is to detail the neurophysiological and behavioural function of acetylcholine in the striatum and the dopaminergic midbrain. Using ChAT::cre Long Evans rats I investigate the direct and the indirect connectivity between brainstem and striatum. Using anatomical, electrophysiological and behavioural experiments, I describe a direct projection to the striatum arising from the cholinergic brainstem and also an indirect pathway through the dopaminergic midbrain. I describe anatomical and physiological differences in the modulation of dopaminergic midbrain neurons by cholinergic neurons in the pedunculopontine and the laterodorsal tegmental nucleus. I also show a novel cholinergic pathway to the striatum arising from the brainstem. Based on anatomical, physiological and behavioural results, I show a strong functional modulation of striatum by cholinergic transmission.

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Contents

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Glossary

µm: micrometres

5-CSRTT: 5 choice serial response time tasks

6-OHDA: 6-hydroxy dopamine

ACh: acetylcholine

AID: agranular insular dorsal cortex

AIV: agranular insular ventral cortex

AVP: arginine vasopressin

BG: basal ganglia

BLA: basolateral amygdala

CA1: CA1 field of the hippocampus

CCK: cholecystokinin

CE: central amygdala L, lateral; M, medial; C, capsular

Cg: cingulate cortex

ChAT: choline acetyltransferase

CINs: cholinergic interneurons

Cli: central linear nucleus

CNS: central nervous system

CS: conditioned stimulus

D1: dopamine receptor subtype 1

D2: dopamine receptor subtype 2

DA: dopamine

DAergic: Dopaminergic

Den: dorsal endopiriform nucleus

DhβE: dihydro-β-erythroidine

DLS: dorsolateral striatam

DMS: dorsomedial striatum

DS: dorsal striatum

EPSP: excitatory postsynaptic potential

fmi: external capsule

FSI: Fast spiking interneurons

GAD: glutamate decarboxylase

gcc: genu of the corpus callosum

GI: granula insular cortex

GPe: external globus pallidus

GPi: internal globus pallidus

Gpr6: orphan G-protein coupled receptor six

HCN: hyperpolarisation-activated cyclic nucleotide-gated

HDB: horizontal limb of the diagonal band of Broca

Hz: Hertz

IEn: intermediate endopiriform nucleus

IF: interfascicular nucleus

Ih: hyperpolarised-activated cation current

InG: intermediate gray layer superior colliculus

IPN: interpeduncular nucleus c, caudal; r, rostral

IPSP: inhibitory post synaptic potential

LDL: laterodorsal thalamic nucleus lateral part

LDT: laterodorsal tegmental nucleus

LENt: lateral enthorinal cortex

lHb: lateral habenula

Ls: lateral septum

LSD: lateral septum dorsal part

M: motor cortex

M1: muscarinic receptor subtype 1

M4: muscarinic receptor subtype 4

MD: mediodorsal thalamic nucleus M, medial; L, lateral

MEPP: miniature end plate potential

mHb: medial habenula

MOA: monoamine oxidase

MoDG: molecular layer dentate gyrus

Min: minute

MS: medial septum

ms: Milliseconds

MSNs: medium spiny neurons

mV: millivolt

NA: nucleus accumbens

0.49NA: 0.49 Numerical aperture

NAc: nucleus accumbens core

nAChRs: nicotinic receptors

NAsh: nucleus accumbens shell

NBM: nucleus basalis of Meynert

NOS: nitric oxide synthase

NPY: neuropeptide Y

NT: neurotensin

Op: optic nerves layer superior colliculus

PBN: parabigeminal nucleus

PBP: parabrachial pigmented area

PD: Parkinson's disease

pf: Parafascicular thalamic nucleus

PFC: prefrontal cortex

PFR: parafasciculus retroflexus area

Pir: piriform cortex

PLTS: low threshold spiking interneurons

PN: paranigral nucleus

PoDg: polymorph layer dentate gyrus

PPN: pedunculopontine nucleus

PPNc: caudal segment of the pedunculopontine nucleus

PPNr: rostral segment of the pedunculopontine nucleus

PV: parvalbumin

Rli: rostral linear nucleus

RMTg: rostromedial tegmental nucleus

RRF: retrorubral field

RSGa: retrospinal granular cortex

Rt: reticular thalamic nucleus

s/sec: second

S: somatosensory cortex

S1: somatosensory cortex 1

scp: superior cerebellar peduncle

SN: substantia nigra

SNc: substantia nigra pars compacta

SNpl: substantia nigra pars lateralis

SNr: substantia nigra pars reticulata

SOM: somatostatin

STN: subthalamic nucleus

Str: striatum

SuG: superficial gray superior colliculus

SWA: slow wave activity

TAN: tonically activate neuron

TH: Tyrosine hydroxylase

tu: olfactory tubercle

V3: third ventricle

V4: fourth ventricule

VAChT: vesicular acetylcholine transporter

VDB: vertical limb of the diagonal band of Broca

VIP: vasoactive intestinal polypeptide

VP: ventral pallidum

VPL: ventro-posterior thalamic nucleus lateral part

VTA: ventral tegmental area

Zi: Zone incerta

Chapter 1 : Introduction

General introduction

An alteration of acetylcholine (**ACh**) neurotransmission within the central nervous system (**CNS**) has been associated with several neurological disorders (Parkinson's disease, Schizophrenia, Alzheimer or drug addiction) (Schliebs and Arendt, 2011; Cuello and Bruno, 2007). Some of these disorders, such as obsessive-compulsive disorder or addiction, have been shown to be associated with an increase of the activity of cholinergic neurons within many brain nuclei (Ishibashi et al., 2009); others, such as Parkinson's disease (**PD**), with a decrease in the number of cholinergic neurons or their function (Hall et al., 2014). It has been demonstrated that most of those conditions (addiction, PD, schizophrenia) are related to the interaction between the mesostriatal pathway (see below) and ACh, in particular the modulation of dopamine (**DA**) release in the striatum (**Str**) (Schultz, 1998; Koob, 2008; Chang, 1988). While a lot is known about the dopaminergic (**DAergic**) pathway, much is still unknown about its modulation by ACh. In this thesis, I will first describe the cholinergic nuclei within the CNS. Next I will focus on the nuclei of the basal ganglia and their relation to the cholinergic systems.

Acetylcholine in the central nervous system

The synthesis of ACh is initiated by a reaction involving choline and coenzyme A. The reaction is catalysed by the enzyme choline acetyltransferase (**ChAT**). Availability of choline is the rate-limiting step in the synthesis of ACh, and thus the choline uptake system is critical (Searl et al., 1991). At least half of the choline used for ACh synthesis is provided by the recycling of released and

metabolised ACh; the other half is coming from general glucose metabolism. While ChAT is the enzyme responsible for the synthesis of ACh, the vesicular acetylcholine transporter (**VAChT**) concentrates ACh in synaptic vesicles.

The complexity of cholinergic nuclei, receptors and projections make cholinergic systems some of the most complex within the whole brain (Wannacott, 1997; Luchicchi, 2014). In the early description of the brain, the entire cholinergic system was divided into three main groups "forebrain, midbrain, and brainstem" cholinergic nuclei. Immunostaining for ChAT or choline acetyl esterase revealed many discrete nuclei with dense and widespread projections across the entire CNS. Mesulam and colleagues (Mesulam et al., 1976; Mesulam et al., 1983a; Mesulam et al., 1983b) described several discrete cholinergic structures and proposed a nomenclature which is still used today. Numerous novel nomenclatures have been proposed, based mostly on subdivision of cholinergic nuclei. Mesulam divided the cholinergic system into seven major nuclei named Ch1 to Ch7 (Mesulam, 1976), later adding additional nucleus (Ch8) (Mesulam, 1983a).

In these early studies, with immunolabelling of cholinergic neurons, the largest cholinergic structure was identified as the striatal complex. However, Mesulam and colleagues (Mesulam et al., 1983a,b) excluded from their reports the interneurons expressing the marker for ACh (located in the cortex and the Str) and the peripheral nervous system neurons (cranial nerve 5) in order to focus on the projecting cholinergic neurons. The second biggest structure is located in the basal forebrain, ventrally and medially to the Str and includes several dense and large cholinergic nuclei. The cholinergic basal forebrain is made up of four ChAT-immunopositive nuclei, Ch1 to Ch4. The first structure, Ch1 is located in the medial

septal (**MS**) and projects massively to the cortex; these neurons are involved in context-place memory (association between context and environment) (Easton et al., 2011). The second (Ch2) is located in the vertical limb of the diagonal band of Broca (**VDB**). The third one (Ch3) is situated in the horizontal limb of the diagonal band of Broca (**HDB**). Ch2 and Ch3 are anatomically similar structures often called together the nucleus of Broca (Palacios et al., 1991). Neurons of these nuclei have the densest axonal tree, innervating several cortical, hippocampal and thalamic structures. Projections to the hippocampus appear to be crucial for theta rhythm generation (Bland et al., 1999). The fourth cholinergic basal forebrain structure (Ch4) is located in the nucleus basalis of Meynert (**NBM**) (also including the ventral pallidum (**VP**) and the substantia innominata in some nomenclature) (Armonda et al., 1991). Mesulam described cholinergic neurons of the NBM as long and complex projecting neurons; they target mostly the cortex and the hippocampus and they have been described as important for attentional functions (Galani et al., 2002).

The rodent brainstem contains a long band of sparsely distributed cholinergic neurons easily visible in sagittal sections immunostained for ChAT. This cholinergic strip located in the ventral brainstem begins below the cerebellum and follows the superior cerebellar peduncle (**scp**). Mesulam described two nuclei, Ch5 and Ch6, positioned rostral to the reticular formation. The first group of neurons, Ch5, is a heterogeneous structure located in the pedunculopontine nucleus (**PPN**) and extends into the cuneiforms and parabrachial nuclei. The second nucleus, Ch6, is confined within the periventricular gray and is localised in the laterodorsal tegmental nucleus (**LDT**).

In the anatomical description of Mesulam, the basal forebrain and the

brainstem nuclei constituted the majority of cholinergic neurons, and also the majority of cholinergic axons within the entire brain. Two small nuclei were also described in the classification of Mesulam and colleagues. One, Ch7, is located in the dorsal midbrain and corresponds to a very small number of cholinergic neurons localised in the medial habenula (**mHb**). These cholinergic neurons are bordered by the lateral habenula (**lHb**) and the third ventricle (**V3**). The mHb contains about 300 to 500 cholinergic neurons in rats. They are distributed along a band following the antero-posterior axis.

The last structure is a small nucleus, formed by a very dense group of cholinergic neurons located in the lateral midbrain. Named Ch8, this structure is localised in the parabigeminal nucleus of the pontomesencephalic region (**PBN**), bordered dorsally by the colliculus and caudally by the nucleus of the pons.

Basal ganglia

Composition

The BG are a group of highly interconnected subcortical nuclei. A role for the entire BG, not just the mesostriatal pathway, has been proposed in many diseases (Brenner et al., 1947; Jus and Jus, 1948; Blandini et al., 2000). In humans, the BG includes the caudate nucleus, the putamen, the external globus pallidus (GPe), the internal globus pallidus (GPi), the subthalamic nucleus (STN), the substantia nigra (SN) and the VP. In addition, a further part of the BG (named ventral basal ganglia) includes the nucleus accumbens (NA), the olfactory tubercle (tu) and its DAergic input from the ventral tegmental area (VTA) (Humphries and Prescott, 2010). In rodents, the caudate/putamen is often referred to as the Str (Fig. 1).

Function

The nuclei of the BG are involved in motor control functions (Marsden, 1982; DeLong and Wichmann, 2009), motivational processes (Stathis et al., 2007), and cognitive functions (Redgrave et al., 2011). A current theory proposes that the cortex, thalamus and BG are interconnected in parallel and functionally segregated loops (Alexander and Crutcher, 1990; Nauta and Domesick, 1978; Haber, 2003). The main function of these loops is to transmit and integrate cortical information (Deng et al., 2015; Robertson et al., 2015). Inputs from motor or limbic cortical areas project directly to certain parts of the striatum (Wichmann and Dostrovsky, 2011). While the cortical inputs to striatum are topographically organised, some parts of the striatum receive overlapping, convergent inputs from several, often related, cortical areas (Brown et al., 1998; DeLong and Wichmann, 2007).

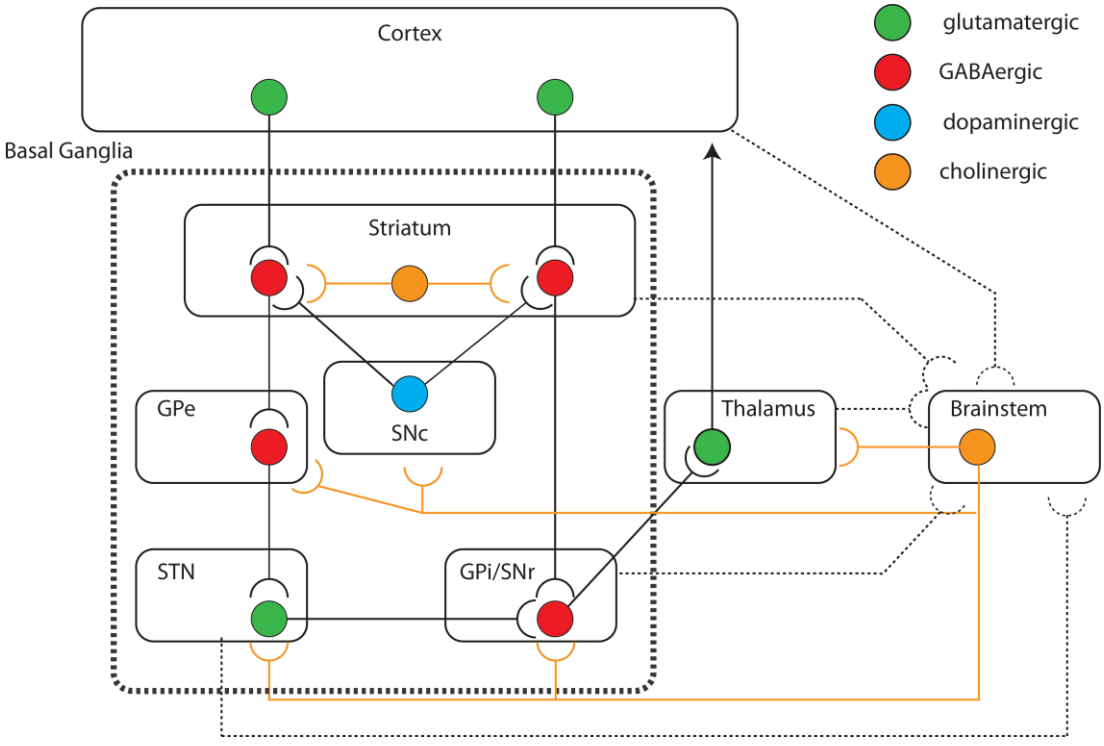

Figure 1: Simplified illustration of the structures and major connections of the basal ganglia. See legends for cell types involved. Inputs to the brainstem are represented with dot lines. Abreviations, GPe: globus pallidum external segment; GPi: globus pallidus internal segment; SNc: substantia nigra pars compacta; STN:

subthalamic nucleus; SNr: substantia nigra pars reticulata. Adapted from Mena-Segovia et al., 2004 and Tepper et al., 2007.

This simplified model of the BG positions the Str as the main "entry structure" as it receives excitatory input from the cortex and thalamus as well as inputs from midbrain structures (Alexander et al., 1986). It has been demonstrated that corticostriatal projections specifically target and activate the main population of neurons within the Str, the medium spiny neurons (**MSNs**) (Somogyi et al., 1981; Lachandani et al., 2013; Huerta-Ocampo et al., 2013). Cortical inputs to the Str affect behaviour differentially depending on types of MSNs contacted, location in the matrix vs. striosomal compartments of the striatum, or location in anterior, posterior, medial or lateral striatum (Wilson et al., 1983; McGeorge and Faull, 1989; Flaherty and Graybiel, 1991; Chudler et al., 1995). The corticostriatal projections show a high degree of divergence and convergence, allowing striatal neurons to integrate inputs from cortical subdivisions (Nagy et al., 2005). Indeed, discrete tracer injections in striatal regions revealed overlapping clusters of corticostriatal projections suggesting that different cortical layers or different cortical neuron types target discrete striatal regions or striatal neuron types (Kincaid and Wilson, 1996; Kincaid and Wilson, 1998).

The mesostriatal pathway innervates either the dorsal striatum (**DS**) (mostly arising in the SNc but also the VTA neurons) or the NA (arising in the VTA and to some extent the SNc) (Ikemoto, 2007). Some DAergic neurons project to prefrontal cortex (PFC) and show collaterals in the NA (Thierry et al., 1973; Beier et al., 2015; Lerner et al., 2015). Those DAergic projections to the cortex have been called mesocorticolimbic pathway, and have been implicated in motor, motivational,

memory, attention and stress functions associated to the BG (Oades, 1987; Deutch et al., 1990; Abercrombie et al.; 1989; Schultz et al., 1993). Other projections arising from midbrain DAergic neurons have been found to innervate the thalamus (Melchitzky et al., 2006), the lateral habenula (Phillipson and Griffith, 1980), hippocampus (Gasbarri et al., 1997), and amygdala (Loughlin et al., 1983).

Model of Basal Ganglia

Cortex

Cortico-striatal pathway

Cortical projections arise from different areas and target topographically the Str and the thalamus (Kemp and Powell, 1970; Bolam et al., 2000; Middleton and strick, 2000). It has been shown that about 60% of the inputs to MSNs arise from corticostriatal neurons (Wall et al., 2013; Lacey et al., 2005; Raju et al., 2006). A proposed model of the corticostriatal pathway is that the function of the BG is mainly to direct the cortical input to appropriate downstream targets (Stocco, Lebiere and Anderson, 2011). In this model, different cortical regions communicate with each other by cortico-cortical connections and send information to their respective subdivision of the Str, which can be a different type of neuron or different striatum regions (Gerfen, 1989; Van Dongen et al., 2005; Huerta-Ocampo et al., 2013). Another marker for MSNs involved in direct and indirect pathways is based on the membrane expression of DAergic receptor subtypes 1 or 2 (respectively D1 and D2). These two pathways have opposite effects on thalamic activity; whereas the direct pathway reduces the inhibition on thalamus activity (by inhibiting the output nuclei), the indirect pathway enhances the thalamic inhibition (by activating the output nuclei) (Albin et al., 1989; DeLong, 1990; Stocco et al., 2010). As direct and indirect

pathways give rise to opposing responses on thalamic activity, the balance between the activation of D1 and D2 MSNs will determine the functional effect (Wei, Rubin and Wang, 2015; Logan et al., 1984). This balance between D1/D2 seems to be important for the flexibility of action selection. Retrograde tracer studies suggested that all regions of the cortex target direct and indirect pathway neurons (Wall et al., 2013) as well as interneurons (Ding et al., 2010; Reynolds et al., 2004). The major cortical regions projecting to the Str are the sensory cortex, the motor cortex and the parietal cortex (McGeorge and Faull, 1989; Goldman and Nauta, 1977).

Thalamus

Thalamostriatal pathway

The thalamostriatal projections represent 20 to 25 % of the entire input to MSNs (Wall et al., 2013). Those excitatory projections follow a mediodorsal organisation, with projections from the mediodorsal and parafascicular (**pf**) nuclei going to the dorsolateral Str (**DLS**) (Schwab et al., 1977; Smith et al., 2009) and projections from the intralaminar, midline thalamic and pf nucleus projecting to the dorsomedial and the ventral Str (Gimenez-Amaya et al., 1995). The ventral thalamic nuclei receive inputs from GP and the substantia nigra pars reticulata (**SNr**) and relay that information to the prefrontal and motor cortices (Druga et al., 1991; Alexander et al., 1990; Wichmann and DeLong, 1996; Schell and Strick, 1984; Matelli and Luppino, 1996). Three main thalamostriatal pathways have been described in monkies, based on their functions: motor, associative and limbic. The “motor thalamostriatal pathway” involves connections between the putamen, the GPi and the medial two-thirds of the centro-median thalamic nucleus; the “associative pathway” has been described between the caudate nucleus, the SNr and the caudal two-thirds of pf, finally the “limbic pathway” showed connections between the NA, GPi and the

rostral third of the pf (Sidibé et al., 1997; Sidibé et al., 2002; Smith et al., 2004; Smith et al., 2010; see review: Smith et al., 2009). Thalamic projections form synaptic contacts with MSNs (Dubé et al., 1998; Xu et al., 1991; Wall et al., 2013) and cholinergic interneurons (CINs) (Laper and Bolam, 1992; Doig et al., 2014). Lesions of thalamic nuclei showed a role of thalamostriatal pathway in reward-guided actions (Yu and al., 2010), learning (Parker et al., 1997; Mitchell et al., 2007a, b), action-outcome associations (Ostlund and Balleine, 2008), fear conditioning (Herry et al., 1999) or recognition memory (Hunt and Aggleton, 1998a), which are believed to be behaviours associated with striatal functions. Following thalamostriatal pathway stimulation, a large population of MSNs increases their firing rate and can induce either an increase or decrease in ACh release in the striatum based on MSNs collaterals and glutamatergic receptors (Nanda et al., 2009; Consolo et al., 1996).

Globus pallidus

Anatomy

In primates, the globus pallidus is comprised of two segments: the external (or lateral, **GPe)** and the internal (or medial, **GPi)**. In rodents, the GPi is named entopeduncular and constitutes a group of GABAergic neurons that extend into the SNr. Both segments have different functions, anatomy and connectivity within the BG.

Inputs/outputs

The GPi is an output structure of the basal ganglia. It is the smallest nucleus of the basal ganglia circuit which is bordered by the internal capsule and receives inputs from Str, GPe and the subthalamic nucleus (**STN**). The GPi has been described as similar to the SNr, sharing similar histologic and functional properties (Nambu et al.,

2002). The GPi projects to the ventral thalamic nucleus (motor thalamus), and gives off several collaterals to thalamic projecting neurons and GABAergic interneurons (Ilinsky et al., 1997; Kuo and Carpenter, 1973; Kim et al., 1976). These GPi GABAergic projections to the thalamus tend to inhibit the thalamocortical feedback. Other GPi projections reach the lateral habenula and the PPN (Carter and Fibiger, 1978; Nauta, 1979). GPi receives a combination of inhibitory and excitatory inputs from striatum, GPe, STN, PFC, intralaminar thalamus, PPN or SNc (Lavoie and Parent, 1990; Lavoie and Parent, 1994; Naito and Kita, 1994a; Parent and Hazrati, 1995b). *In vitro* studies show that GPi neurons are enriched in GABA and glutamate receptors compared to other neurons (Wisden et al., 1992; Albin et al., 1992). GPi neurons express a strong hyperpolarisation-activated cyclic nucleotide-gated (HCN) current supporting the high frequency spontaneous firing (Nakanishi et al., 1990). *In vivo*, GPi neurons also show a homogenous neuronal population exhibiting a short action potential (<1ms) and a high firing rate (>15Hz) (Benhamou and Cohen, 2014).

The GPe is centrally located within the BG, sending inhibitory outputs to virtually every other BG nuclei (Kita and Kitai, 1994; Bevan et al., 1998). The GPe sends projections outside the basal ganglia, and does not always seem to give rise to local collaterals (Parent et al., 2001; Parent and Parent, 2004). GPe efferents have been reported in the STN, the SNc, the PPN, the thalamus, SNr and the cortex (Heimer et al., 1995; Parent and Hazrati, 1995; Deschenes et al., 1996; Yasukawa et al., 2004). Indeed, a subpopulation of GPe neurons projects to all regions of the basal ganglia including the Str and influences the activity of GABAergic and nitric oxide synthesizing (NOS) interneurons (Bevan et al., 1998). Those pallidal neurons projecting to the Str have the potential to regulate basal ganglia processing of cortical information. The main sources of afferent projections to the GPe are the striatum

MSNs (GABAergic) and the STN glutamatergic fibres (Parent and Hazrati, 1995b). Like the GPi, the GPe is enriched in GABA and glutamate receptors (Wisden et al., 1992; Albin et al., 1992). Some D1 and D2 dopamine receptors are also present in the GPe (Yung et al., 1995). The GPe seems to contain three main types of neurons: Arkypallidal, parvalbumin (PV) and Lhx6. Arkypallidal neurons are a subset of GPe neurons that make exclusively strong projections back to the Str innervating MSNs and interneurons (Mallet et al., 2012). Lhx6 pallidal neurons selectively innervate fast spiking interneurons in the Str (Kita and Kita, 2001) and PV neurons show very weak projections to GABAergic interneurons of the Str (Mastro et al., 2014). Arkypallidal and Lhx6-GPe neurons give rise to a topographical organisation. Lhx6-GPe neurons seem to project mostly to the SNr and Str, Arkypallidal neuron project only to the Str, while PV neurons send strong projections to the STN and pf thalamic nucleus (Mastro et al., 2014). Lhx6 neurons are more concentrated in the medial region of the GPe which have been described as associative; and PV neurons are more densely distributed in the lateral region of the GPe, which is described as sensorimotor (Mogenson et al., 1983; Haber et al., 1995; Kita et al., 2007). *In vivo*, a high proportion of GPe neurons show a high frequency tonic firing while Arkypallidal neurons show phasic activity and lower firing rates (Schmidt et al., 2014). The GPe is the principal source of GABAergic inhibition of the STN (Smith et al., 1988) through a very dense synaptic innervation (Baufreton et al., 2009). In animal models of Parkinson's, the GPe has been described as abnormally hypoactive while the STN shows hyperactivity (Galvan and Wichmann, 2008). Hyperactivity of the indirect pathway observed in PD models has been supposed to explain the hypofunction of the GPe-STN pathway (Gerfen and Surmeier, 2011; Fan et al., 2012).

Subthalamic nucleus

Anatomy

The STN is a small structure of the BG, showing dysfunction in PD (Wichmann and DeLong, 1996; Bergman et al., 1998; Hollermann and Grace, 1992; Magill et al., 2001). The STN presents excitatory outputs projecting to most of the basal ganglia (Nauta and Cole, 1978). Indeed, projections to the SN, the GPe, motor cortex or the Str have been described (Kita and Kitai, 1987; Smith et al., 1990), but the main afference is clearly the GPi/SNr.

Inputs/outputs

The principal inhibitory input to the STN arises from the GPe, and acts with GABAa receptors. However, STN neurons also receive inputs from the VP, the ventral striatum and cortical projections (hyperdirect pathway). The STN also receive excitatory projections from the thalamus (Kita et al., 1983; Canteras et al., 1990; Fujimoto and Kita, 1993). In addition to glutamatergic (cortex) and GABAergic (GPe) inputs, the STN receives dopaminergic (SN), serotoninergic (dorsal raphe) and cholinergic inputs (PPN, LDT), but very little is known about the role of 5-HT or ACh in STN function (Wang and Morales, 2009; Nambu et al., 2002).

Neuronal populations

The principal types of neurons in the STN are glutamatergic and present as long spiny dendrites (Afsharpour, 1985; Rafols and Fox, 1976). There is also a small number of GABAergic interneurons (Levesque and Parent, 2005). Both GABA and glutamate receptors are highly expressed in the STN. Few D1, D2 and D3 receptors are also observed in the STN (Flores et al., 1999).

Electrophysiology

STN neurons show a rhythmic neuronal activity (Beurrier et al., 2000; Bevan and

Wilson, 1999). Slowly inactivating voltage-gated Na^+ channels are involved in the silent phase of the oscillation; while calcium channels control the period and the precision of the oscillation (Bevan and Wilson, 1999). During cortical slow wave activity (**SWA**), self-generated movement or passive movement STN and GPe fire differently in a complex manner (Nini et al., 1995; Wichmann et al., 1994).

Functions

STN seems to be involved in the inhibition of motor responses (Frank et al., 2007). The hyperdirect pathway enables STN to update performance during behaviour tasks (Frank et al., 2007; Nambu et al., 2002). STN neurons modulate response inhibition in conflict tasks (Schroeder et al., 2002; Aron et al., 2007) by shunting cortico-striatal-STN information to slow down or cancel an action (stop-start behaviour). However, the STN is involved only in tasks with high cognitive value, not in simpler cognitive tasks (Rektor et al., 2009). Unilateral lesion of the STN was shown to induce postural asymmetry, gait and axial disorders (Andren et al., 1995; Su et al., 2002; Lozano and Snyder, 2008) and increase premature responses in 5 choice serial response time tasks (5-CSRTT) (Baunez and Robbins, 1999b) or go no-go performance (Ballanger et al., 2009). Various non-motor functions (motivational, learning) have been associated with STN activity (Mallet el al., 2007; Voon et al., 2009; Brittain et al., 2012; see review: Baunez and Lardeux, 2011).

In PD, abnormal synchronous activities between STN and GPe (and other parts of the basal ganglia) have been observed (Filion, 1979; Levy et al., 2001; Raz et al., 2000). Due to the central role of the STN in basal ganglia function and because STN lesions were found to be very effective in parkinsonian primates (Bregman et al., 1990), stimulation of the STN in PD patients was used as an experimental therapy. Indeed, deep brain stimulation (**DBS**) of the STN reduces symptoms on Parkinson's

patients (Okun and Foote, 2005; Okun and Vitek, 2004).

Striatum

The DS and the NA share a very similar anatomical structure and similar input patterns. I will describe the NA and further focus on the dorsal part of the Str. While it has been accepted that the olfactory tubercle (**tu**) is a part of the ventral Str, only a few experiments describe the role and an anatomical description of this nucleus (Next, striatum will refer to ventral and dorsal striatum, dorsal striatum to lateral and medial part, ventral striatum to accumbens and olfactory tubercle).

Anatomy

In primates, the Str is easily divisible into the caudate nucleus and putamen, both sectors are separated by a white matter tract called the internal capsule. However, in rodent research, such clear anatomical divisions have not been observed. Only a combination of antibodies (calretinin, calbindin and ChaT) can perfectly separate the dorsal and the ventral Str, the NA core and shell (Cragg et al., 2002). An interesting debate appeared when using the term NA when describing the ventral part of the Str:

Is the nucleus accumbens equivalent to the ventral part of the Str?

Anatomical descriptions of the NA appeared very early (Pennartz et al., 1994), but it has been accepted that the first detailed anatomical, physiological and behavioural description was made later (Zahm, 1999). The NA shell was described as a complex region with higher neuroanatomical diversity than the core, and also receiving greater inputs from the thalamus and the DAergic midbrain (Voorn et al., 2004; Bossert et al., 2007). At a molecular level, core and shell show differences in the distribution of a number of neuroactive substances and receptors, including

serotonin (Deutch and Cameron, 1992), DA (Patel et al., 1995) and calretinin (Prensa et al., 2003), with these substances preferentially located in the shell. There is also evidence to suggest that the core is the main dopaminergic target of the NA as it receives densest inputs from the VTA and the SNc (Mogenson et al., 1980; Phillipson and Griffiths, 1985).

Inputs/outputs

The major neural input to the striatum is excitatory. Glutamatergic projections from all cortical areas, from the thalamus, hippocampus and from the amygdala have been observed (Kelley, 1982). Other inputs arise from the SN/VTA, dorsal raphe nucleus, locus coeruleus or brainstem (Flaherty and Graybiel, 1994; Pan et al., 2010; Beier et al., 2015), hypothalamus and many more. MSNs constitute the only output of the DS and target the GPe, the GPi and the SNr.

Neuronal populations

The Str and the Na are both structures with a heterogeneous neural composition, very complex interconnections and a wide variety of receptors. In adult mice, the Str contains approximately 700,000 neurons in each hemisphere, while in adult rats the number is about 1,500,000 neurons (Fentress et al., 1981). The primary population of striatal neurons is the GABAergic MSNs. These projection neurons, also known as spiny projection neurons, are the only output of the Str and represent up to 95% of the total population. In rats, approximately 6% of the three million MSNs in the Str express both D1 and D2 receptors, this giving them the potential to modulate the basal ganglia network bi-directionally (Perreault et al., 2012). In adult mice, no differences were observed in the ratio of D1, D2 and D1/D2 MSNs (Fentress et al., 1981).

The second biggest population of striatal neurons is the GABAergic

interneurons, which consist of several subpopulations based on expression or co-expression of molecular markers (see review: Tepper et al., 2010). The third striatal cell population is the large aspiny CINs often referred to in humans/monkies as tonically active neurons (**TAN**). Originally described as the only cholinergic source of ACh in the Str (Woolf and Butcher, 1981) (Woolf and Butcher used ACh esterase staining), the CINs constitute up to 2% of the striatal neurons (Bolam et al, 1984) and the Str has the highest level of ChAT in the brain (MacIntosh, 1941; Hebb and Silver, 1961; Woolf et al., 1984).

Functions

The Str is a subcortical brain structure crucial for motivation and movement (Da Cunha et al., 2012). Str structure and composition appear to be highly conserved across the evolution of several species (Stephenson-Jones et al., 2011), indicating a fundamental functional role. Str dysfunctions have been described in a wide variety of human neurological and psychological disorders, such as addiction (Koob, 1992), obsessive-compulsive disorder (Chudasama and Robbins, 2006), eating disorders (Norgren et al., 2006), dyskinesia (Barroso-Chinea and Bezard, 2010) and PD (Ellens and Leventhal, 2013). The DS is implicated in action selection (Balleine, 2007), in motor behaviours and decision-making while ventral Str mediates motivational processes (Graybiel et al., 1994; Yin et al., 2006). Behavioural experiments using similar lesion techniques suggest a role of the dorsomedial Str (DMS) in opposite function, such as decreasing behaviour updating (Okada et al., 2011; Ragozzino et al., 2002a; Aoki et al., 2015). These behavioural effects depend on testing different protocols or animal strains (Aoki et al., 2015). Nonetheless, the complexity of the Str and its inputs has also been used to explain such across studies.

Medium spiny neurons

The Str contains a large number of small to medium sized neurons, less than 20 µm in diameter (Mehler, 1981; Ramon y Cajal, 1911; Vogt and Vogt, 1920), which constitute the only output of the Str (Bolam et al., 1981); they are identified as MSNs. MSNs bear a large number of spines on their dendrites. They receive synaptic input from an important number of structures, such as cortex, thalamus, hippocampus and DAergic neurons of the midbrain (Wall et al., 2013; Chuhma et al., 2011). Cortical inputs to the Str activate both D1 and D2 MSNs, their activation results in different behaviours (DeLong and Wichmann, 2007; Huerta-Ocampo et al., 2013).

It has been shown that D1 MSNs also express the muscarinic receptor subtypes 4 (M4) and 1 (M1), and the neuropeptides dynorphin and substance P. In contrast, D2 MSNs were expressing the adenosine receptor 2a, M1 and the orphan G-protein coupled receptor six (**Gpr6**) (Gerfen, 1992; Heiman et al., 2008; Bernard et al., 1992; Lobbo et al., 2007).

The optogenetic/pharmacologic activation of the direct pathway results in an inhibition of the GPi and SNr, and therefore results in a release of the inhibition of the thalamocortical pathway, facilitating motor functions. In contrast, activation of the indirect pathway inhibits the GPe, which releases the inhibition on GPi and SNr; and therefore increases the inhibition of the thalamocortical pathway (Ferguson et al., 2014; Kravitz et al., 2012; Lobo et al., 2010). Finally, direct pathway activation will facilitate movement while the indirect pathway activation leads to a decrease in the locomotion (Delong, 1990; Penny et al., 1986).

Interestingly, basal ganglia models propose that in addition to controlling movement, striatal projection neurons also influence motivational and social

behaviour. However, the position of the MSNs (dorsomedial, dorsolateral or ventral Str) and whether they are part of the direct or indirect pathway appears to be important for different behaviours. Activation of the direct pathway in the DLS increases locomotion, in the DMS MSNs regulate the response latency and goal-directed behaviour (Fukabori et al., 2012) and in the accumbens MSNs modulate reward-related learning (Lobo et al., 2010; Kravitz et al., 2012). Activation of the indirect pathway in the DLS modulates the response accuracy in audio discrimination tasks (Nishizawa et al., 2012). In the DLS, MSNs decrease motor behaviour (Kravitz et al., 2010; Freeze et al., 2013) and in the accumbens MSNs increase aversive association (Danjo et al., 2014; Hikida et al., 2010). Finally, activation of dorsomedial MSNs affects action selection with no differences observed between D1 and D2 MSNs (Tai et al., 2012).

Fast spiking interneurons (FSI)

In the early 1990s, reports described three distinct subtypes of GABAergic interneurons in the Str: 1) fast spiking interneurons (FSI) expressing the calcium-binding protein parvalbumin (PV), 2) low threshold spiking interneurons expressing the neuropeptide Y (NPY), the neuropeptide somatostatin (SOM) and NOS and 3) low threshold calcium spike interneurons expressing the calcium channel marker calretinin (Kawaguchi, 1993; Kubota et al., 1993; Vincent and Johansson, 1983; Bennett and Bolam, 1993). New subpopulations of GABAergic interneurons have been described recently: Tyrosine hydroxylase (TH) FSIs (Xenias et al., 2015; Ibáñez-sandoval et al., 2010), PV^+/$secretagogin^+$, PV^+/$secretagogin^-$, PV^-/$secretagogin^+$ or 5HT-3a expressing interneurons (Faust et al., 2015; Cains et al., 2012). However, these groups comprise a small number of neurons (around 300 TH^+ FSI interneurons in rat) and have a poorly understood or unknown function (Tepper

et al. 2010). Unbiased stereological counting studies in mice have shown that 0.7 % of Str neurons are PV^+, 0.5 % are CR^+ and 0.6 % are NPY/NOS/SOM^+ (Rymar et al., 2004). Immunolabelling for PV revealed a sparse population of interneurons (Gerfen et al., 1990) that give rise to five to eight aspiny dendrites (Cowan et al., 1990; Kita, 1993) with a compact and roughly spherical field that extends 200 to 300 µm around the soma (Kawaguchi, 1993). During *in vitro* whole cell recording or intracellular recording, PV interneurons show a distinctive electrophysiological profile (Taverna et al., 2007; Bracci et al., 2002), are strongly hyperpolarised and do not display spontaneous activity. *In vivo*, PV interneurons show a very fast phasic firing rate with short spike duration (Sharott et al., 2012; Bean, 2007). An additional characteristic of PV^+ interneurons is their relative synchronicity due to the presence of GAP junctions (Koós and Tepper, 1999). PV interneurons receive thalamic and cortical inputs (Kita, 1993; Bennet and Bolam, 1994; Ramanathan et al., 2002). Strong excitatory cholinergic inputs have been observed (Chang and Kita, 1992) mostly through nicotinic receptors (Koós and Tepper, 2002). However, no direct effect of CINs activation on PV^+ interneurons has yet been observed. FSI can exert strong control over striatal output neurons; indeed a single FSI makes synapses onto hundreds of surrounding MSNs (Kita et al., 1990).

The second population of neurons described are the low threshold spiking (**PLTS**) interneurons expressing NPY/NOS/SOM markers (English et al., 2011; Hope et al., 1991). PLTS-interneurons have a medium sized soma with three to five aspiny dendrites, which extend over 600 µm diameter (Aoki and Pickel, 1988). *In vitro*, whole cell recordings reveal a low threshold Ca^{2+} spike and long-lasting plateau potentials in responses to excitatory synaptic inputs (Kawaguchi, 1993). While no *in vivo* recording of NPY^+ PLTS-interneurons have been published, these interneurons

contribute to the modulation of MSNs *in vitro* (Gittis et al., 2010).

The last main groups of GABAergic interneurons are the CR-interneurons, representing about 0.5 % of the neurons in the Str (Rymar et al., 2004). Information on this group of interneurons is limited; they were described as medium sized aspiny neurons, with a diameter between 12-20 µm, the dendrites of which branched into thin processes (Bennet and Bolam, 1993). However, studies have shown the subdivision of these CR-interneurons into three to four morphogically distinct types (Schlosser et al., 1999; Rymar et al., 2004). Other striatal interneurons have been observed and recorded recently, such as TH^+ interneurons (Dubach et al., 1987; Busceti et al., 2008), cholecystokinin (**CCK**) or vasoactive intestinal polypeptide (**VIP**) expressing (Takagi et al., 1984b; Adams and Fisher, 1990). However, these interneurons represent a very small amount of neurons and little information is available about them.

Cholinergic interneurons (CINs)

It has been proposed that CINs provide the major cholinergic innervation of the Str (Bolam, 1984). CINs constitute a small number of neurons within the Str; total numbers of CINs in the brain was estimated to lie between 30.000 and 50.000 neurons (1 to 3% of striatal neurons) and they innervate the entire Str (Contant et al., 1996; Woolf and Butcher, 1981, Oorschot, 1996; Oorschot, 1997). CINs have large soma (up to 40 µm) and are readily distinguishable from other striatal neurons. CINs receive an important number of inputs (Straub et al., 2014; Ravel et al., 1999). Glutamatergic inputs from thalamic and sensorimotor cortex have been observed (Lapper and Bolam, 1992; Calabresi et al., 1999; Cepeda et al., 2001; Bell et al., 2002) or from cholinergic interneurons that co-release glutamate (Straub et al., 2014;

Martella et al., 2009). CINs receive monoaminergic inputs from DAergic midbrain neurons (Aosaki et al., 1998; Ding et al., 2011b), TH-interneurons (Tandé et al., 2006) and the locus coeruleus (Deng et al., 2007a; Chuhma et al., 2014). However, the main inhibitory inputs to the CINs were provided by the MSNs, the GABAergic interneurons and the GABAergic projecting neurons of the midbrain (Gonzales et al., 2013; Brown et al., 2012; DeBoer and Westerink, 1994). CINs present synaptic contacts with MSNs and other cholinergic interneurons (English et al., 2012). Presynaptic contacts have been observed on DAergic (Nelson et al., 2014) and glutamatergic (Oldenburg and Ding, 2012) synapse in the Str.

CINs express different receptors types that mediate their activity by wide variety of inputs. D1 and D5 receptors have been observed to increase CINs activity, while D2 receptors slow down their firing (Chuhma et al., 2014; Kreitzer, 2009). The ratio of these receptors determines their main response and its duration. Up to 85% of the DAergic receptors expressed on the surface of CINs are type D5, while 90% of the glutamate receptors are kainate (KA) receptors and lead to excitation of CINs (Vorobjev et al., 2000; Gras et al., 2002; Higley et al., 2011). It has been suggested that CINs received a major GABAergic input, mediated only by $GABA_A$ receptors (Persohn et al., 1992) and arising mostly from MSNs (Martone et al., 1992; Chuhma et al., 2011; Gonzales et al., 2013). CINs are the only striatal cells expressing the $GABA_A$ α3 subunit (Rodriguez-Pallares et al., 2000). The GABAergic local inhibition can be reduced by using the GABA receptor antagonist, biccuculline (De Rover et al., 2002; Sullivan et al., 2008; Sato et al., 2014), and no evidence for GABAergic effect modulated by $GABA_B$ receptors has been demonstrated on CINs (DeBoer and Westerink, 1994; Oswald et al., 2015). A weak inhibitory effect mediated by PV interneurons has been observed (Szydlowski et al., 2013). Finally,

GABAergic neurons from the VTA also project to CINs (Brown et al., 2012). *In vitro*, activation of GABA or DA projections from the midbrain leads to a pause-rebound response in CINs (Brown et al., 2012; Straub et al., 2014). This effect might be mediated by DAergic neurons co-releasing GABA (Tritsh et al., 2012) but had also been proposed to be an artefact of certain animal models (Lammel et al., 2015). In addition, CINs express the muscarinic autoreceptors M2 and M4 which affect ACh release (Calabresi et al., 1999; Ding et al., 2006). A very important part of the CINs population is immunopositive for glutamatergic receptors; half of the CINs express the ionotropic receptors GluR1, GluR2 or GluR4 (Bernard et al., 1997; Tallaksen-Greene et al., 1998; Richardson et al., 2000; Pisani et al., 2001; Berg et al., 2007; Deng et al., 2007), while almost all of them express the kainate receptors GluR5, GluR6 or GluR7 (Chen et al., 1997). While metabotropic glutamate receptors decrease cell excitability by either a cation current or an HCN channel (Berg et al., 2007; Diraddo et al., 2014; Bell et al., 2002; Martella et al., 2009), ionotropic receptors increase the excitability of CINs (Calabresi et al., 1999; Cepeda et al., 2001). Most of the glutamatergic inputs to CINs arises in the cortex and the pf thalamic nucleus (or centro-median thalamic nucleus for the CINs located in the NA) (Gonzales et al., 2013; Lapper and Bolam, 1992; Thomas et al., 2000; Ding et al., 2010; Doig et al., 2014); a weak source of glutamate arises in the SNc (Tritsch et al., 2012) or the VTA (Stuber et al., 2010). Stimulation of thalamic projections (Doig et al., 2014) provided strong activation of CINs followed by a pause (Ding et al., 2010); this effect seems to be mediated by a modulation of DA release. Stimulation of the cortex (Doig et al., 2014) provides a moderate activation of the CINs firing (Ding et al., 2010), but stimulation of the thalamus enhances the corticostriatal release of glutamate (Ding et al., 2010). All pauses observed during CINs inputs stimulation

were shown to facilitate the cortical drive on MSNs to suppress an action (Ding et al., 2010). Early observations reported that ACh levels rise as DA release decreases and CINs firing decreases depending on bursting activity of DAergic neurons (Zhao-Shea et al., 2010; Joshua et al., 2009; Morris et al., 2004), suggesting a strong relation between DA and ACh in the Str. DA release in the Str seems to be mostly non-synaptic (Rice and Cragg, 2008; Fuxe et al., 2012; Taber and Hurley, 2014) and some evidence suggests that DAergic projections provide synaptic contacts to CINs (Pisani et al., 2000; Freund et al., 1984).

In vitro, during patch clamp recording CINs display a tonic firing at a rate of 3 to 10 Hz (Wilson et al., 1990; Witten et al., 2011), a medium-high input resistance (200 MΩ) (Calabresi et al., 1997), a hyperpolarised-activated cation current (**Ih**) (Deng et al., 2007) and a long action potential duration (Threlfell et al., 2012). In contrast to most striatal neurons, *in vitro* CINs show a spontaneous spiking activity (Bennet and Wilson, 1999) also referred to as "pacemaker firing". The basal activity of CINs appears to be driven by interplay of voltage-dependent sodium channels, cyclic adenosine monophosphate dependent cation channels (**HCN**) and low conductance calcium/potassium channels (Maurice et al., 2004; Wilson, 2005). *In vivo*, CINs have often been described as tonically active, which is why CINs are often thought to be analogous to TAN recorded in monkeys (Inokawa et al., 2010). Pathological changes in striatal CINs physiology has been linked to motor dysfunction such as dyskinesia (Hauser and Olanow, 1993; Linazasoro, 1994).

SNc/VTA

The development of monoaminergic staining using histochemical techniques (Falck et al., 1962) offered a precise description of monoaminergic structures within

the brain of humans, rodents and other species (Dalhstrom and Fuxe 1964). The first nomenclature for the distribution of monoamine neurons identified more than sixteen groups referred to as A1 to A16. Of these, three were located in the ventral midbrain and described as DAergic. The first one, A9, is located in the SN. The second, A8, is more medial and located in the VTA and the third group, lies more caudal and is located in the retrorubral field (**RRF**). While this nomenclature is still used as a reference, even more detailed subdivisions of these nuclei have been identified (Paxinos and Franklin, 2007; Ikemoto, 2007).

The catecholamines include DA, adrenaline and noradrenaline. The A-amino acid phenylalanine is converted to tyrosine by a reaction catalysed by the phenylalanine hydroxylase enzyme. Then a hydroxide anion is added and a decarboxylation converts tyrosine to DA (tyrosine to DOPA then to dopamine), the reaction is mostly catalysed by the enzymes tyrosine hydroxylase (**TH**) and aromatic L-amino acid decarboxylase. At this stage, DA can be transported along the axons or hydroxylated by dopamine-β-hydroxylase to noradrenaline. Noradrenaline may then be directed along the axon for direct use or converted into adrenaline by the enzyme phenylethanolamine N-methyltransferase (Daubner et al., 2011).

In this section I will briefly address the anatomy of the substantia nigra and mostly of the ventral tegmental area. I will also describe the function of the mesostriatal system and the modulatory role of ACh.

Anatomy of the substantia nigra

In the rat, immunostaining for TH revealed up to 40,000 DAergic neurons in the ventral midbrain. With 20-25,000 only in the SN (including the substantia nigra pars compacta (**SNc**), the substantia nigra pars lateralis (**SNpl**) and the substantia

nigra pars reticulata (**SNr**)). The SNc contains more than half of the SN DA neurons. The rest of these DA neurons located in the SN lie within the SNpl and few are located in the SNr. The other neurons of the SN were defined as being mostly GABAergic and are located in the SNr, some were observed in the SNpl and very few in the SNc (Nair-Roberts et al., 2008).

Anatomy of the ventral tegmental area

The VTA is a structure with no clearly defined borders, formed by heterogeneous nuclei and including several neuron types, functions and outputs. The VTA was first described in the opossum by Tsai (Tsai, 1925) which gave it temporarily the name of ventral tegmental area of Tsai (Nauta et al., 1958). The VTA was then described in detail using TH immunostaining (Phillipson, 1979). The number of sub-nuclei within the VTA remains a matter of debate, and has variously been proposed to consist of 2 to 5 sub-nuclei with various borders and nomenclatures including the parabrachial pigmented area (**PBP**), paranigral nucleus (**PN**) (Swanson, 1982), the parafasciculus retroflexus area (**PFR**), the rostromedial tegmental nucleus (**RMTg**) (Ikemoto, 2007), the interfascicular nucleus (**IF**), rostral linear nucleus (**Rli**) and central linear nucleus (**Cli**) (Phillipson, 1979b). Depending on the species, the VTA contains between 10.000 to 20.000 DAergic neurons per hemisphere (Swanson, 1982; Margolis et al., 2006). The VTA was described as being the main DAergic nucleus in the CNS (Nair-Roberts et al., 2008). Three main types of neurons, DAergic, GABAergic and glutamatergic, have been described on the basis of immunohistochemistry and *in situ* hybridization. Recent *in vitro* studies have shown the release of GABA in the NA/Str by TH^+ neurons (Tritsch et al., 2012) and by GABAergic projecting neurons (Brown et al., 2012; Fields et al., 2007). However, the absence of immunostaining for glutamate decarboxylase (**GAD**), an enzyme

needed for GABA synthesis, in TH^+ neurons suggests that GABA release can be due to reuptake of local GABA (Tritsch et al., 2012). In addition, a subset of catecholamine and non-catecholamine neurons within the VTA express the vesicular transporter type 2 (VGLUT2) (Méndez et al., 2008; Yamagushi et al., 2011) and constitute a group of VTA neurons projecting to both the PFC and the NA (Gorelova et al., 2012).

Inputs/Outputs

Early studies have described the VTA as a nucleus receiving dense GABAergic input. Using retrograde tracers injected into the VTA, Phillipson (Phillipson, 1979) described in detail VTA inputs arising from prefrontal cortex, NA, bed nucleus of the stria terminalis, amygdala, diagonal band of Broca, substantia innominata, preoptic area, hypothalamus, lateral habenula, pf thalamic nucleus, superior colliculus, dorsal raphe nucleus, locus coeruleus and cerebellum but without a description of the molecular identity of those inputs. One year earlier, Nauta (Nauta et al., 1978) described the same inputs to the VTA (the paper included the medial basal forebrain as an additional input) and using *in situ* hybridization characterised their GABAergic nature. With improving anatomical methods, the caudal part of the VTA called RMTg has been described as an individual nucleus (Ikemoto, 2007). It sends GABAergic projections to the VTA (Jhou et al., 2009; Kaufling et al., 2010a). There is also strong evidence for GABAergic interneurons within the VTA which can modulate the activity of local DA neurons (Tolu et al., 2012). However, most of the tracer studies used injection of retrorograde tracers (fluorogold, CTb, retrobeads, etc) which can be taken up "*en passage*" fibres (Omelchenko and Sesack, 2009; Cruz et al., 2008; Vrang et al., 1995; Chen and Aston-Jones, 1995). Several studies showed that excitatory inputs to the VTA arises from glutamatergic neurons in the PFC

(Harden et al., 1998), the brainstem (PPN: Floresco et al., 2003; LDT: Omelchenko and Sesack, 2005; Lammel et al., 2012), the dorsal raphe (Weissbourd et al., 2014), the BNST (Georges and Aston 2002; Herzog et al., 2004), the lateral habenula (Goncalves et al., 2012) and the lateral hypothalamus (Poller et al., 2013). The third major type of input to the VTA is cholinergic. Tracers injected into the VTA showed retrogradely labelled neurons within the PPN and the LDT which were also positive for ChAT (Oakman et al. 1995). Pharmacological studies showed an excitatory effect of ACh on DAergic neurons of the VTA (Mameli-Engvall et al., 2006; Tolu et al., 2012). Electron microscopy combined with staining for the vesicular acetylcholine transferase (VAChT) confirmed the presence of cholinergic synapses within the VTA (Bolam et al., 1991).

Very few differences between inputs to midbrain DAergic or GABAergic neurons have been observed (Watabe-Uchida et al., 2012; Beier et al., 2015). However, oxytocin and arginine vasopressin (**AVP**) neurons in the paraventricular hypothalamic nucleus mostly target GABAergic neurons of the VTA, while lateral hypothalamus orexin and neurotensin neurons target DA neurons (Beier et al., 2015).

Electrophysiology and anatomy of the VTA/SNc neurons

In vitro recording of DAergic neurons shows low frequency pacemaker firing (Sanghera et al., 1984; Grace and Onn, 1989). The action potential shows a large after-hyperpolarisation phase and an HCN current (Shi, 2009). *In vivo*, the characteristic of the action potential of DAergic neurons were first described by Grace and Bunney and consisted of a broad action potential waveform and two modes of discharge (Grace and Bunney, 1980; Grace and Bunney, 1984). Grace and Bunney showed the presence of high frequency bursting activity (>15Hz) (Grace and

Bunney, 1984), a long action potential waveform (>2.2 ms), a very regular firing rate (pacemaker) (1-5Hz) and inhibition by D2-autoreceptors. These characteristics have been identified in other species including primates (Scultz and Romo, 1988). Dorsolateral VTA DAergic neurons and SNc DAergic neurons display similar firing patterns; on the other hand ventromedial DA VTA neurons display a higher firing rate (<20Hz), a slightly smaller after-hyperpolarisation phase and also silent phases for several seconds (Margolis et al., 2008). *In vitro*, the characteristic spontaneous burst firing state seen *in vivo* is absent (Lacey et al., 1987; Grace and Onn, 1989).

The major description of DA output of the midbrain is derived from tracer studies (Bjorklund and Dunnet, 2007) or single cell reconstructions (Matsuda et al., 2009). Recent advances in molecular labelling tools allowed targeting inputs and outputs in a cell-specific manner (Wickersham et al., 2007b; Callaway, 2008; Ugolini, 2011). Projections from DAergic neurons of the VTA were found in NA (Ikemoto, 2007), DMS (Ikemoto, 2007), lateral septum (Louilot et al., 1989), motivational and motor cortex (Hosp et al., 2011), amygdala (Loughlin et al., 1983), dorsal hippocampus (Gasbarri et al., 1997), entorhinal area (Caruana and Chapman, 2008), lateral habenula (Stamatakis et al., 2013), locus coeruleus (Deutch et al., 1986) and parabrachial nucleus (Swanson, 1982; Margolis et al., 2006a). DAergic neurons projecting to the amygdala and the prefrontal cortex (**PFC**) have the shortest AP duration, the highest firing rates and an absence of response to D2 agonist (Lammel et al., 2008; Margolis et al., 2008).

The second population of neurons identified in the VTA is GABAergic and was first described by *in situ* hybridization for GAD. Estimations suggest that GABAergic neurons represent up to 30-35% of the total number of neurons in the

VTA (Nair-Roberts et al., 2008; Olson and Nestler, 2006; Gonzales-Hernandez et al., 2001). These neurons also contribute to the mesocorticolimbic network (Carr and Sesack, 2000). *In vitro,* GABAergic neurons in the VTA were classically divided into two subgroups, based on their firing rate (Korotkova et al., 2003). One group displays a slow firing rate (0.7 Hz), while the other group fires at a relatively high frequency (>9 Hz). No apparent difference was found in the spike duration or the firing regularity (no bursts or pauses were observed) (Klink et al., 2001). *In vivo*, the firing rate of non-DA VTA neurons depends on the phase of sleep-wake cycle (Steffensen et al., 1998). Putative GABAergic neurons of the VTA and the SNc recorded *in vivo* in anaesthetized rodents showed several firing patterns (Ungless et al., 2004; Diaz et al., 2000) but no differences were observed between non-DAergic neurons projecting to amygdala, PFC or NA (Margolis et al., 2008).

The majority of VTA cells show an irregular firing including a bursting phase, while a smaller number of neurons fires in a pacemaker-like fashion. Following partial lesion of DA neurons using 6-hydroxy dopamine (**6-OHDA**) an increase of the firing of DA-bursting neurons has been observed while the number of regular firing neurons was not affected (Koob et al., 1981). This result suggested a regulation of the bursting activity of DA neurons through local DA release (Llorens-Cortes et al., 1979; Adell and Artigas, 2004). Recent anatomical papers described a subpopulation of GABAergic neurons co-expressing TH (Nair-Robert et al., 2008; Olson and Nestler, 2007; Gonzales-Hernandez et al., 2001). These neurons GAD^+/TH^+ represent 20 to 25% of all the GAD^+ neurons. However, the cell labelling methods used in these studies are not suitable for an accurate analysis and no co-expression has been found using other techniques (Merril et al., 2015).

The third group of neurons in the VTA express the molecular marker VGLUT2 (Kawano et al., 2006; Nair-Roberts et al., 2008). About 15% of the VTA neurons described as $VGLUT2^+$ were also TH^+, while about 40% of the $VGLUT2^+$ neurons were also able to synthetize GABA (Yamaguchi et al., 2013). The VTA-glutamate neurons showed a small Ih, low sensitivity to DA and a fast firing rate (Hnasko et al., 2010). Glutamatergic neurons project to the VP, the amygdala, the lateral habenula, the NA and the PFC (Hnasko et al., 2010; Gorelova et al., 2012; Yamaguchi et al., 2011).

Functions

DAergic neurons of the VTA play a role in reward-related learning for natural outcomes (food, water) or addictive substances (coffee, alcohol or drugs of abuse) (Schultz et al., 1997; Liu et al., 2012). DA has been implicated in learning for both rewards and punishments (Brischoux et al., 2009; Joshua et al., 2008). In a classical set of studies, Schultz and colleagues (Schultz et al., 1997) showed that putative DAergic neurons in untrained monkeys increased their firing rate during reward delivery in a cue-reward association task (**CS**). Following training of the animals, DA neurons increased their basal activity following the CS rather than the reward. DA neurons suppress firing on reward omission and increase firing onsuprising reward presentation. This is termed a reward prediction error signal (Schultz et al., 1997; Lerner et al., 2015; Schoenbaum et al., 2013). However, the response of putative DAergic neurons to a reward or a punishment is not always consistent (Brischoux et al., 2009; Eddine et al., 2015). During recording of TH^+ neurons of the VTA, hind paw pinches (putative aversive stimulus) produced an increase in firing rate in 80% of recorded neurons, while 20% of the neurons showed a significant decrease of their activity (Brischoux et al., 2009). *In vitro*, pharmacological or

anatomical analysis of these neurons does not show any specific pattern related to the response to aversive or rewarding stimuli (Lammel et al., 2012). Interestingly, some putative DAergic neurons respond in the same direction to reward and punishment (Matsumoto and Hikosaka, 2009). Recent recordings in untrained animals suggests that the same neuron can respond differently to a wide range of punishment intensity (air puff, pinch, electric chock) or reward (sugar pellet, sucrose liquid, nutriment) (Joshua et al.,2008; Stauffer et al., 2015; Lak et al., 2014). As a consequence, DA release in the NA will be modulated based on previous experiences of reward and punishment (Wanat et al., 2010; Adamantidis et al., 2010). In this context, the DAergic neurons projecting to the NA, a primary component of the mesolimbic system have been conceptualized as teaching signal, encoding the memory of aversive and rewarding environmental stimuli (Pecina and Berridge, 2013).

Mesostriatal pathway

Functions of the BG-thalamo-cortico-BG loops can be divided into three systems, the sensorimotor, the associative and the limbic (Redgrave et al., 2011; Gerfen and Surmeier, 2011). One important pathway, present in the sensorimotor, associative and the limbic system, is the connection between midbrain and Str through the DAergic afferents, the mesostriatal pathway (Alexander et al., 1990). Most of the motor symptoms of PD have been associated with a dysfunction of the pathway connecting the SN to the DS (Carlsson, 1959), while symptoms of drug addiction are induced by at least the dysfunction of the connection between VTA and NA (see review: Koob and Le Moal, 2008). As far as we know, PD, like other neurodegenerative diseases, is specific to humans.

The nigrostriatal pathway has generally been associated with movement

regulation, whose dysfunction leads to neurodisorders such as PD or dystonia, and the mesolimbic pathway has been associated with motivation and reward (Ikemoto and Panksepp, 1999; Wise, 2009). However, recent studies suggest that motor behaviour might also be controlled by the mesolimbic pathway while the nigrostriatal pathway could also be involved in motivation and reward (Ilango et al., 2014; Rossi et al., 2013) (Fig. 2).

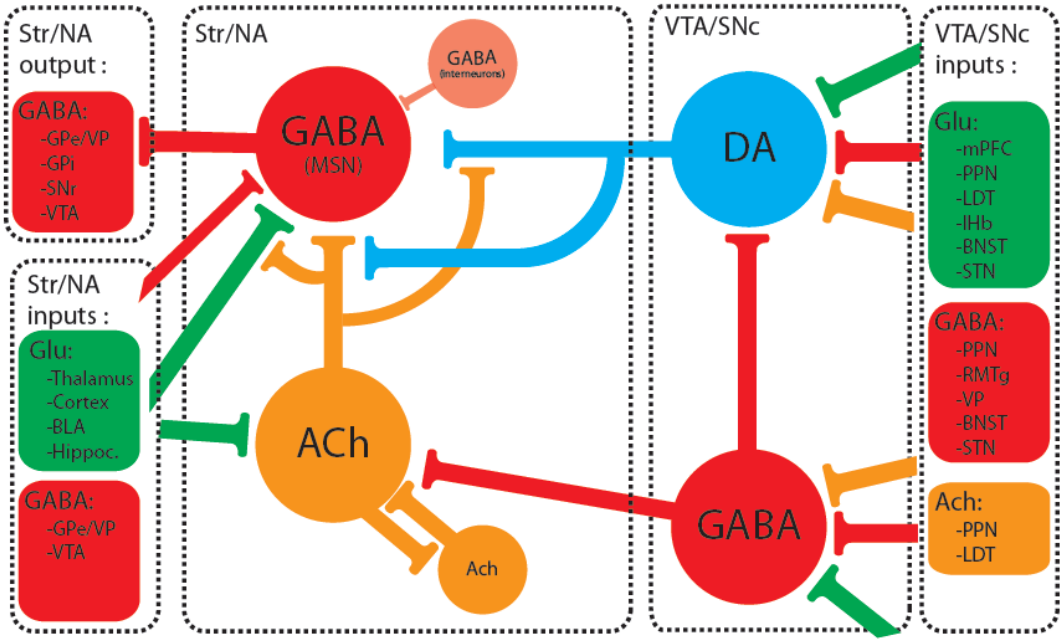

Figure 2: Illustration of the main neurons and major connections in the mesostriatal pathway. The main confirmed connection between identified cell type: glutamatergic (glu) inputs are represented in green, the main DAergic in blue, the main GABAergic (GABA) in red and the main cholinergic (ACh) in orange. In VTA, GABA release can be due to GABAergic interneurons or collaterals from GABAergic projecting neurons. Abreviations, GPe: globus pallidum external segment; GPi: globus pallidus internal segment; SNc: substantia nigra pars compacta; STN: subthalamic nucleus; SNr: substantia nigra pars reticulata; VTA: ventral tegmental area; BLA: basolateral amygdala; hippoc: hippocampus; VP: ventral pallidum; BNST: bed nucleus of the stria terminalis; lHb: lateral habenula; mPFC: medial prefrontal cortex; PPN: pedunculopontine nucleus; LDT: laterodorsal tegmental nucleus; NA: nucleus accumbens; Str: striatum; RMTg: retro medial tegmental nucleus. Adapted from Silberberg and Bolam, 2015 and Vella and Giovanni, 2013.

The function of DA neurons seems to be modulated at the level of the dendrites and soma (firing rate) and by DA release in Str terminals. Indeed, decrease of DA spiking activity or DA neuron number is associated with a decrease of DA release in the Str (Venton et al., 2006; Floresco et al., 2003; Parsons and Justice, 1992).

The nigrostriatal and the mesolimbic pathways are both highly regulated by ACh (Threlfell et al., 2012; Exley et al., 2013; Lester et al., 2010). DAergic neurons at the level of the VTA/SNc receive cholinergic inputs only from, the PPN and the LDT (Mena-Segovia et al., 2008; Beninato and Spencer, 1988; Bolam et al., 1991). At striatal terminals of DA neurons, cholinergic input arises from cholinergic interneurons and as recently described from the PPN/LDT (see Chapter 2; Pereira et al., 2015).

Cholinergic receptors

ACh is present in both the central and peripheral nervous system. Cholinergic receptors (**AChR**) have been characterised in soma, neuromuscular junctions and postsynaptic membrane (see review: Popot and Changeux, 1984). Nicotinic AChRs (**nAChR**) are composed of 5 units that can be similar (homopentamer) or different (heteropentamer). They respond to binding of ACh by an extensive change of conformation (Kistler et al., 1982). NAChR showed higher affinity for nicotine in tobacco than muscarinic receptors (Schuller and Orloff, 1998; Gustav and Auerbach, 1999). 12 nAChR subunit genes have been sequenced in the nervous system: α2-10 and β2-4. In situ hybridization studies showed that α4 and β2 are the most widespread and abundant subunits in the CNS; α6 is mostly located in DAergic nuclei (Novere et al., 1996); β3 located in Str, cerebellum and substantia nigra

(Sargent, 1993). So far, α9 and α10 have been observed only in the cochlear nuclei. Homomeric receptors showed a faster desensitisation and higher calcium permeability than heteromeric α/β receptors.

Muscarinic receptors (mAChR) are widely distributed in the human body, where 5 subtypes have been described (M1 to M5). In the brain, M1 and M4 receptors are mostly found in the hippocampus, Str and cortical structures; M2 and M5 are found throughout the entire brain, while M3 staining is seen at low levels in the hippocampus, the Str and the thalamus (Volpicelli and Levey, 2004). While all nAChRs give rise to an excitatory post synaptic potential (**EPSP**), only M1, M3 and M5 lead to a decrease of the K^+ conductance (Iahp, Im, Ileak) and an EPSP. Activation of M2 and M4 receptors leads to a decrease in the $Ca2^+$ and increase in K^+ conductance, which produce inhibitory effects (**IPSP**) (Allen and Burnstock, 1990; Rouse et al., 2000).

Iontophoretic injections of ACh in the SNc/VTA leads to an activation of DA neurons which can be suppressed by dihydro-β-erythroidine (nAChR β receptor competitive antagonist), but not by the muscarinic antagonist atropine. These results suggest that ACh modulation is mediated by nAChRs rather than mAChRs (Lichtensteiger et al., 1982). Nevertheless, a muscarinic effect has been described in the VTA (Yeomans and Baptista, 1997). M1, M2, M3, M4 and M5 mAChRs have been found in the VTA, SNc and SNr (Vilaro et al., 1990; Weiner et al., 1990). M1 and M4 are present at low levels in the VTA/SNc but knock out of these receptors suggests a role in the control of the activity of DA neurons in the midbrain, and a role in movement and motivation (Tzavara et al., 2004). M2 muscarinic receptors in the VTA show dendritic distribution mainly on non-DAergic neurons and presynaptically

on most of the cholinergic-like terminals found in the VTA (Garzon and Pickel, 2006). The presynaptic distribution of M2 suggests a muscarinic modulation of cholinergic release (Garzon and Pickel, 2006). M3 receptors have been found on non-DAergic neurons in the SNc, possibly GABAergic interneurons, and seem to play a weak role in the activity of DA neurons (Miller and Blaha, 2005). A knock out of the major muscarinic receptor found in the SNc/VTA (M5) produced a decrease in drug-induced locomotion (Vilaro et al., 1990; Steidl and Yeomans, 2008; Steidl et al., 2011). NAChRs have been described on DAergic neurons (α4, α6, α5, α7, β2, β3), on glutamatergic terminals (α7) and on non-DAergic neurons (α4, α7, β2) (Dani and Bertrand, 2007; Grady et al., 2007; McGehee and Role, 1995; Keath et al., 2007; Mansvelder and McGehee, 2002; see review: Faure et al., 2014). The β2 subunit mediates the main nicotinic modulation on DAergic activity (Mameli-Engvall et al., 2006; Mansvelder et al., 2002) and was described as being mostly involved in the regulation of bursting (Maskos et al., 2005). On non-DAergic neurons, the β2 subunit shows a faster desensitisation than on DA neurons (Yin and French, 2000) suggesting that α subunits play a more prominent role in non-DAergic neurons (Dani et al., 2000). In α7 knockout animals, a small difference in the spontaneous activity on both DA and non-DAergic neurons has been observed (Mameli-Engvall et al., 2006) and a strong glutamate-mediated response has been described (Placzek et al., 2009; McKay et al., 2007; see review: Quick and Wonnacott, 2011; Naudé et al., 2014).

In the Str, the expression of mAChRs and nAChRs has traditionally been studied by autoradiography and both have been found on several types of striatal neurons. CIN inputs from others CINs are mediated by M2 and M4 receptors, showing a strong inhibition (Ding et al., 2006; Smiley et al., 1999). Almost all CINs express the β2 subunit, while only half of the CINs present α7 and less than 10%

present α3 and α4 subunits, suggesting that nicotinic-mediated effects on CINs are β2 dependant.

A strong effect of ACh on DA release has been observed, an effect that can be abolished by administration of the β2 antagonist, DHβE (Threlfell et al., 2012). Direct activation of nAchRs on MSNs seems to have almost no significant effects (Luo et al., 2013; Liu et al., 2007). The direct modulation of MSNs by ACh is abolished by atropine administration, suggesting a muscarinic mechanism (Hsu et al., 1996; Galarraga et al., 1999). Two mAChRs have been described in MSNs: M1 is present in all MSNs, while M4 is present in all direct pathway MSNs and less than half of indirect pathway MSNs (Bernard et al., 1992; Yan et al., 2001). M1 mAChRs mediate a decrease in the potassium channel Kv7 current and lead to inhibition of calcium entry through N/P/Q type channels (Perez-Rosello et al., 2005; Shen et al., 2005). M4 mAChRs activation decreases MSNs excitability by decreasing calcium influx. It is believed that the balance between M1 and M4 density in direct pathway MSNs influences the overall striatum output.

ACh reduces striatal GABA release (Marchi et al., 1990). M2 receptors have been observed at the surface of NPY-interneurons (Sugita et al., 1991) mAchRs on FSI mediate synaptic transmission between FSI and MSNs (Koós and Tepper, 2002). These NPY-interneurons in the Str have a high expression of the α7 nAChR and the M2 mAChR (Beggiato et al., 2013; Bernard and Bolam, 1998). In awake animals, activation of both α7 and M2 receptors leads to a decrease in GABA levels in the Str (Campos et al., 2010; Marchi et al., 1990). Str GABA is believed to come from the release of GABA by interneurons and by local MSNs axon collaterals (Sugita et al., 1991). This decrease in GABA release in the striatum suggests that activation of α7

and M2 receptors leads to inhibition of GABAergic interneurons and MSNs.

Activation of nAChRs on glutamatergic terminals modulates the release of glutamate and the excitatory drive onto MSNs. The presynaptic modulation of glutamate release has been shown to be mainly controlled by the α7 subunit (Gray et al., 1996; Campos et al., 2010; Carpenedo et al., 2001). Despite the presence of α4β2 receptors on glutamatergic terminals in the Str, their activation has a weaker effect on the release of glutamate than α7 receptors activation (Xia et al., 2009). Glutamate release is also negatively modulated by M2 receptors and positively modulated by M3 receptors (Levey et al., 1991; Hersch et al., 1994).

Dense cholinergic projections have been observed in **STN**, mostly arising from PPN and LDT (Bevan and Bolam, 1995), and ACh action is modulated by nicotinic receptors (Nashmi et al., 2007; Drenan et al., 2008; Xiao et al., 2009a). The group of STN neurons projecting to the SNr GABAergic inhibitory neurons showed a strong expression of α4β2 receptors (Wooltorton et al., 2003; Xiao et al., 2015). Neurons projecting to SNc DA neurons express α7 subunit (Xiao et al., 2009a; Xiao et al., 2015; Nambu et al., 2002).

The **GPe** is a basal ganglia structure, where a single GPe neuron can project to several targets (Kita and Kitai, 1994; Bevan et al., 1998; Kita et al., 2007; Aceves et al., 2011; Takakusaki, 2013). While PPN and LDT cholinergic neurons constitute major inputs to the GPe (Charara and Parent, 1994; Chan and Surmeier, 2005), it has been suggested that the basal forebrain provides a weaker cholinergic innervation of the GPe (Bengtsone and Osborne, 2000) and it has been hypothesized that some projecting neurons of the GPe are cholinergic (Rodrigo et al., 1998; Eid et al., 2015; Abdi et al., 2015). M1 muscarinic receptors activation modulates the IPSC from

MSNs and facilitates GABA release in the GPe (Hernandez-Martinez et al., 2015).

The pf and CL **thalamus** are two main components of the intralaminar thalamus (Deschenes et al., 1996a) and constitute the main part of the thalamostriatal connection (Smith and Parents, 1986; François et al., 1991; Raju et al., 2006). Both nuclei receive a strong cholinergic innervation from the brainstem (PPN, LDT) and from the basal forebrain (medial septum, diagonal band of Broca, nucleus basalis of Meynert) (Capozzo et al., 2003; Kha et al., 2000; Kobayashi and Nakamura, 2003). Injection of atropine in the thalamus produces a significant impairment in the control of voluntary movement (Yan et al., 2008) suggesting a muscarinic mechanism. In fact, M1, M2, M3 and M5 muscarinic receptors have been described in the thalamus (Clarke et al., 1985). M1 and M3 muscarinic receptor activation leads to control of the medium-fast modulation of the thalamostriatal pathway. M5 muscarinic receptors modulate the slow response of the thalamostriatal and thalamocortical pathway while M2 inhibit the thalamocortical pathway (Zhu and Uhlrich, 1998; Valera and Sherman, 2007). The M1 receptor is mostly found on GABAergic neurons while the M2 is found on glutamatergic neurons (Ye et al., 2010). The α3β2 is the only described nAChR in the intralaminar thalamic nucleus (Rubboli et al., 1994) and will participate in the fast activation of the thalamostriatal and thalamocortical neurons (Dossi et al., 1991; Capozzo et al., 2003). The modulation of the pathway will be managed first by nicotinic receptors, followed by M1/M3, and then activation is maintained by M5 or stopped by M2.

In vivo, exposure to nicotine, along with other drugs of abuse such as cocaine and alcohol, results in long-term potentiation (LTP) of excitatory glutamatergic inputs to VTA DA neurons (Ungless et al., 2001; Saal et al., 2003). Nicotine (thought via a7

nAchRs (Mansvelder et al., 2007), a6 nAchRs (Berry et al., 2015) or B2 nAchRs (Picciotto et al. 1998)) helps to initiate synaptic plasticity in the VTA.

Cholinergic Brainstem

Many studies have described the relationship between basal ganglia and cholinergic neurons in both anatomical and physiological contexts. As previously explained, DA release is strongly linked to the firing activity of DA neurons and is modulated *inter alia* by cholinergic neurons. It has been known for a long time that basal ganglia and the cholinergic brainstem share anatomical connections (Bolam, 1991; Mena-Segovia 2008; Lammel et al., 2012), functions and dysfunctions (Alderson et al., 2004; Wilson et al., 2009; Steidl et al., 2015). However, recent studies suggest a more complex interconnection between brainstem cholinergic neurons and DAergic neurons. The only described common cholinergic input to the entire basal ganglia nuclei arises from the pons. Cholinergic inputs from the PPN or the LDT to the STN, the DAergic midbrain, the Str and to the GP have been reported (Semba and Fibiger, 1992; Lavoie and Parent, 1994; Parent et al., 2014).

The following section will describe the anatomical and physiological structures of the two cholinergic brainstem formations, the PPN and the LDT. Description of their involvement in behaviour and descriptions of its main inputs and outputs will be provided.

The pedunculopontine nucleus (PPN)

Anatomy

The PPN is a long heterogeneous nucleus located in the upper brainstem. It is often described as closely interacting with the basal ganglia through direct or indirect connections (Pahapill and Lozano, 2000; Garcia-Rill, 1991; Mena-Segovia et al.,

2004). In its first description, the PPN was described as a homogeneous structure (in human: Olszewski and Baxter, 1954; Olszewski and Baxter, 1982; in rodent: Jacobsohn, 1909; Mesulam, 1983a). Commonly, PPN borders are delimited by the distribution of its cholinergic neurons. The PPN is not a homogeneous nucleus; several types of neurochemical neurons with different phenotypes (sizes, input, output patterns or firing rates) have been described. The rodent PPN can best be seen in parasagittal sections the PPN can be delimited rostrally by the posterolateral SN, the pontine cuneiform and sub-cuneiform nuclei dorsally by the retrorubral field (**RRF**) borders and ventrally by the pontine reticular formation (Mesulam et al., 1983; Vincent et al., 1986).

However, it is commonly accepted that the PPN has two subdivisions in the human brain, the pars compacta (**PPNc**) and the pars dissipata (**PPNd**) (Olszewski and Baxter, 1954; Manaye et al., 1999; Spann and Grofova, 1992) based on cholinergic neuron distribution. Rodent and monkey studies using molecular markers of different neurotransmitter types have given rise to a different nomenclature based mostly on the brain axis. Here, the PPN is divided into rostral and caudal parts (Noback, 1959; Spann and Grofova, 1992; Geula et al., 1993). The caudal subdivision (**PPNc**) is bordered by the dorsolateral border of the superior cerebellar peduncle, whereas the rostral part (**PPNr**) is surrounded by the trochlear nucleus (Lavoie and Parent, 1994a; Rye et al., 1996). An anatomical segregation based on a radial segmentation of the PPN from the centre of the SNr has been described, and is still used to describe PPNc and PPNr (Mena-Segovia et al., 2009).

Inputs/outputs

PPN neurons provide strong glutamatergic and cholinergic excitatory inputs to DAergic neurons in the midbrain (Mena-Segovia et al., 2008; Oakman et al., 1995;

Pan and Hyland, 2005) and to thalamic nuclei (Holmstrand and Sesack, 2011). Moreover, projections to the basal forebrain (Hallanger and Wainer, 1988), the hypothalamic or the prefrontal cortex formation have been observed (Crawley et al., 1985; Satoh and Fibiger, 1986).

The central position of the PPN within the basal ganglia and the limbic system suggests a key role in the fronto-striatal information processing. PPN neurons receive inputs from periaqueductal gray, dorsal raphe nucleus, superior colliculus, zona incerta, lateral habenula, prefrontal cortex, central nucleus of the amygdala, nucleus of the solitary tract, the midbrain, the basal forebrain, subthalamic nucleus or GPi (Beninato and Spencer 1988; Cornwall et al. 1990; Heimer et al. 1991; Steininger et al., 1992; Semba and Fibiger, 1992; Matsumura et al. 2000; Chiba et al. 2001; Chivileva and Gorbachevskaya, 2008; Zahm et al. 2011; Beier et al., 2015). Projections from cerebellar nuclei were found in the PPN (Hazrati and Parent, 1992). Most of these inputs to PPN are GABAergic (Noda and Oka, 1986; Granata and Kitai, 1991). However, pallidal and midbrain GABAergic projections to PPN do not seem to target cholinergic neurons (Shink et al., 1996; Rye et al., 1995b; Granata and Kitai, 1991). Furthermore, glutamatergic inputs to the PPN were found to arise in the STN (Hammond et al., 1983; Jackson and Crossman, 1983; Kita and Kitai, 1994; Bevan et al., 1995) and the cortex (Monakow et al., 1979; Moonedley and Graybiel, 1980; Edley and Graybiel, 1983; Matsumura et al., 2000). The presence of D1 receptors on PPN neurons, and particularly cholinergic neurons, suggests a DAergic feedback from VTA and SNc (Winn et al., 1997; Reese et al., 1995). Activation of the SNr induces IPSPs in PPN neurons with a very short-latency (Noda and Oka, 1984). These multiple feedback connections place the PPN (also the LDT) at a central modulatory position of the basal ganglia (Winn, 1998).

The outputs of the PPN are divided into ascending (toward thalamus, forebrain and colliculus) and descending (toward gigantocellular nucleus and reticular nuclei) components. The ascending projections of the PPN are much denser than the descending projections. The descending pathway has been described as a reticular pathway, projecting to the medullary reticular nuclei (Rye et al., 1988; Grofova and Keane, 1991). Functionally, electrical stimulation of the PPN elicits controlled locomotion (Eiderlberg et al., 1981; Garcia-Rill, 1990) suggesting a strong connectivity of the PPN with reticular locomotor-associated formations. The direct output of the PPN to the spinal cord seems to be responsible for the initiation of locomotion (Grillner, 1985) and for the maintenance of movement (Di Prisco et al., 1997). All of these connections between lower brainstem reticulospinal neurons and mesopontine centres appear to be important to adapt the motor pattern to external events, indeed a single brainstem neuron can project to several motor associated structures. The ascending pathways follow two routes. The first, the dorsal-ascending, targets the LDT, and then axons arborise in the parabigeminal nucleus (PBN), projections follow the colliculus and arborise in many thalamic nuclei. The second route, ventral-ascending, follows the scp, arborises in the VTA and SN, follows the lateral hypothalamus, arborises in the STN, ventral pallidum, DS and then cortex (Spann and Grofova, 1991; Lavoie and Parent, 1994b). The ascending output of the PPN, mostly the cholinergic projections to the thalamus, may also take part in the modulation of sensory information (Grunwerg et al., 1992; Reese et al., 1995, Hylden et al., 1985). Projections to the VTA show no preference for DAergic and non-DAergic neurons (Beier et al., 2015) (Fig 3).

Neuronal populations

Using *in situ* hybridization combined with classic immunohistochemistry, the

anatomy of the pontine nucleus revealed several types of neurons (Wang and Morales, 2009). GABAergic neurons are one of the most important populations in the PPN, mostly concentrated in the PPNr. GABAergic neurons delimit the caudal part of the substantia nigra (Mena-Segovia et al., 2009). A negligible proportion of GABAergic neurons co-express the cholinergic neuron marker, ChAT (Wang and Morales 2009; Ford et al., 1995). The PPNr contains almost twice as many GABAergic neurons as cholinergic neurons (Wang and Morales, 2009; Takakusaki et al., 1996). Putative GABAergic neurons recorded *in vivo* with subsequent juxtacellular labelling were described as having a tonic and irregular firing (Ros et al., 2010). However, few studies have described the function and the electrophysiology of the GABAergic neurons in the PPN. Some tracer studies have shown that GABAergic neurons are not only interneurons, but also project to brain structure such as the midbrain (Mena-Segovia et al., 2009; Martinez-Gonzalez et al., 2014).

The second most important population of neurons in the PPN is positive for VGLUTs mRNA and are thus glutamatergic. They are mostly located in the PPNc, with complementary distribution to that of the GABAergic neurons in the rostrocaudal axis (Wang and Morales, 2009; Mena-Segovia et al., 2009). Almost two percent of the VGLUT2-positive neurons also co-express ChAT (Wang and Morales, 2009). The calcium-binding proteins, calbindin and calretinin, are co-expressed with VGLUT2 and GAD, suggesting distinct subpopulations (Côté and Parent, 1992; Dun et al., 1995; Fortin and Parents, 1999; Martinez-Gonzales et al., 2012). In addition, two glutamatergic subtypes have been described based on their *in vivo* physiological characteristics. The first group presents a fast firing activity associated with cortical slow oscillations while the second shows very slow firing activity that is not related to the cortical activity (Mena-Segovia et al., 2008; Ros et al., 2010).

The last type of neuron was described as bipolar-shaped (Koch et al., 1993). These neurons were immunopositive for ChAT. A higher concentration of cholinergic neurons is observed in the PPNc (Wang and Morales, 2009). About two-thirds of the cholinergic neurons of the PPN co-express nitric oxide synthase (**NOS**), the synthetic enzyme for a gaseous neurotransmitter involved in several behaviours normally associated with ACh (Vincent and Kimura, 1992). Two types of cholinergic projections have been observed, ascending (toward the thalamus, pretectal area and tectum) and descending (toward pontomedullary reticular formation) (Takakusaki and Kitai, 1997; Mena-Segovia et al., 2008). *In vitro*, cholinergic neurons of the PPN fire spontaneously due to voltage-dependent oscillations and three types of cholinergic neurons have been described based on their membrane properties: 1) those expressing an A-type potassium current (Ia), with rapidly inactivated voltage-dependant potassium currents able to regulate the action potential generation; 2) those expressing low threshold $Ca2^{+}$ spikes; and 3) those expressing both low threshold $Ca2^{+}$ and an Ia current (Saitoh et al., 2003; Takakusaki and Kitai, 1997; Kang and Kitai, 1990). However, the cholinergic nature of these three groups of physiologically different neurons has been questioned several times (Kang and Kitai, 1990; Kamondi et al., 1992; Takakusaki et al., 1996). Interestingly, cholinergic neurons recorded *in vivo* show two types of firing patterns. A third of the cholinergic neurons recorded have a phasic synchronous activity with the cortex, while the second group showed a bursting activity during the up-phase of the cortical SWA (Mena-Segovia et al., 2008).

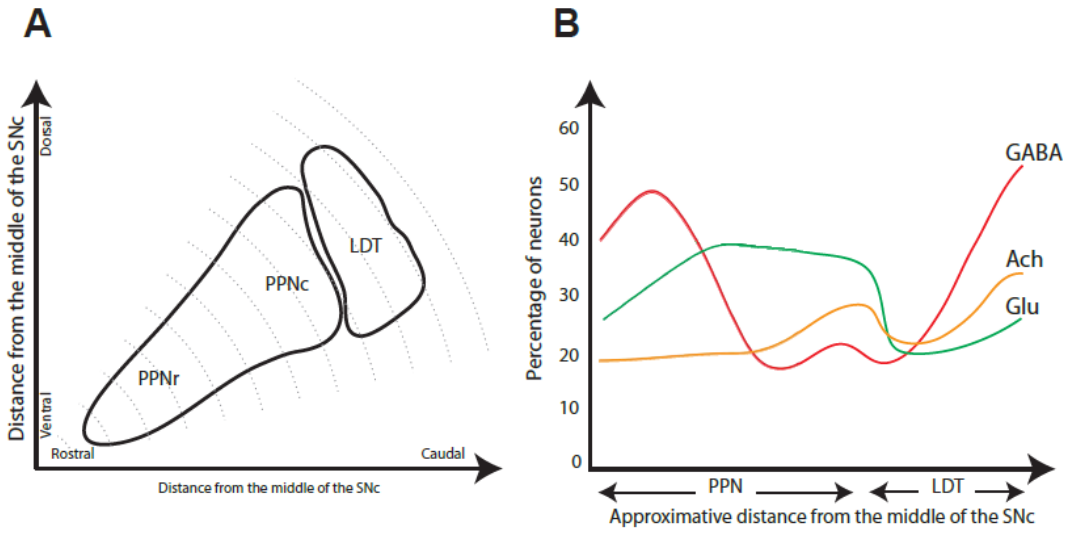

Figure 3: Simplified illustration of the PPN and LDT organisation. (A) Topographical division and organisation of the differents segments of the PPN/LDT from the centre of the SNc as described by Mena-Segovia et al., 2009. (B) Representation of the approximate density of GABAergic (red), glutamatergic (green) and cholinergic (orange) neurons throughout the brainstem on a rostrocaudal extend. Based on Mena-Segovia et al., 2009 and Wang and Morales, 2009.

Electrophysiology

In vivo recording of PPN activity during locomotion reveals a group of neurons that display a tonic firing pattern in synchronicity with the locomotion which decreases or even stops entirely with the cessation of movement (Garcia-Rill and Skinner, 1988; Garcia-Rill and Skinner, 1991; Petzold et al., 2015). Conversely, recording in behaving cats (Dormont et al., 1998) and monkeys (Matsumura et al., 1997) reveals two types of PPN neurons directly related to movement. One group has a low frequency firing and a significant increase of activity during the movement; the second group has a higher basal firing rate and firing increases during the movement initiation (Matsumura et al., 1997; Okada et al., 2015).

Functions

The functions of PPN cholinergic projections have been inferred from lesion experiments and injection of various pharmacological agents. Small injections of

cholinergic agonists into the SNc increase the activity of DAergic neurons, mostly due to a potentiation of their response (Winn et al., 1983; Teo et al., 2004). Self-administration of cholinergic agonists directly into the VTA provides evidence for a role of ACh input in addiction and learning (Ikemoto and Wise, 2002; David et al., 2006). Several studies using non-specific excitotoxic lesions of the PPN observed PD-related symptoms, such as bradykinesia, reduction of the motor activity or hypokinesia (Kojima et al., 1997; Aziz et al., 1998). The choice of cell-specific lesions or pharmacological modulation of PPN neuron activity has enabled more precise behavioural observations. Within the tegmentum, only cholinergic neurons express the receptor for urotensin II (Clark et al., 2001; Wilson et al., 2009) and so the use of diphtheria toxin conjugated to urotensin II (**DTx-UII**) allows a lesion with high cellular specificity (Steidl et al., 2014; MacLaren et al., 2015). Lesions of cholinergic neurons in the PPN showed no significant effect on locomotion or exploratory behaviour when the animals were placed in a novel or familiar environment (Inglis et al., 1994; Wilson et al., 2009a; Steiniger and Kretschmer, 2004). However, new studies using a more specific method for activation/inhibition are need for testing the role of cholinergic neurons in more discrete locomotor behaviour. Similarly, no effect was observed on anxiety-like behaviour (Homs-Ormo et al., 2003), food consumption (Inglis et al., 1994) or natural sleep (Deurveilher and Hennevin, 2001). However, lesions of cholinergic neurons in the PPNc produced deficits in the reward-related behaviour (Alderson and Winn, 2005). In rodents, the startle acoustic response is tested by producing a loud and unexpected sound and recording freezing. Studies show that the initial sound response can be reduced by an early presentation of short and quieter acoustic stimuli (Fendt et al., 2001). This reflex, naturally present in most species, is considered as an indicator of normal

sensorimotor gating (Wu et al., 1984). Non-specific PPN lesions lead to a significant reduction of the acoustic startle reflex (Swerdlow and Geyer, 1993a; Diederich and Koch, 2005) and a decrease of prepulse inhibition (Koch et al., 1993). Other behavioural studies show impairment in classical operant tasks correlated with non-specific lesions of the PPN. Rats were first trained to associate an action (lever pressing) with a reinforcer (sugar, cocaine or nicotine). In animals with non-specific lesions of the PPN, a reduction in lever pressing has been observed in this type of analysis (Corrigal et al., 2001; Floresco et al., 2003; Diederich and Koch, 2005). The study demonstrates that even if motivation was not affected by PPN lesions (Taylor et al., 2004) the adaptation to new paradigms based on previously learned behaviours is impaired (Samson and Chappel, 2001). This abnormal behaviour can be interpreted as a learning impairment, and therefore an inability to update the association between action and outcome (Alderson et al., 2004). Most of these studies did not produce any effects during inappropriate responses, while operant choice with risk (same mean reward but higher variance) showed that PPN non-specific lesions reduce risk aversion (Leblond et al., 2014).

PPN is implicated in locomotion (Bechara and Van de Kooy, 1992; Dormont et al., 1998), action-outcome learning (Satorra-marín et al., 2001; Alderson and Winn, 2005; Pan and Hyland, 2005), place preference (Lammel et al., 2012) or reward prediction error (Kobayashi and Okada, 2007; Okada et al., 2009). The results of these studies strengthen the hypothesis that the PPN is part of the basal ganglia (Mena-Segovia, 2004).

The laterodorsal tegmental nucleus (LDT)

Anatomy

The LDT is a small nucleus situated in the medial brainstem, bordered

dorsally by the fourth ventricle (V4) and ventrally by the cerebro-pontine formation; the LDT is positioned caudally of the PPN (Cornwall et al., 1990; Mesulam et al., 1986; Maley et al., 1988; Woolf, 1991). The LDT is the second cholinergic input to the DAergic midbrain. Very little is known about the anatomy and the function of the LDT. Indeed, most studies describe the LDT as a continuum of the PPN.

Inputs/outputs

The LDT has been described as mainly projecting to medial DA midbrain formation (PBN and Rli; Lammel et al., 2012). Retrograde tracer injections into the VTA show a significant number of VGLUT2-positive and ChAT-positive projecting neurons (Lammel et al., 2012; Clement and Grant, 1990; Oakman et al., 1995). Tracer injections show that the LDT projections are very similar to those of the PPN (Cornwall et al., 1990; Satoh and Fibiger, 1986). LDT neurons mostly target the medial midbrain DAergic structure, the cholinergic basal forebrain including the ventral pallidum, the medial septum and the nucleus of Broca (Cornwall and Phillipson, 1989). LDT projections are also found in the cingulate cortex, the thalamus (Holmstrand et al., 2010; Gonzalo-Ruiz et al., 1995; Holmstrand and Sesack, 2011), the lateral habenula (Semba and Fibiger, 1992), the GP (Eid et al., 2014) and the subthalamic nucleus (Bevan and Bolam, 1995). However, in most of the studies injections were located in the dorsal tegmental nucleus (Lammel et al., 2012), which contains a very low number of cholinergic neurons (Morest, 1961; Clements and Grant, 1990; Satoh and Fibiger, 1986).

Afferent neurons for the LDT are located in the reticular formation, the medial preoptic nucleus, the hypothalamus, basal forebrain and medial prefrontal cortex (Satoh and Fibiger, 1986; Semba and Fibiger, 1992). There is no evidence at present that allows the integration of the LDT into the basal ganglia network as

discussed for the PPN (Mena-Segovia et al., 2008). However, its interactions with most of the nuclei of the BG and the role in BG-related cognitive processes suggests at least a strong relationship.

Neuronal populations

Immunohistochemal analyses reveal that the LDT has a similar molecular composition as the PPN. Three distinct populations of neurons are found within its borders (Wang and Morales, 2009). The first population of neurons express glutamatergic markers, the second major subpopulation of neurons, similar in numbers to the glutamatergic neurons, express GABAergic markers (Ford et al., 1995; Wang and Morales, 2009). In rodents, glutamatergic and GABAergic neurons represent more than 80% of the total neurons located within the LDT. The third subpopulation of neurons express cholinergic markers (Wang and Morales, 2009).

Electrophsyiology

In vitro whole cell recordings of cholinergic neurons in the LDT revealed that two firing patterns can be observed, a regular activity or a bursting activity following current injection (Kamii et al., 2015). These two populations appear to be differentially represented in the LDT: two-thirds of the neurons recorded were regular (Kamii et al., 2015). The majority of LDT putative cholinergic neurons express an A-type potassium channel and persistent sodium channel (Nav1.9) (Leonard and Llinas, 1990; Kamondi et al., 1992), like PPN type I and II neurons (Saitoh et al., 2003). *In vivo*, putative cholinergic neurons have a very short spike duration (<1ms) and a large range of firing rates (0.08 to 5 Hz), while non-cholinergic neurons have a long spike duration (±2ms) and a regular firing frequency (2 Hz) (Koyama et al., 1999; Mansari et al., 1989; Kayama et al., 1992; Mansari et al., 1990).

Functions

Classically, the LDT is considered to have a strong role in limbic (Kamii et al., 2015; Lester et al., 2008; Nelson et al., 2007; Lammel et al., 2012) and sleep/awake functions (Monti and Jantos, 2015; Kohlmeier et al., 2013; Datta et al., 2009). Inactivation of the cholinergic LDT neurons leads to a significant decrease of locomotion under baseline conditions (Dobbs and Mark, 2012) and also a decrease of locomotion produced by drugs (nicotine: Alderson et al., 2005; amphetamine: Laviolette et al., 2000; methamphetamine: Dobbs and Cunningham, 2014). An increase of stereotypical behaviours related to drug injections have been found in animals with LDT non-specific lesions (nicotine: Forster and Blaha, 2000; Ishibashi et al., 2009). Pharmacological blocking of cholinergic neurons (with microinjections of the M2 cholinergic agonist) of the LDT results in motor (Dobbs and Cunningham, 2014), learning (Shinohara et al., 2014) and behavioural state deficit (Kohlmeier and Kristiansen, 2010). Drug-related behaviour is mostly associated with the strong connectivity between DAergic neurons of the VTA and the cholinergic LDT (Lester et al., 2010). Significant variation in the self-administration of nicotine, methamphetamine or cocaine was observed in LDT non-specific lesioned animals (Dobbs and Cunningham, 2014; Alderson et al., 2004; Lanca et al., 2000b; Laviolette et al., 2002). Recent studies using specific lesions of cholinergic neurons have failed to detect a role of the LDT in learning (Steidl et al., 2014).

The development of transgenic animals expressing specific genes allows the activation, inhibition or lesion of a specific subpopulation of neurons. Recent reports using an optogenetic approach of the brainstem confirmed the role of cholinergic neurons in REM sleep (Van Dort et al., 2015) and in operant behaviour (Steidl and Veverka, 2015).

Summary and aims

PPN and LDT are two brainstem cholinergic nuclei interconnected with most of the BG nuclei. Despite their functional differences, PPN and LDT share common anatomical, electrophysiological and behavioural functions.

PPN and LDT have been proposed to influence activity within the striatum by two routes: by innervation of midbrain dopaminergic neurons or by innervation of the intralaminar and midline thalamus. However, earlier tract-tracing studies suggest that brainstem neurons may project directly to the striatum, but no studies of the nature of these neurons have been made.

The aims of this thesis are to define the anatomical, electrophysiological and behavioural functions of cholinergic input to VTA and striatum. This thesis addresses several current issues on the cholinergic modulation of the mesostriatal pathways:

1) Define the cholinergic inputs to the striatum (Chapter 2)

2) Define the anatomical, electrophysiological and behavioural contribution of PPN and LDT cholinergic axons in the VTA (Chapter 3)

3) Define the anatomical, electrophysiological and behavioural contribution of PPN and LDT cholinergic axons in the striatum (Chapter 4)

4) Define *in vivo* the electrophysiological contribution of cholinergic interneurons in the striatum (Chapter 4)

5) Compare the behavioural function of cholinergic interneurons and cholinergic brainstem projections in the DMS and DLS (Chapter 4)

Chapter 2: Cholinergic innervation of the mesostriatal pathway

The following chapter is a summary of the two following papers

A major external source of cholinergic innervation of the striatum originates in the Brainstem

Daniel Dautan, Icnelia Huerta-Ocampo, Ilana B. Witten, Karl Deisseroth, J. Paul Bolam, Todor Gerdjikov and Juan Mena-Segovia

The Journal of Neuroscience, March 26, 2014. 34(13):4509-4518.4509

Evaluation of potential extrinsic sources of cholinergic innervation to the striatal complex: a whole brain mapping analysis

Daniel Dautan, Husniye Hacioğlu Bay, J. Paul Bolam, Todor Gerdjikov and Juan Mena-Segovia

Frontiers in Neuroanatomy, 2016, doi: 10.3389/fnana.2016.00001

DD, TG, PB and JMS designed the experiments, analysed the data and wrote the manuscript. DD performed the surgeries and experiments (excluding EM). IHO performed the electron microscopy experiments. HBH helped with the immunohistochemistry. IW and KD provided the ChAT::cre line.

Abstract

Cholinergic innervation of the mesostriatal pathway is critical for the modulation of the DA release. In the VTA/SNc release of ACh has been considered to originate exclusively from the cholinergic brainstem (PPN and LDT). In the striatal complex, the cholinergic inputs are believed to arise exclusively from a subtype of striatal interneurons that provide widespread innervation of the Str. Here we show that cholinergic neurons in the brainstem also provide a direct innervation of the striatal complex. We also confirmed that VTA/SNc but also Str received cholinergic innervation only from the brainstem. By the expression of fluorescent proteins in ChAT::cre $^{+}$ transgenic rats, we selectively labelled cholinergic neurons in all cholinergic nuclei. We show that with the exception of the PPN/LDT no others cholinergic structures innervate of the DA midbrain formation or the striatal complex. We also show that cholinergic neurons of the brainstem topographically innervate large areas of the striatal complex: PPNr preferentially innervates the DLS, while LDT preferentially innervates the DMS and the NA. Retrograde labelling combined with immunohistochemistry in wild-type rats confirmed the exclusive origin of the cholinergic innervation of the mesostriatal. Furthermore, transynaptic gene activation and conventional double retrograde labelling suggest that LDT neurons that innervate the NA also send collaterals to the thalamus and the DAergic midbrain, thus providing both direct and indirect projections to the striatal complex. The exclusive origin of the cholinergic innervation of the mesostriatal pathway, the differential activity of cholinergic interneurons and cholinergic neurons of the brainstem during reward-related paradigms suggest that the two systems play different but complementary role in the processing of information in the basal ganglia.

Key words: cholinergic; laterodorsal tegmental nucleus; pedunculopontine nucleus; striatum; VTA; SNc; Ch1 to Ch8

Introduction

Nicotinic and muscarinic receptors are widely distributed throughout the CNS. ACh constitutes one of the major modulators of neuronal excitability and synaptic communication, explaining the central role played by cholinergic nuclei in several behaviours (Everitt and Robbins, 1997; Micheau and Marighetto, 2011; Davies and Maloney, 1976). Early descriptions of the cholinergic system in the CNS proposed a nomenclature based on eight projecting cholinergic nuclei, so named Ch1 to Ch8 (McGeer et al., 1984; Schwaber et al., 1987; Geula et al., 1993).

Cholinergic innervation of the brain is widely distributed and predominantly originates from eight anatomically segregated nuclei: medial septum (Ch1), the vertical limb of the diagonal band of Broca (Ch2), the horizontal limb of the diagonal band of Broca (Ch3), the nucleus basalis of Meynert (Ch4), the PPN (Ch5), the LDT (Ch6), the medial habenula (Ch7) and the parabigeminal nucleus (Ch8) (Mesulam et al., 1983a, b; Mesulam and Geula, 1988; Mufson et al., 1986; Mesulam, 1990). For long, tracer studies described projections to the cortex, the thalamus or the hippocampus that arise from the cholinergic nuclei (Cobb et al., 1999; Dutar et al., 1995; Cornwall et al., 1990; Mena-Segovia et al., 2008). PPN and LDT have been shown to influence the basal ganglia structures, and mostly the midbrain (Bolam et al., 1991; Lacey et al., 1990) or the thalamus (Parent and Descarries, 2008). Basal forebrain cholinergic neurons have also shown important projections to the thalamus (Deiana et al., 2011) and the GP (Záborszky et al., 2012). All these routes modulate indirectly the striatal complex (Erro et al., 1999).

Data from earlier tracer studies suggest that some cholinergic neurons could influence the Str by direct innervation (Saper and Loewy, 1982; Hallanger and

Wainer, 1988; Nakano et al., 1990). Suggesting that cholinergic interneurons were not the only source of ACh in the Str as considered before (Wang et al., 2006; Ding et al., 2010; Goldberg et al., 2012). In view of the importance of ACh mechanism in the regulation of DA release (Cachope et al., 2012; Threlfell et al., 2012) and network activity in the Str (Carrillo-Reid et al., 2009; Goldberg et al., 2012), we aimed to determine whether cholinergic neurons in the entire CNS directly innervate the Str.

Some retrograde tracing experiments showed that some neurons located in the main cholinergic nuclei project directly to the striatum. No characterisation of the nature of these projections have been made. In order to determine whether any of these systems provide a source of ACh in the Str, we used a combined anterograde and retrograde analysis of populations of cholinergic neurons to obtain a detailed mapping of their axonal distributions. Using Cre-driver line to genetically target cholinergic neurons and induce the expression of a fluorescent marker in their axons (Witten et al., 2011) we were able to characterize specific responses on sub-population of DA neurons. Following whole brain mapping of individual cholinergic nucleus projections and retrograde tracer injections, we observed that the DS and the NA receive a dense and highly organised cholinergic innervation arising exclusively from the brainstem. Our results suggest further the existence of a direct and indirect cholinergic modulation of the Str.

Materials and methods

Animals

Adults (250-450g) Long Evans (LE) wild-type and ChAT::cre^{+} (Witten et al., 2011) male (n=31) and female (n=15) rats were used for all experiments. Rats were maintained on a 12h light cycle (light on 07:00) and *ad libitum* access to water and food. All procedures were performed in accordance with the Society for Neuroscience policy on the use of animals in neuroscience and the animals (Scientific Procedures) Act, 1986 (United Kingdom), under the authority of a Project Licence approved by the Home Office and the local ethical committee of the University of Oxford.

Stereotaxic injections

All surgical procedures were performed during deep isoflurane anesthesia (2% in O2; Isoflo, Schering-Plough). For the anterograde tracing studies, LE ChAT::cre^{+} rats (n=21) were injected with adeno-associated virus stereotype 2 (AAV2) carrying the fusion genes for channelrhodospin 2 (ChR2) and the enhanced yellow fluorescent protein (eYFP) (AAV2-EF1a-DIO-hChR2-eYFP; Gene Therapy Centre Virus Vector Core, University of North Carolina). The viral vectors were injected in a volume of 300 nl for the forebrain cholinergic structures (Ch1 to Ch4) to avoid spreading over contiguous cholinergic structures, whereas a volume of 500 nl was used for the other cholinergic nuclei (Ch5 to Ch8). The injection sites were randomized for hemisphere and fluorescent reporter. Viral injections were delivered in 8 different locations corresponding to the 8 cholinergic groups described by Mesulam et al. (1983a, b) using the following stereotaxic coordinates (from bregma, in mm; DV ventral to the dura): Ch1 (medial septum) AP +0.7, ML +0.2, DV-4.5; Ch2 (vertical limb of the

diagonal band) AP +0.5, ML +0.4, DV -7.5; Ch3 (horizontal limb of the diagonal band) AP +0.1, ML +1.6, DV -8.5; Ch4 (nucleus basalis of Meynert) AP +0.9, ML +2.5, DV -7.0; Ch5 (PPN) AP -7.3, ML +1.8, DV -7.2; Ch6 (LDT) AP -8.5, ML +1.0, DV -6.0; Ch7 (medial habenula) AP -3.5, ML +0.3, DV -4.0; and Ch8 (parabigeminal nucleus) AP -4.5, ML +4.3, DV -5.5 (Paxinos and Watson, 2007). For striatum mapping viral vectors were injected in the **dorsomedial striatum** (900nl over 20min; from the bregma in mm: AP -0,5; ML +1,8, DV -4,5 ventral of the dura); in the **dorsolateral striatum** (900nl over 10min; from the bregma in mm: AP -0,5; ML +3,0, DV -4,5 ventral of the dura), or in the **accumbens** (800nl over 10min; from the bregma in mm: AP +1,5; ML +1,8, DV -6,7 ventral of the dura).

For the retrograde tracing injections, wild-type LE rats (n=10) were injected with cholera toxin b (CTb 2,5% in distilled water, 400nl over 10min; Sigma-Aldrich) and fluorogold (FG; 2,0% in distilled water, 300nl over 10min; Fluorochrome), each in one of the following areas: dorsolateral striatum (as above), dorsomedial striatum (as above), the accumbens (as above), the **lateral shell of the nucleus accumbens** (from the bregma in mm: AP +1,2; ML +2,7, DV -6,8 ventral of the dura), the **medial shell of the NA** (from the bregma in mm: AP +1,5; ML +0,9, DV -7,2 ventral of the dura), or the **medial core of the NA** (AP +1,5; ML +0,9, DV -6,5 ventral of the dura). Each animal received two injections, and the combinations of tracers/targets were varied. Finally, for the combined retrograde/anterograde tracing studies, wild-type LE rats (n=6) were injected with AAV-EF1a-IRES-WGA-Cre-mCherry in the DLS or the NA and with AAV2-EF1a-DIO-eYFP in the PPNr or LDT, respectively. The wheat germ agglutinin (WGA) fusion was transcellularly transported to the afferent neurons, and retrogradely transported to the soma, in which it produced the recombination of AAV2-EF1a-IRES-eYFP and the expression of eYFP. Thus, we

specifically labelled the brainstem neurons projecting to the Str and their axon collaterals (Gradinaru et al., 2010). For all experiments, injections were made using 1µl syringes (Neuros 7001, Hamilton) at a rate of 50 nl/min and left to diffuse for 5min before retraction of the syringe. Approximately 4 weeks following AAV2-EF1a-DIO-eYFP injections, 10-15 days following tracers injections, and 2-4 weeks after the AAV-EF1a-IRES-WGA-Cre-mCherry, the rats were humanely euthanized using a lethal dose of pentobarbital (>200 mg/kg, i.p.) and perfused transcardially with 0,05 M phosphate buffer solution (**PBS**) pH 7.4 (approximately 50 ml over 5 min), followed by 300 ml of 4% w/v paraformaldehyde in phosphate buffer (0.1 M, pH 7.4) containing 0.1% glutaraldehyde (TAAB Laboratories) over about 20 min. Brains were stored in PBS at 4°C until sectioning.

To corroborate findings from the WGA-Cre study, an additional group of wild-type (n=4) rats were used in double retrograde tracers labelling experiments. FG was injected into the NA (see above) and CTb was injected in either the ventral tegmental area (VTA; 300nl over 10 min; AP -5,2 ML +0,9, DV -7,5 ventral of the dura) or the mediodorsal thalamus (500nl over 10 min; AP -3,0; ML +1,4, DV -5,0 ventral of the dura). Retrogradely labelled neurons were revealed after perfusion fixation as described above.

Immunohistochemistry

Brain blocks were formed using 2% agarose gel in PBS (Agarose, BIO-41025, Bioline). For whole brain mapping, coronal or parasagittal sections of the entire brain were used. For Str projections mapping sagittal sections of the right hemispheres were collected. Brains were cut at 50µm thickness in PBS using a vibrating microtome (VT1000S, Leica). For each experiment, the sites of injection were

verified by fluorescent microscopy and only those with on-target injections were processed further. For the anterograde tracing study, sections from the entire brain were incubated overnight in a solution containing antibodies against green fluorescent protein (GFP; which also detects eYFP; 1:1000 dilution, raised in rabbit, A21311; Invitrogen) and either ChAT (raised in goat; 1:500 dilution; AB144P, Millipore) or tyrosine hydroxylase (TH; to define the accumbens, Str and VTA/SNc borders; raised in chicken, 1:500 dilution; Abcam) in 1% normal donkey serum (NDS) and 0,03% Triton X-100 in PBS. After several washes in PBS, the sections were incubated for a minimum of 4h in Alexa Fluor 488-conjugated donkey anti-rabbit antibody (1:1000, Life Technologies) and either Cy3-conjugated donkey anti-chicken antibodies(1:1000; Jackson Immunoresearch) or Cy3-conjugated donkey anti-goat antibodies (1:1000; Jackson Immunoresearch). Additional sections were incubated with an antibody against the vesicular ACh transporter (VAChT; raised in guinea pig, 1:500 dilution in 1% NDS and 0,03% Triton X-100 in PBS; AB1588; Millipore Bioscience Research Reagents) to detect the presence of VAChT in the eYFP-labelled axons. Some sections were incubated with antibodies against μ-opioid receptor (MOR; raised in guinea pig, AB1774; Millipore) to define the striosomes (Graybiel and Ragsdale, 1978). These incubations were followed by their corresponding fluorophore-conjugated secondary antibodies, as described above.

Sections of the brains that had received tracer injections were first incubated in antibodies against ChAT (1:500) and CTb (raised in mouse, 1:500 dilution in 1% NDS and 0,03% Triton X-100 in PBS; Abcam), washed in PBS, and incubated in Cy3-conjygated donkey anti-mouse antibody (1:1000; Jackson ImmunoResearch). For FG detection, no additional processing was required.

For whole brain mapping a section every 300µm was selected. For Str mapping all sections were used. Brain sections were mounted on slides using VectaShield and then examined in a fluorescent (ImagerM2; Zeiss) or confocal (LSM-510; Zeiss) microscope using the following filters: 504nm for FG and Alexa Fluor-488, 560nm for Cy3 and 650nm for Cy5 (20x, 0.8 numerical aperture dry objective or 40X, 1.4 numerical aperture oil immersion). Confocal images were processed using Huygens Professional deconvolution software (version 4.1; Scientific Volume Imaging) with a maximum of 40 iterations. The brightness and contrast of captured images were adjusted in Photoshop (Adobe Systems). All brain sections were scanned and distribution of labelled axons in the entire brain or retrogradely labelled neurons in the cholinergic nuclei was digitized offline using StereoInvestigator (MicroBrightField).

Whole brain mapping

The processed sections were mounted on slides using VectaShield and then examined in a confocal microscope (LSM-510, Zeiss) at two distinct magnifications (10X, 0.32 NA and 20X, 0.8 NA), using the corresponding filters (504 nm for FG and YFP, 560 nm for Cy3 and mCherry, and 650 nm for Cy5). Brightness and contrast of captured images were adjusted in Photoshop. AAV-injected sections were contoured and fully scanned on a single Z-stack using StereoInvestigator (MicroBrightField; 10X, 0.25 NA). For every scanned site, the top and bottom of the section were manually delimited based on the section surface and single pictures were captured (2048 X 1056 pixel resolution) at 10 µm below the surface of the section to ensure that the antibody completely penetrated the section (frame size 860 X 660µm). Scanning sites were selected using the StereoInvestigator system incorporating a XYZ stage controller and a 25% overlap was selected to facilitate

reconstruction. To avoid photobleaching, an inter-acquisition interval of 50 ms was applied during scanning. Exposure time was automatically adapted for every site in order to keep same intensity threshold.

Analysis of the distribution of cholinergic axons and density quantification

Scans from AAV-injected brains were reconstructed offline based on the 2D serial section reconstruction module of StereoInvestigator. Injections with GFP cell body expression lower than 30% of the total number of $ChAT^{+}$ neurons within the diffusion area, or with positive soma located outside the borders of the nucleus for contiguous cholinergic structures, were excluded from the analysis. Scans were then overlapped with outlines of the Rat Brain Atlas (Paxinos and Franklin, 2007) using TH and ChAT staining.

The amount of AAV positive fibres in each nucleus was semi-quantitatively assessed offline. Representative levels for each main brain nuclei/region were scored using a predetermined density rating. The relative density was scored *4+* for many labelled fibres covering approximatively more than 50% of the selected image surface (very dense). Nuclei were labelled as *3+* or *2+* when projections covered approximatively more than 25% (dense) or less than 25% (moderate) of the surface, respectively. Structures were noted *1+* (few) when only few terminals were visible. All brains were scanned and only nuclei presenting similar scores in at least 2 animals were considered. Fibres without terminals or arborisation, defined as fibres "*en passage"*, were excluded from the mapping.

Analysis of the distribution of retrogradely labelled neurons

For whole brain mapping: brains injected with retrograde tracers were sectioned and

scanned as described above. Parasagittal and coronal sections comprising the Str and all cholinergic nuclei were used further. All single optical sections containing $ChAT^+$ neurons were fully scanned in high resolution (20X, 0.8 NA, step size 1µm, 2056 X 1056 pixels) for channels corresponding to ChAT, FG and CTb labelling. Analysis of distribution of retrogradely labelled neurons was done using StereoInvestigator functions: the cholinergic nuclei borders were outlined based on ChAT signal using *contour* tools, an overlay projection of the Z-stack was obtained based on *average intensity* tools, colocalisation of ChAT, Ctb and FG was quantified using the *markers* plugin. A minimum of two single optical sections were examined. Neurons within the borders of each cholinergic nucleus were classified as follows: (1) $ChAT^+/CTb^+$; (2) $ChAT^+/FG^+$; (3) ChAT-/CTb^+, (4) ChAT-/FG^+. Data were confirmed in a minimum of 4 animals where Ctb and FG injections were alternated between DS and NA.

The number of neurons in the PPNr, PPNc, and LDT projecting to the DLS, DMS and lateral, medial shell, and core of the NA were quantified according to their rostrocaudal distribution. Neurons within the PPN and LDT, as delimited by the ChAT-immunopositive cell bodies, were classified as follows: (1) $ChAT^+/CTb^+$; (2) $ChAT^+/FG^+$; (3) ChAT-/CTb^+; (4) ChAT-/FG^+. Three representative ML levels of the brainstem were selected for the analysis corresponding to 1.55, 1.13 and 0.9 mm from the midline (Paxinos and Franklin, 2007). To define the boundaries between PPNr and PPNc, we adapted a method based on the subdivision of the PPN into equally spaced segments in the sagittal plane using concentric circles, as described previously (Mena-Segovia et al., 2009). Thus, using the centre of the substantia nigra pars reticulata (SNr) as a reference point, two equally sized segments of 1400µm were defined. The first segment represents the PPNr (0.6 to 2.0 mm from the midpoint of the SNr), and the second segment represents the PPNc (2.0-3.3 mm from

the midpoint of the SNr). The LDT were quantified in a single segment (>3.3 mm from the SNr). Results are expressed as total numbers of neurons that are positive for each tracers and the normalised number of retrogradely traced neurons that are immunopositive for ChAT.

Results

Conditional labelling and mapping of cholinergic axons

Transduction of cholinergic neurons in Ch1 to Ch8 areas in ChAT::cre^{+} rats (n=12) following the insertion of the reporter transgene produced strong and discrete eYFP or mCherry signals in neurons immunopositive for ChAT (**Fig. 4**). Coordinates were based on previous experimentations in rats and coordinates from the Rat Brain Atlas (Paxinos and Franklin, 2007). Following three to four weeks, immunopositive-terminals were charted in the entire brain. Reporter labelling was observed in cell bodies, dendrites and local axons within the sites of injection. No differences were observed in the labelling produced by eYFP or mCherry (see Chapter 3), nor in the labelling specificity among cholinergic structures. The labelling of cholinergic neurons with fluorescent reporters was confirmed with immunohistochemistry for ChAT in the following structures: DLS (**Fig. 4A**), NA (**Fig. 4B**), medial septum (Ch1; **Fig. 4C**), the vertical limb of the diagonal band of the nucleus of Broca (Ch2, **Fig. 4D**), the horizontal limb of the diagonal band of the nucleus of Broca (Ch3, **Fig. 4E**), the nucleus basalis of Meynert (Ch4, **Fig. 4F**), the PPN (Ch5, **Fig. 4G**), the LDT (Ch6, **Fig. 4H**), the medial habenula (Ch7, **Fig. 4I**) and the parabigeminal nucleus (Ch8, **Fig. 4J**). Injections in the Str and NA produced labelling of interneurons whose axons were contained within the striatal borders but extended ventrally or dorsally beyond the site of injection.

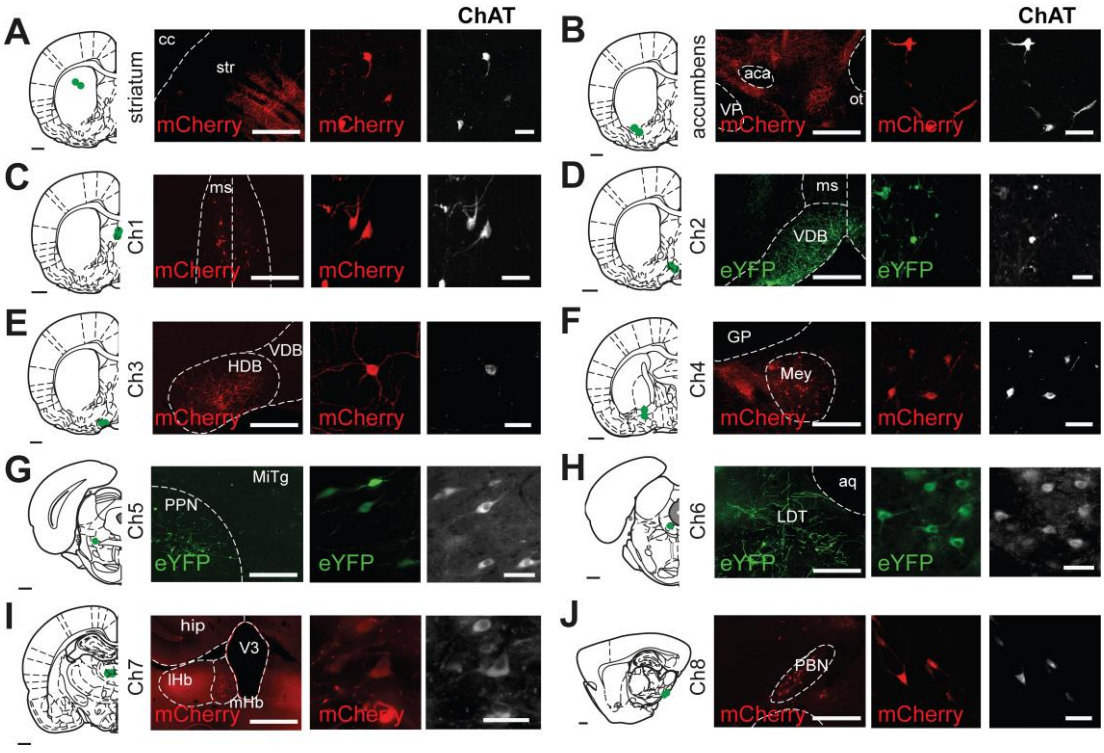

Figure 4: Transduction of cholinergic neurons across the brain. Coronal sections showing the sites of cholinergic neuron transduction following injection of AAV2-eYFP or AAV2-mCherry into the Str and the cholinergic nuclei (Ch1 to Ch8). eYFP or mCherry expression was observed in the dorsal Str (A), NA (B), medial septum (Ch1; C), the vertical limb of the diagonal band of Broca (Ch2; D), the horizontal limb of the diagonal band of Broca (Ch3; E), the nucleus basalis of Meynert (Ch4; F), the PPN (Ch5; G), the LDT (Ch6; H), the medial habenula (Ch7; I) and the parabigeminal nucleus (Ch8; J). All the injections were confined to their corresponding anatomical borders, as defined by Paxinos & Watson (1986). Abbreviations: aca, anterior commissure; NA, nucleus accumbens; aq, aqueduct; cc, corpus callosum; GP, globus pallidus; hip, hippocampus: HDB, horizontal limb of the diagonal band of the nucleus of Broca; LDT, laterodorsal tegmental nucleus; lHb, lateral habenula; Mey, nucleus basalis of Meynert; mHb, medial habenula; MITg, microcellular tegmental nucleus; ms, medial septum; ot, olfactory tubercle; PBN, parabigeminal nucleus; PPN, pedunculopontine nucleus; Str, striatum; V3, third ventricle; VDB, ventral limb of the diagonal band of the nucleus of Broca; VP, ventral pallidum. Scale bars: brain outlines, 1000 µm; low magnification panels (left), 500 µm; high magnification panels (centre and right), 50 µm.

However, no overlap between the axons from each region was detected, suggesting that the area of innervation of cholinergic axons is restricted within the functional domains. Medial septum injections were targeted to its mediodorsal region to avoid overlap with the diagonal band of the nucleus of Broca, which resulted in strong

bilateral expression (**Fig. 4C**). The vertical limb (**Fig. 4D**) and the horizontal limb (**Fig. 4E**) are very ventral structures surrounded by the ventral pallidum dorsally and the olfactory tubercle laterally; both show a high density of small cholinergic neurons. The nucleus of basalis of Meynert, situated ventral to the globus pallidus, contained loosely distributed, small cholinergic neurons (**Fig. 4F**), consistent with Mesulam (1983 a, b).

Cholinergic projections to the Central nervous system

Medial septum (Ch1; **Fig. 5**) cholinergic projections were mainly observed in the cingulate cortex (3+; **Fig. 6**), the diagonal band of the nucleus of Broca (3+), the lateral septum (3+), the ventral pallidum (3+), hippocampus (4+) and the reticular thalamic nucleus (2+; **Fig. 7**) (Senut et al., 1989; Kalèn and Wiklund, 1989; Nyakas et al., 1987). No labelled axons were visible in the Str or NA.

The axons and terminals of cholinergic neurons located in the vertical limb of the diagonal band of Broca (Ch5; **Fig. 5**) were found predominantly in the medial prefrontal cortex (3+; **Fig. 6**), the cingulate cortex (4+), the orbital cortex (4+), the motor cortex (4+),the piriform cortex (4+), the lateral and medial septum (4+), the ventral pallidum (3+), the amygdala (4+), the rostral and caudal hippocampus (4+), zona incerta (3+; **Fig. 7**), mediodorsal and reticular thalamic nucleus (3+) (Kalèn and Wiklund, 1989; Henny and Jones, 2008; Nyakas et al., 1987; Nelson et al., 2000). Injections in Ch2 did not produce axon labelling in the Str or the NA. However, a few projections were visible in the olfactory tubercle.

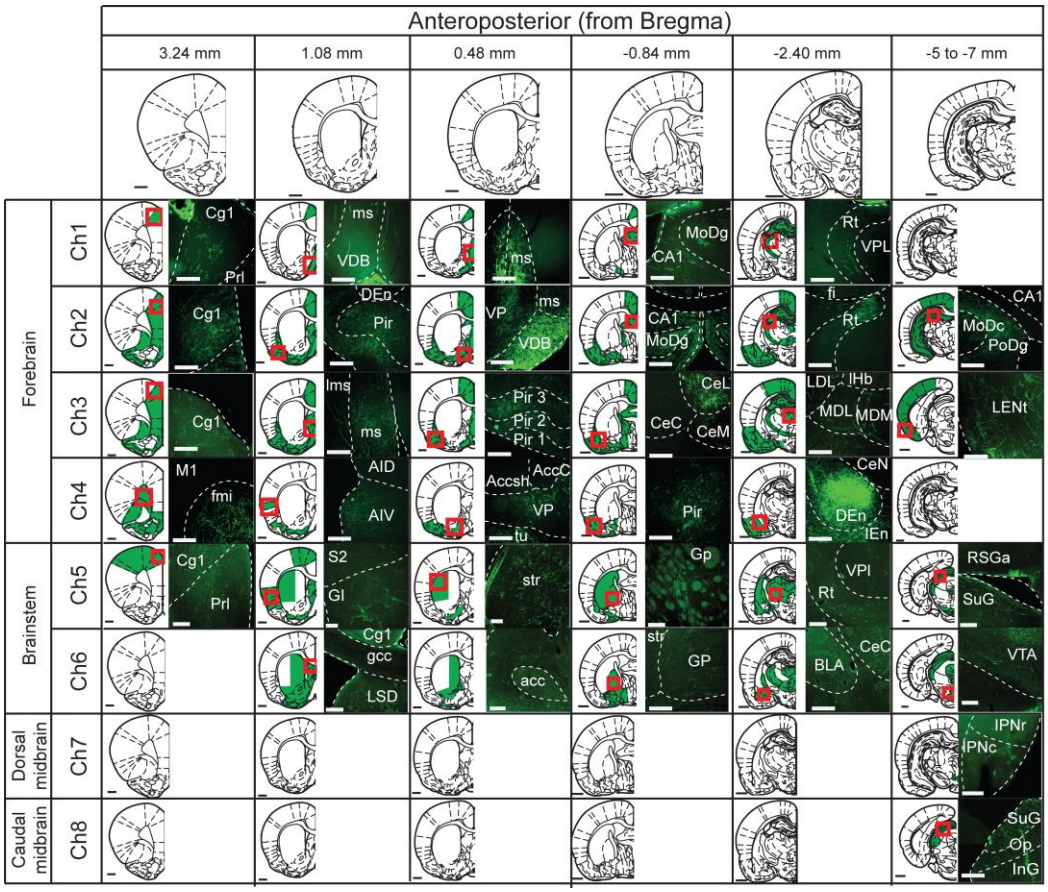

Figure 5: Mapping of cholinergic axons in the whole brain. Six representative coronal sections (anteroposterior bregma levels, columns) throughout the brain were selected to map the innervation of the cholinergic nuclei (Ch1 to Ch8; rows). For each cholinergic group, each row depicts a schematic summary where green shaded areas indicate high density of fluorescently-labelled axons. Red squares indicate the site where the fluorescent images on the right were obtained. Abbreviations (if not defined previously): NAsh, nucleus accumbens shell; accC, nucleus accumbens core; AID, agranula insular dorsal cortex, AIV, agranular insular ventral cortex; BLA, basolateral amygdala; CA1, CA1 field of the hippocampus; CE, central amygdala (L, lateral; M, medial; C, capsular); Cg, cingulate cortex; Den, dorsal endopiriform nucleus; fmi, external capsule; GI, granula insular cortex; gcc, genu of the corpus callosum; IEn, intermediate endopiriform nucleus; InG, intermediate gray layer superior colliculus; IPN, interpeduncular nucleus (c, caudal; r, rostral); LDL, laterodorsal thalamic nucleus lateral part; LENt, lateral enthorinal cortex; Ls, lateral septum; LSD, lateral septum dorsal part; M, motor cortex; MD, mediodorsal thalamic nucleus (M, medial; L, lateral); MoDG, molecular layer dentate gyrus; Op, optic nerves layer superior colliculus; Pir, piriform cortex; PoDg, polymorph layer dentate gyrus; RSGa, retrospinal granular cortex; Rt, reticular thalamic nucleus; S, somatosensory cortex; SuG, superficial gray superior colliculus; VP, ventral pallidum; VPL, ventro-posterior thalamic nucleus lateral part; VTA, ventral tegmental area. Red arrows represent discrete cholinergic axons. Scale bars: brain outlines, 1000 µm; fluorescent images, 200 µm.

The cholinergic neurons of the horizontal limb of the diagonal band of the nucleus of Broca (Ch3; **Fig. 5**) gave rise to similar projection pattern as Ch2 cholinergic neurons. YFP-positive terminals were found mainly in the cingulate cortex (4+; **Fig. 6**), medial prefrontal cortex (3+), motor cortex (3+), somatosensory cortex (3+), piriform cortex (4+), insular cortex (3+) and prelimbic cortex (3+). This population gave rise to projections to the ventral pallidum (4+), the amygdala (4+), hippocampus (4+), reticular and mediodorsal thalamic nucleus (3+; **Fig. 7**) (Kalèn and Wiklund, 1989; Henny and Jones, 2008; Záborszky et al., 1986; Gaykema et al., 1990; Gritti et al., 1997). Analysis of the Ch3-injected rats never showed positive axons within the Str or NA. However, *en-passage* fibres within the olfactory tubercle were observed.

The nucleus basalis of Meynert (Ch4; **Fig. 5**) revealed cholinergic projections primarily to the orbital cortex (3+; **Fig. 6**), the peduncular cortex (3+), the insular cortex (2+), the piriform cortex (3+), the ventral pallidum (4+) and the amygdala (4+) (Baskerville et al., 1993; Nagai et al., 1982; Pearson et al., 1983; Saper, 1984; Woolf and Butcher, 1982; Schauz and Kock, 1999; Záborszky et al., 2015). No visible terminals were observed in the Str, the NA or the olfactory tubercle.

Injections in the PPN (Ch5; **Fig. 5**) revealed weak axonal labelling in the cingulate (1+), motor cortex (1+; **Fig. 6**) and the insular cortex (2+). A much stronger signal was observed in the ventral pallidum (3+), the medial and lateral septum (2+), the globus pallidus (2+), the amygdala (2+), the ventral and dorsal lateral thalamus (3+; **Fig. 7**), the reticular thalamic nucleus (3+), the superior colliculus (3+), the DAergic ventral midbrain nuclei (3+), the LDT (3+) and the gigantocellular tegmental field (3+) (Oakman et al., 1999; Mitani et al., 1988; Semba and Fibiger, 1992; Lavoie and

Parent, 1994; Futami et al., 1995; Mena-Segovia et al., 2004; Mena-Segovia et al.; 2008). Abundant labelled axons were observed in the DLS (3+), NA (3+) and olfactory tubercle (2+) (Dautan et al., 2014).

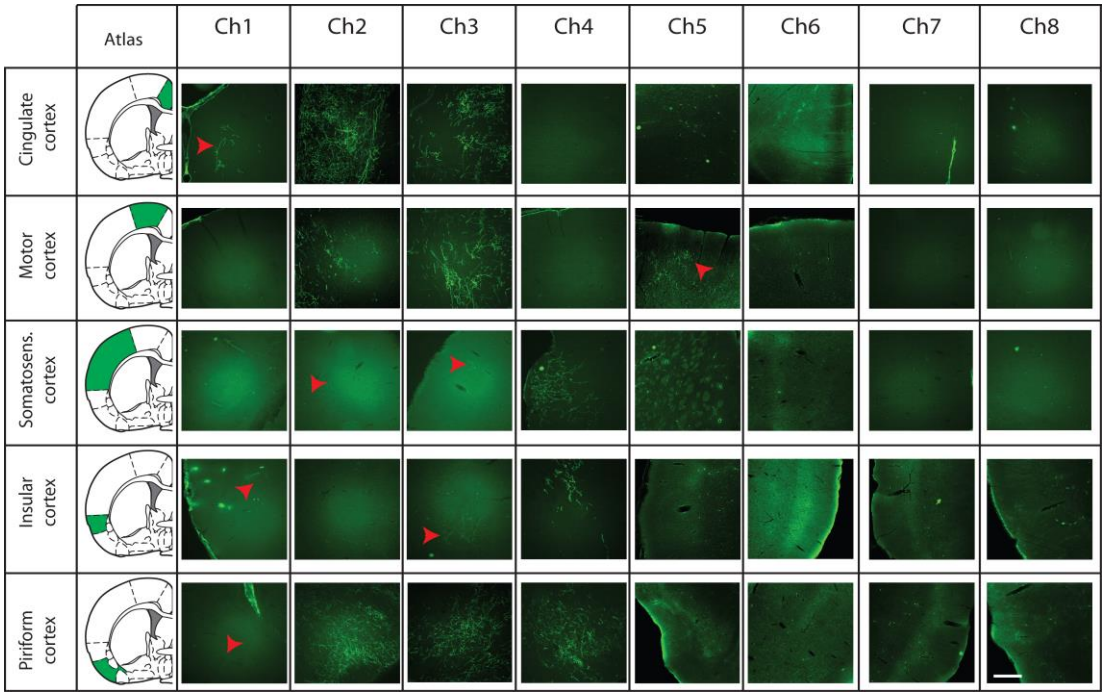

Figure 6: Mapping of cholinergic axons in the cortex. Five representative coronal sections of the cortex (anteroposterior bregma levels, rows) throughout the brain were selected to map the innervation of the cholinergic nuclei (Ch1 to Ch8; column). For each cholinergic group, each row depicts a schematic summary where green shaded areas indicate where the fluorescent images on the right were obtained. Red arrows represent discrete cholinergic axons. Scale bars: brain outlines, 1000 µm; fluorescent images, 200 µm.

LDT (Ch6; **Fig. 5**) injected animals revealed labelled axons and terninals in the ventral pallidum (3+), medial and lateral septum (3+), globus pallidus (3+), amygdala (3+), reticular and medial thalamic nucleus (3+; **Fig. 7**), inferior colliculus (2+), dorsal raphe (2+), gigantocellular tegmental field (3+) and the midbrain DAergic nuclei (3+) (Dautan et al., 2014; Motts et al., 2008; Holmstrand and Sesack, 2011; Hallanger and Wainer, 1988). YFP-positive axons were observed in the DMS (2+), NA (3+) and olfactory tubercle (4+).

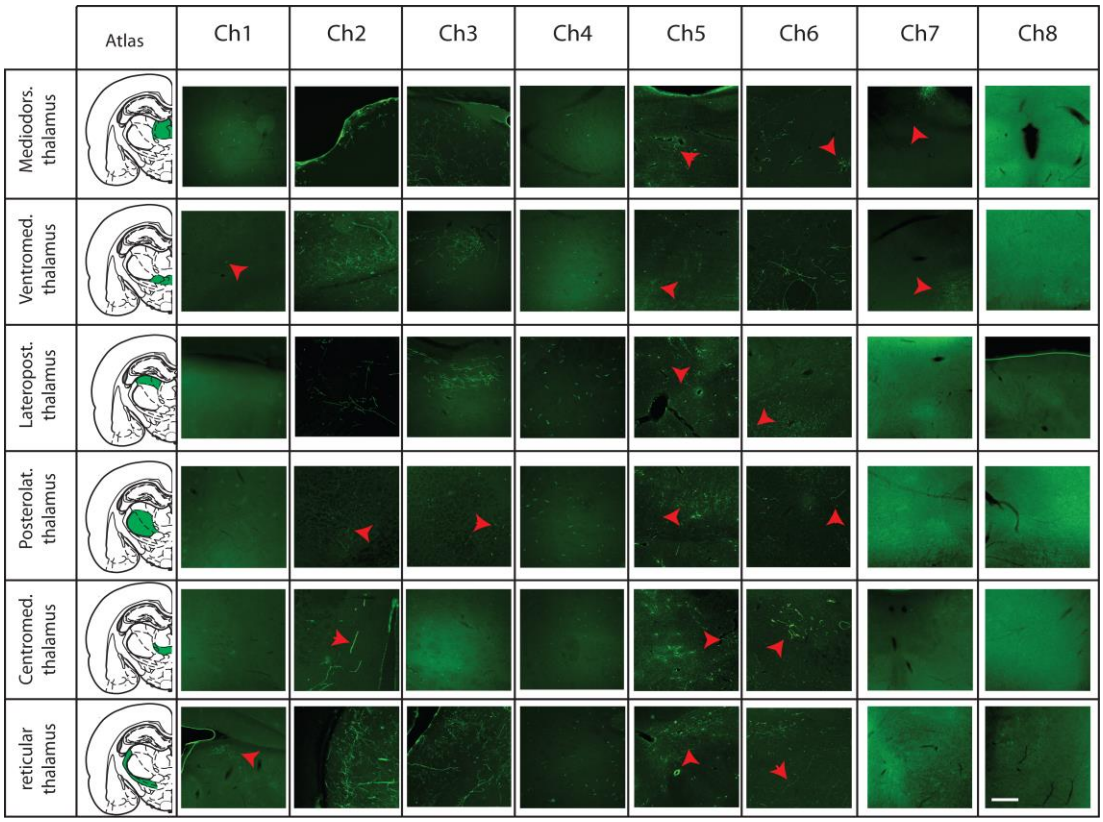

Figure 7: Mapping of cholinergic axons in the thalamus. Six representative coronal sections of the thalamus (anteroposterior bregma levels, rows) throughout the brain were selected to map the innervation of the cholinergic nuclei (Ch1 to Ch8; column). For each cholinergic group, each row depicts a schematic summary where green shaded areas indicate where the fluorescent images on the right were obtained. Scale bars: brain outlines, 1000 µm; fluorescent images, 200 µm.

Medial habenula-injected animals (Ch7; **Fig. 5**) showed a strong and discrete descending pathway that followed the fasciculus retroflexus and terminate in the interpeduncular nucleus (3+) (Cuello et al., 1978; Kobayashi et al., 2013; Ren et al., 2011). Animals injected in the parabigeminal nucleus (Ch8; **Fig. 5** shows an ascending pathway that spread densely in the inferior (3+) and superior colliculi (4+) (Mufson et al., 1986; Fitzpatrick et al., 1988). No rostral pathway was observed in animals injected either in the medial habenula or the parabigeminal; detailed observation of the Str, accumbens and olfactory tubercle revealed no signal within their borders.

The descriptions above comprise the main areas of innervation for each of the cholinergic groups.

Retrograde tracing

Injections into the Str and NA revealed widespread cell body labelling (**Fig. 8**), predominantly in the deep layers of the motor, somatosensory and the limbic cortex. DS injections also produced a strong signal in the thalamus, predominantly in the anterior thalamic nucleus, the central-median, the parafascicular and the ventro-posterior nuclei. In contrast, NA injections produce labelling mainly in the parafascicular and medial thalamic nuclei. Further labelling following DS injections was observed in the lateral substantia nigra compacta, whereas injections delivered in the NA produced labelling in the ventral tegmental area and medial substantia nigra compacta. Other nuclei with presence of tracers include the globus pallidus, the ventral pallidum, dorsal raphe, ventral hypothalamus and locus coeruleus.

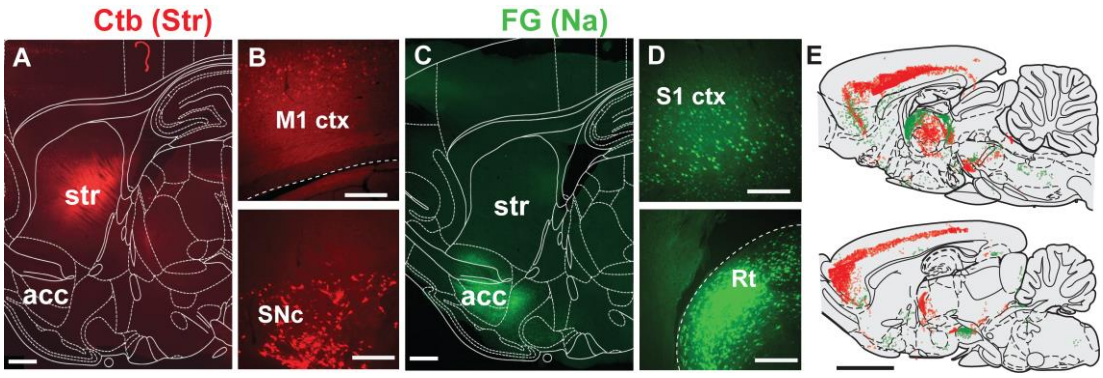

Figure 8: Distribution of neurons projecting to the dorsal striatum and nucleus accumbens. (A, C) Deposits of Ctb and FG were delivered into the DLS and NA, respectively (this was alternated across animals). (B) Representative examples of cell body labelling in the motor cortex (M1 ctx) and substantia nigra pars compacta (SNc) following an injection in the DLS. (D) Representative examples of cell body labelling in the sensory cortex (S1 ctx) and reticular thalamic nucleus (Rt) following an injection in the NA. (E) Mapping of retrogradely labelled neurons across the brain in two sagittal representative levels (lateral to bregma: 0.4 and 1.55 mm; each dot represents a positive cell body; FG, green; Ctb, red). Scale bars: A and C, 1000 µm; B and D, 200µm; E, 5000 µm.

Labelling of neurons within the borders of cholinergic structures (Ch1-Ch8) was only observed in the nucleus basalis of Meynert, PPN and LDT, with no neurons detected within the borders of Ch1, Ch2, Ch3, Ch7 or Ch8 groups (**Fig. 9**). However, ChAT immuno labelling revealed that the majority of the retrogradely labelled neurons in the PPN and LDT are cholinergic, whereas none of the retrogradely labelled neurons in the nucleus basalis of Meynert were immunopositive for ChAT. These results further confirm the presence of a cholinergic projection from the PPN/LDT to the Str and NA and show that no other cholinergic nuclei contribute to the innervation to the striatal complex.

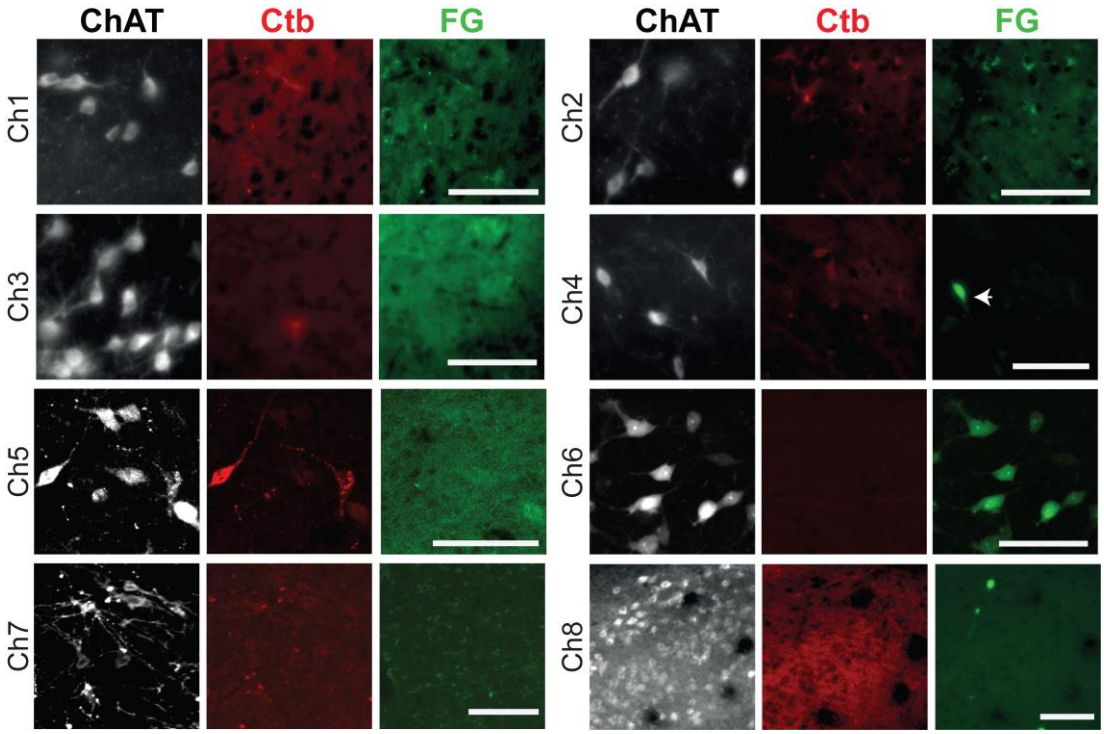

Figure 9: Retrograde labelling in cholinergic Ch1-Ch8 groups. Retrogradely-labelled neurons were absent in Ch1, Ch2, Ch3, Ch7 and Ch8, and present in Ch4, Ch5 and Ch6, but co-expression with ChAT immuno labelling was only observed in Ch5 and Ch6. In these examples, neurons projecting to the Str are the ones positive for FG (green) and neurons projecting to the NA are the ones positive for Ctb (red). The arrow indicates a neuron containing FG that is ChAT negative. Scale bars: Ch1 to Ch8, 100µm.

Innervation of the striatum and NA by cholinergic axons originating in the PPN and the LDT nuclei

Three to four weeks after injections with the AAV vector in the ChAT::cre^{+} rats, clusters of cholinergic neurons, identified by immunofluorescent labelling for the eYFP, were found around the site of injection. Cell bodies, dendrites, and local axonal arbors were labelled, and the clusters of labelled neurons ranged from 0,5 to 1mm in diameter in the sagittal plane.

The axons of the brainstem cholinergic neurons gave rise to widespread projections in the form of beaded axons in the midbrain and forebrain, including the superior colliculus, substantia nigra, thalamus, subthalamus and GP. In addition to this, each of the injections in the PPN or LDT gave rise to eYFP positive axons that occupied most regions of the Str, NA, and substriatal structures, and their distribution correlated with the region of the brainstem injected (**Fig. 10**).

The labelled axons gave rise to frequent varicosities, could follow long straight path or give rise to tortuous arborisation (Fig. 11-13) that mostly avoided patches/striosomes (see below). Furthermore, immunofluorescence labelling for VAChT of some of the YFP-positive axons and boutons confirmed the cholinergic nature of the projection (**Figs. 11C, 12C, 13C**).

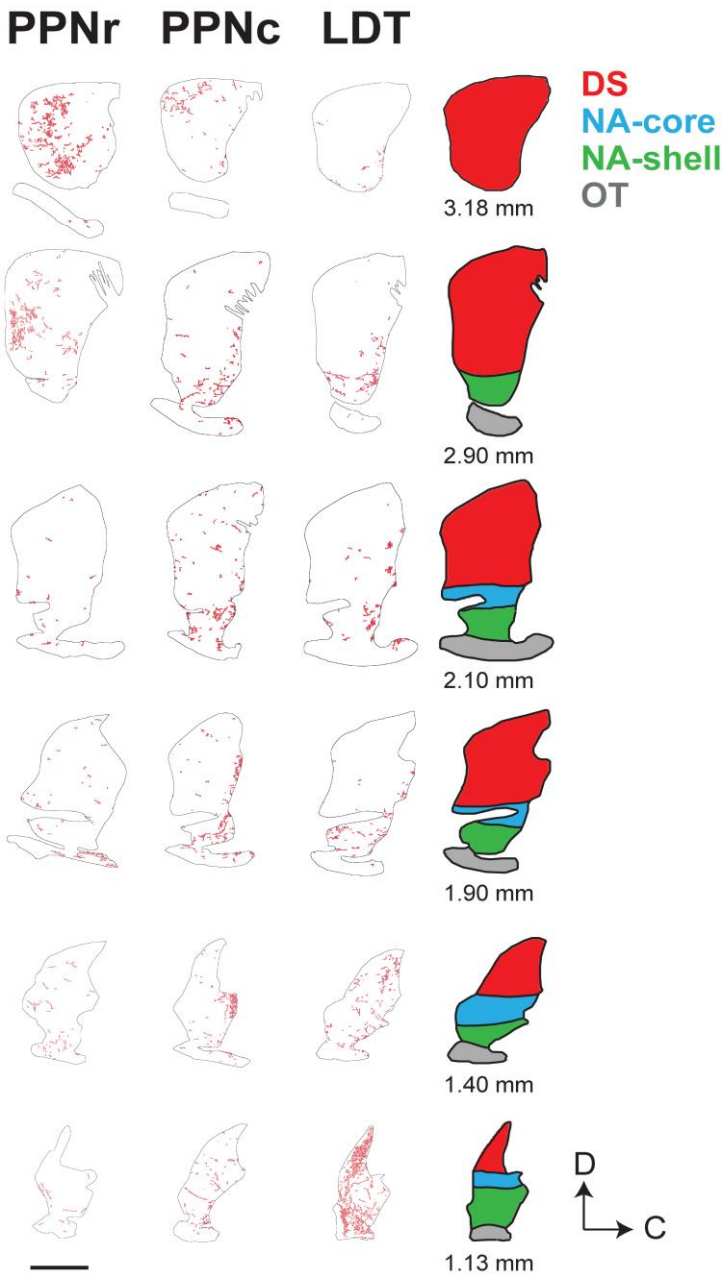

Figure 10:

Distribution of brainstem cholinergic axons in the striatum. Plots in the sagittal plane of the distribution of cholinergic neurons from the brainstem. Plots in the sagittal plane of the distribution of cholinergic axonal profiles from three representative animals that received viral vector injections in the PPNr, PPNc, and the LDT. Injections in the PPNr gave rise to a dense innervation of the lateral Str. Injections in the PPNc gave rise to a sparser innervation, with a tendency to innervate more medial regions of the Str and parts of the NA. In contrast, injections into the LDT led to dense labelling in the most medial Str and the NAcore. Each injection led to labelling in the olfactory tubercle. Each red dot represents at least one immunopositive axonal profile in a bin of 40µm2. The corresponding template (right) illustrates the ML levels (millimeters from the midline) and subdivisions of the Str (DS, dorsal striatum), NAcore (NA core) and shell (NA shell), and olfactory tubercle (OT) according to the stereotaxic Rat Brain Atlas of Paxinos and Watson (1986). D, Dorsal; C, caudal. Scale bar, 2 mm.

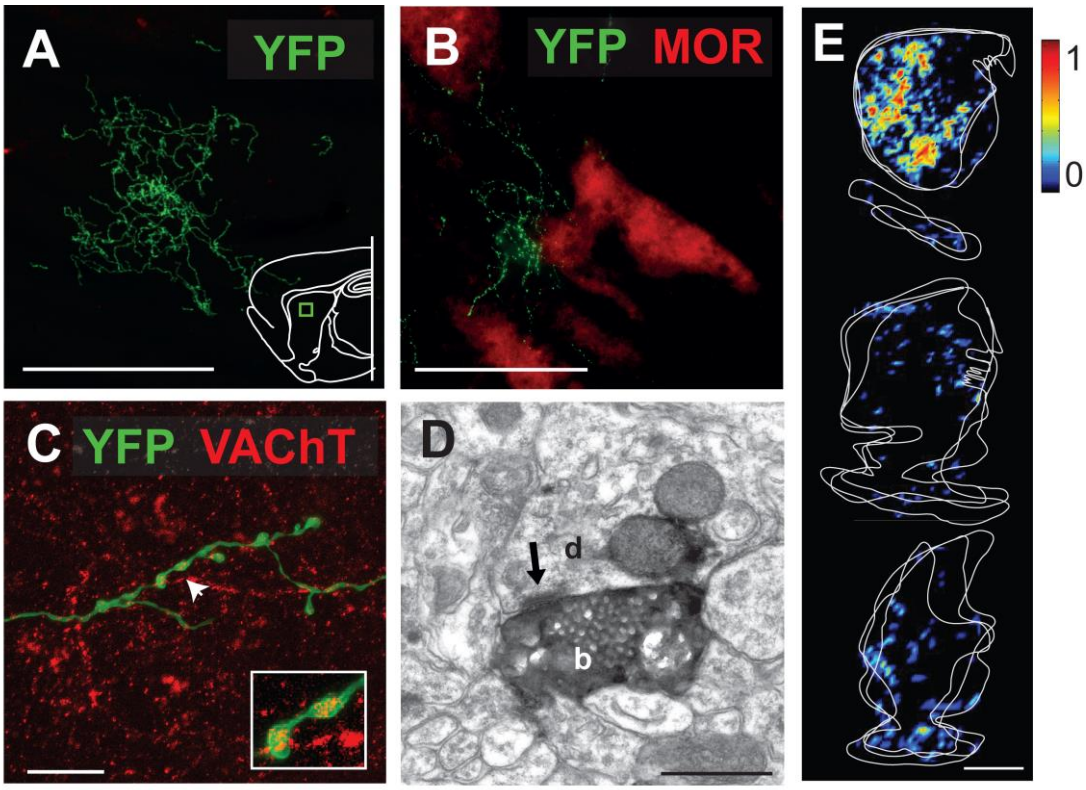

Figure 11: Cholinergic axons arising from the PPNr. Cholinergic axons arising from the PPNr preferentially innervate the lateral Str. **A**, YFP-immunopositive axons arising from cholinergic neurons after the deposit of AAV2–EF1a–DIO– hChR2–YFP in the rostral PPN of a ChAT::Cre rat. The labelled cholinergic axons formed dense regions of innervation in the DLS. The green box in the outline represents the area in which the image was acquired. **B**, MOR immuno labelling to identify the striosomes. The distribution of the cholinergic axons was primarily confined to the MOR-negative matrix compartment. **C**, The cholinergic axons gave rise to a large number of varicosities, some of which were identified as VAChT immunopositive (arrowhead; see inset).**D**, Electron micrograph of a YFP-immunopositive cholinergic bouton (b) forming an asymmetric (Gray's type 1) synapse (arrow) with a dendritic shaft (d). **E**, Probability plot, calculated from all animals (n=3) at three differentMLlevels (3.18, 2.10, and 1.13mmfrom the midline, from top to bottom), of cholinergic axons arising in the PPNr. Cholinergic axons from this region of the PPN were more densely distributed in the lateral aspects of the DS. Scale bars: **A**, 250µm; **B**, 250µm; **C**, 10µm; **D**, 0.4µm; **E**, 1000µm.

The cholinergic projection from the PPN and LDT to the striatal complex was topographically organised such that the rostral part of the cholinergic brainstem innervated more abundantly the lateral levels of the Str, and the caudal part

innervated more abundantly the medial and ventral levels of the Str. The AAV injections in the PPNr produced areas of dense innervation (patches) mainly in the DLS (**Figs. 10, 11A, 11E**).

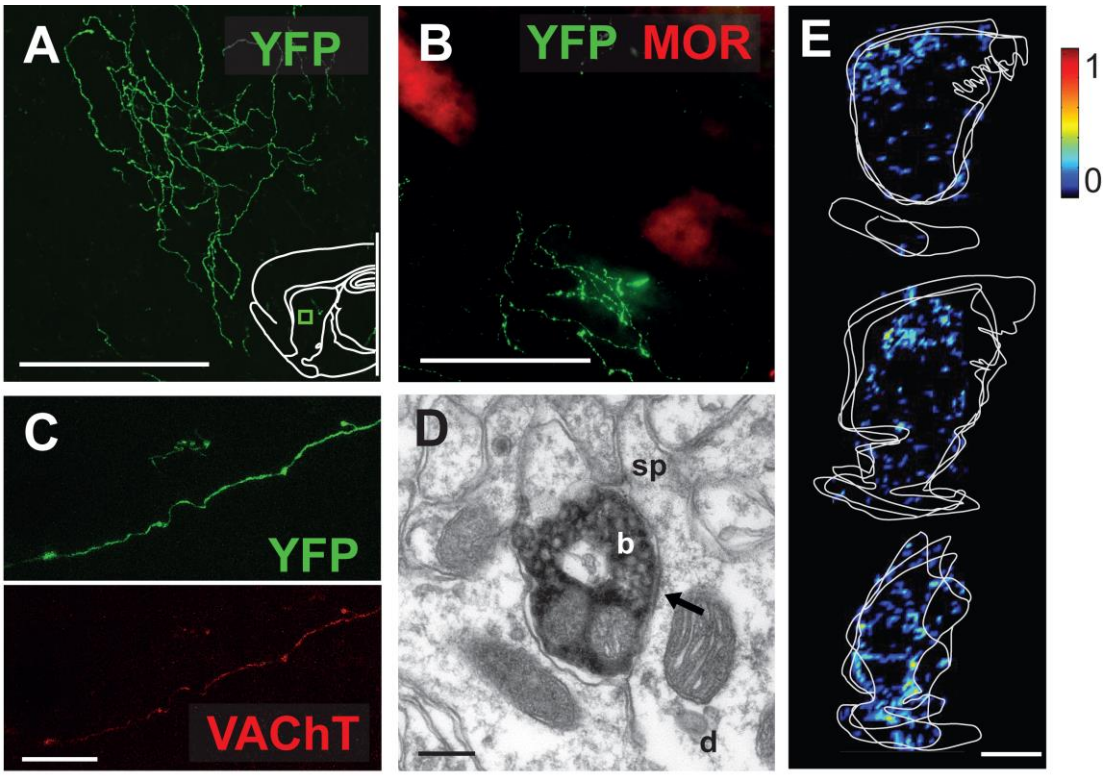

Figure 12: Cholinergic axons arising in the PPNc. Cholinergic axons arising in the PPNc project diffusely across the striatum and NA. **A**, YFP-immunopositive axons arising from cholinergic neurons in the PPNc form small patches mainly in the dorsal part of the Str. The green box in the outline represents the area in which the image was acquired. **B**, Cholinergic axons from PPNc also avoided the striosomes. **C**, These cholinergic axons (top) gave rise to a smaller number of varicosities than those of the PPNr but were always immunopositive for VAChT (bottom). **D**, Electron micrograph of an immunopositive cholinergic (YFP-positive) bouton (b) establishing symmetric (Gray's type 2) synaptic contact (arrow) with the neck or base of a dendritic (d) spine (sp). **E**, Probability plot showing that cholinergic axons arising in the PPNc have a lower density distribution than those arising in the PPNr, with higher values in the lateral Str, mainly in the more dorsal regions, and in the NA core and shell (at three different ML levels as in Fig. 2). Scale bars: **A**, 250µm; **B**, 250µm; **C**, 10µm; **D**, 0.2µm; **E**, 1000µm.

In contrast, deposits in the PPNc gave rise to smaller areas of innervation that were sparser and distributed over different levels of the DS and NA (**Figs. 10, 12A, 12E**).

Cholinergic axons from the LDT showed a level of innervation comparable with that of the PPNr, but it was concentrated in the DMS and the NA (**Figs. 10, 13A, 13E**). In each case, the regions of axonal arborisations in the striatal complex avoided the patches/striosome as defined by the high level of expression of MOR immunoreactivity (**Figs. 11B, 12B and 13B**). These data reveal topographically organised cholinergic projection originating in the brainstem that innervates wide areas of the DS and NA, forming synaptic contacts with different postsynaptic structures.

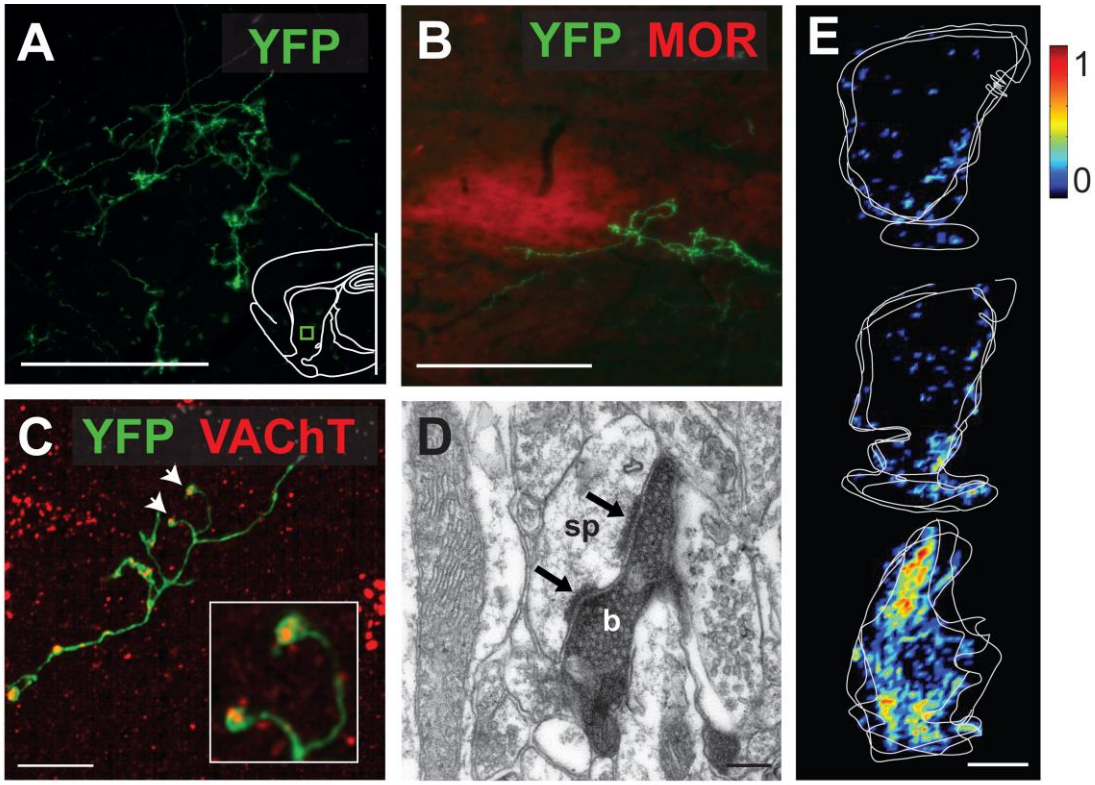

Figure 13: Cholinergic axons arising in the LDT. Cholinergic axons arising in the LDT preferentially innervate the medial Str and the Na core. **A**, YFP-immunopositive axons from cholinergic neurons in the LDT form dense regions of innervation in the most medial levels of the Str andNAcore (depicted here). The green box in the outline represents the area in which the image was acquired. **B**, MOR immuno labelling in the NA shell revealed that cholinergic axons from the LDT tend to avoid striosomes. **C**, Cholinergic axons from the LDT formed large en passant varicosities that were immunopositive for VAChT (arrowheads). **D**, Electron micrograph of a cholinergic axon bouton (YFP-immunopositive; b) forming asymmetric (Gray's type 1) synapses (arrows) with a spine (sp). **E**, Probability plot

showing that cholinergic axons arising in the LDT were more densely distributed in the medial Str and the core and medial shell of the NA (n µ 3; at three different ML levels as in Figs. 2, 3). Scale bars: **A**, 250µm; **B**, 100µm; **C**, 10µm; **D**, 0.2µm; **E**, 1000µm.

EM observations

EM experimentations have been performed by Icnelia Huerta-campo.

EM analysis of the Str revealed that the axons of brainstem cholinergic neurons gave rise to vesicle-filled boutons that often contained one or more mitochondria and formed synaptic contacts with spines (**Figs. 12*D*, 13*D***) and dendritic shafts (**11*D***). Synapses were mostly asymmetric (Gray's type 1; **Figs. 11*D*, 134*D*, 14*A*, *B*, *E*, *F***); in some cases, the postsynaptic density was very prominent (similar to cortical and thalamic synapses) but on other occasions was less dense and more diffuse (Fig. 14*B*). Symmetrical synapses were less frequently observed (Gray's type 2; **Figs. 12*D*, 14*C***). Serial section analysis revealed that synaptic incidence varied depending on the origin (highest for LDT axons and lowest for PPNc axons). The deposits of the viral vector in the Str led to the labelling of striatal cholinergic neurons and their local axon collaterals (**Fig. 14*D***). EM analysis of 39 cholinergic synapses derived from interneurons revealed that approximately one-third form asymmetric synapses (**Fig. 14*E*,*F***; including those with prominent and those with less prominent postsynaptic densities), and two-thirds formed symmetrical synapses (**Fig. 14*G***). This contrasts to cholinergic synapses derived from the PPNr, of which 82% formed asymmetric synapses and 18% formed symmetrical synapses (*n* = 22; **Fig. 14*H***). Similar differences in the postsynaptic targets of PPNr-derived and striatal interneuron-derived cholinergic synapses were observed.

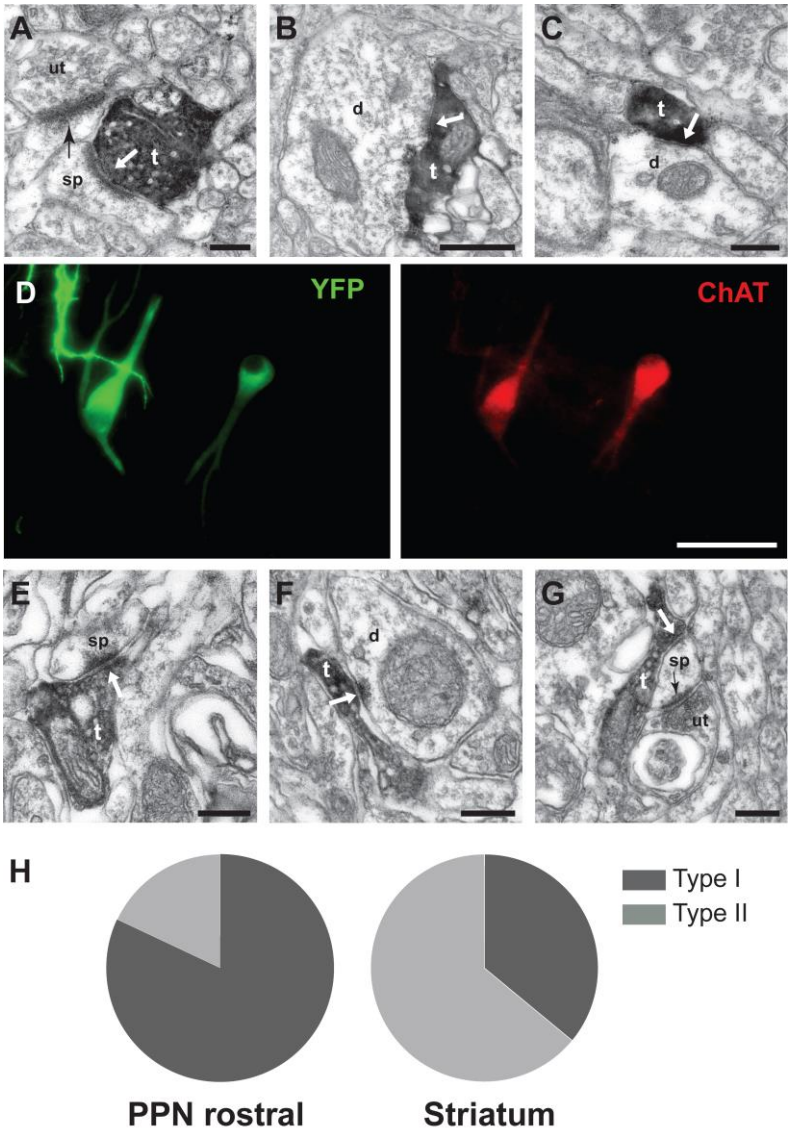

Figure 14: Morphology of synapses differentiates brainstem cholinergic from cholinergic interneurons contacts. (Data collected by Icnelia Huerta-Ocampo and presented for understanding purposes) Morphology of synapses differentiates brainstem cholinergic from cholinergic interneurons contacts. **A–C**, Electron micrographs of cholinergic boutons (b) arising from the PPNr (YFP-immunopositive) forming asymmetric (Gray's type 1) synapses with prominent (**A**) and moderate (**B**) postsynaptic densities onto a spine (sp) and dendritic shaft (d), respectively. **C**, A PPN cholinergic terminal forming a symmetrical (Gray's type 2) synapse with a dendritic shaft. **D**, YFP-immunopositive cell bodies from striatal cholinergic interneurons and their axons after the deposit of AAV2–EF1a–DIO– hChR2–YFP in the DLS of a ChAT::Cre^{+} rat. ChAT immuno labelling confirms their cholinergic nature. **E**, **F**, Electron micrographs of cholinergic boutons arising from striatal cholinergic interneurons (YFP-immunopositive) establishing asymmetrical synapses that possess prominent (**E**) and less prominent (**F**) postsynaptic densities onto a spine and dendritic shaft, respectively. **G**, A striatal cholinergic bouton forming a symmetrical (Gray's type 2) synapse with a spine. Note the unlabelled terminals (ut) forming synapses (black arrows) with unlabelled spines in **A** and **G**. **H**, Synapses from the PPNr are predominantly asymmetric, whereas the synapses from the cholinergic interneurons are predominantly symmetric. Scale bars: **A**, **C**, **E–G**, 0.2µm; **B**, 0.5 µm; **D**, 50µm.

Thus, ~74% of those derived from the PPNr contacted dendritic shafts and ~26% contacted spines, whereas the figures were ~46% in contact with shafts, ~49% with spines, and ~5% with perikarya for synapses derived from cholinergic interneurons. Overall, these findings suggest that cholinergic axons derived from neurons in the brainstem, like striatal cholinergic interneurons, make synaptic contact with the spines of medium spiny neurons, and the asymmetric synapses on dendritic shafts suggest that they also contact striatal interneurons.

Neurons retrogradely labelled from the dorsal striatum and NA are topographically organised in the brainstem

To extend the data from anterograde tracing experiments using viral vectors in transgenic rats, we injected two retrograde tracers in wild-type animals. We injected a combination of targets that included the dorsolateral and dorsomedial areas of the Str and the core, medial shell, and lateral shell of the NA. The presence and distribution of retrogradely labelled neurons were analysed in the PPN and LDT. Immunofluorescence for ChAT was used to define the borders of the brainstem structures and to quantify the proportion of neurons projecting to the striatal complex that were cholinergic. The results confirm the presence of a topographical projection from the cholinergic neurons of the brainstem to the forebrain (**Fig. 15**).

Injections in the core and medial shell of the NA (n=4 and n=3, respectively) led to dense labelling particularly concentrated in the LDT (**Fig. 15B, C**), with some labelling, albeit much sparser, in the PPNc.

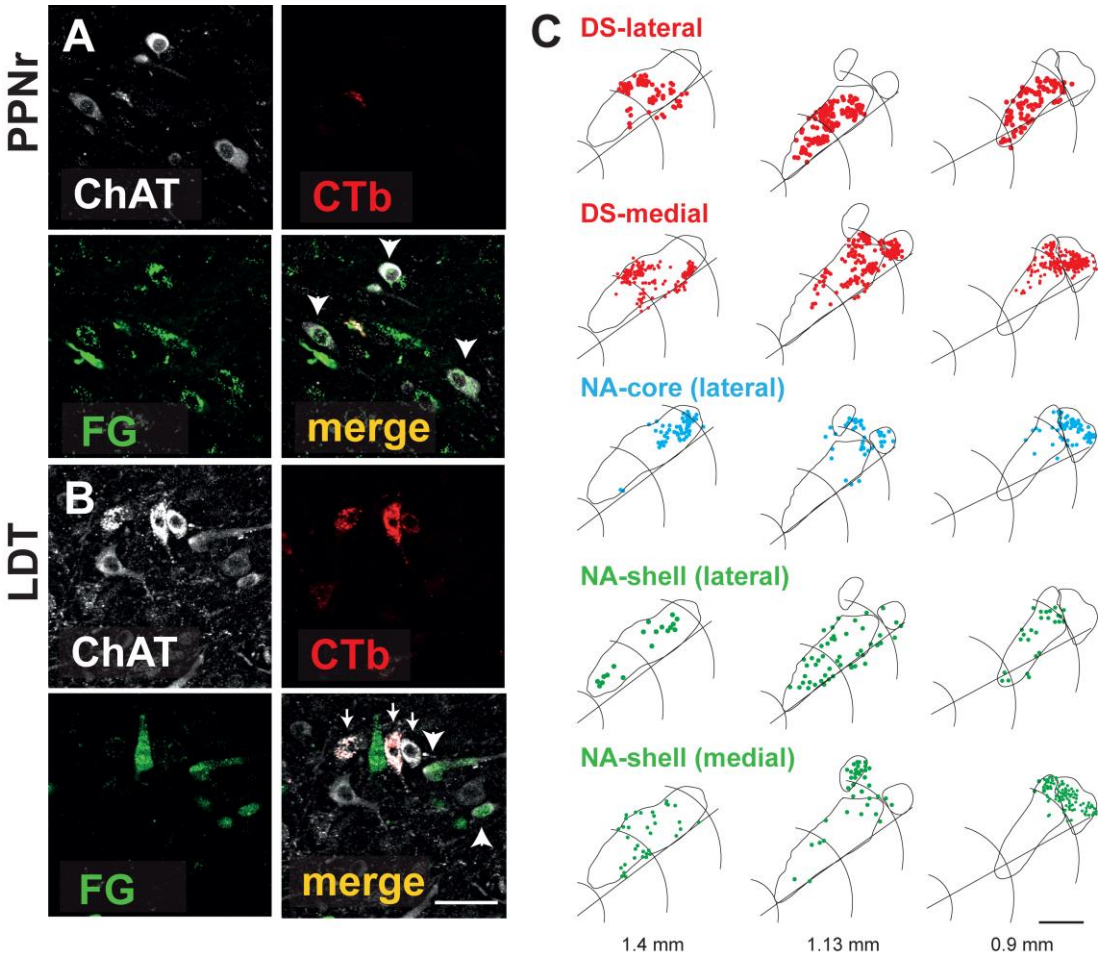

Figure 15: Retrograde labelling from the striatum. Retrograde labelling from the Str and NA shows a topographical distribution of cholinergic neurons in the brainstem. **A**, **B**, Confocal fluorescent images showing triple immuno labelling for ChAT, CTb, and FG in the PPNr (**A**; PPNr) and LDT (**B**). In **A**, CTb was injected in the medial Str and FG was injected in the lateral Str, whereas in **B**, CTb was injected in the NA core and FG was injected in the NA medial shell. In both cases, most of the retrogradely labelled neurons were immunopositive for ChAT. **C**, Plots of the location of retrogradely labelled neurons in the PPN and LDT (at 3ML levels; millimeters from midline) after the injections of tracers in different regions of the striatal complex. Injections in DLS led to retrograde labelling in the PPNr and PPNc. Injections in the DMS led to retrograde labelling in the PPNc and the LDT. Injections in the lateral part of the NA core (NA core) led to a similar pattern of labelling. Injections in the medial shell of the NA (NA shell) produced retrograde labelling mainly in the LDT, whereas injections in the lateral shell produced labelling mainly in the PPNr and PPNc. Scale bars: **A**, **B**, 50µm; **C**, 500µm.

In contrast, injections in the lateral shell (n=4) produced widespread labelling across the PPN and, to minor degree, the LDT **(Fig. 15C**). The proportion of neurons

retrogradely labelled from the NA that were ChAT-immunopositive was more variable (41-78%) than those labelled from the DS. The fact that a proportion of the projection neurons were not ChAT-positive suggests that non-cholinergic neurons (i.e., glutamatergic and GABAergic) are also involved in the striatal projection pathways (**Table 1**).

	Normalized cell count (%)			% retrogradely-traced neurons that were identified as cholinergic		
	Rostral PPN	**Caudal PPN**	**LDT**	**Rostral PPN**	**Caudal PPN**	**LDT**
DS lateral	36.37 ± 6.07	47.32 ± 5.8	16.30 ± 13.53	76.16 ± 6.69	76.62 ± 5.81	61.07 ± 10.37
DS medial	21.78 ± 2.9	32.9 ± 3.65	45.29 ± 3.49	60.61 ± 8.7	77.74 ± 3.7	74.67 ± 3.4
NA core	15.3 ± 3.44	41.8 ± 4.8	42.8 ± 5.46	51.36 ± 5.4	69 ± 2.02	66.8 ± 1.0
NA shell (lateral)	48.8 ± 6.6	46.8 ± 5.5	4.2 ± 1.9	72.25 ± 4.3	77.9 ± 1.4	59.75 ± 20.5
NA shell (medial)	19.8 ± 1.6	45.1 ± 6.1	34.9 ± 5.9	41.15 ± 3.4	63.9 ± 6.8	73.9 ± 8.1

Table 1: Distribution of retrogradely traced neurons in the brainstem. Quantification of retrogradely labelled neurons in the PPNr, PPNc and LDT after the injection of tracers in different regions of the striatal complex: dorsal striatum (DS) lateral (n=3) and medial (n=3), NA core (n=4), and NA shell lateral (n=4) and medial (n=3). Data are expressed as the normalised cell count for the three regions sampled and the percentage of retrogradely traced neurons that were immunopositive for ChAT±SEM.

Axons collaterals of striatal-projecting neurons in the brainstem also innervate the dopaminergic midbrain and the thalamus

Cholinergic neurons of the brainstem are known to innervate DAergic nigrostriatal/mesolimbic regions and the intralaminar thalamic nuclei (see review: Martinez-Gonzales et al., 2011), and as such have the potential to modulate two of the most significant inputs to the striatal complex. To determine whether it is the same brainstem neurons that innervate the striatal complex that, via collaterals, also innervate midbrain and thalamic targets, we injected a viral vector associated with the WGA-Cre fusion protein in the NA core of wild-type rats (n=3). This produced transcellular trafficking of the WGA-Cre fusion protein that was retrogradely

transported to the cell body (Gradinaru et al., 2010; Xu and Südhof, 2013). In combination with the injection of a Cre-dependent vector in the LDT, it induced the expression of the YFP only in those brainstem neurons projecting to the NA that were retrogradely labelled with the WGA-Cre. YFP expressed by these neurons was distributed throughout their somato-dendritic and axonal arbors including their axon collaterals (**Fig. 16A**). These targets (NA core and LDT) were selected on the basis of our previous retrograde and anterograde results (**FIGs. 13, 15B**). We detected the presence of YFP-positive neurons in the LDT (**Fig. 16B**), of which the majority were immunopositive for ChAT, consistent with the other experimental approaches. In addition, we detected the presence of YFP-positive axons in the NA core, spread over the region in which the vector carrying the WGA-Cre sequence was injected. Neurons of the LDT gave rise to abundant collaterals that were detected in the VTA (**Fig. 16C, D**) and the midline thalamus (**Fig. 16 E**), suggesting that LDT neurons that innervate the NA core also innervate the VTA and thalamus. This combined approach was not possible in the PPNr because of the proximity of DA neurons in the substantia nigra pars compacta (SNc), which project densely to the DS. Animals in which one of the two targets was missed did not produce any detectable labelling of the cell bodies or axons. The time between injections and perfusion was shortened compared with previous studies (see Materials and Methods, Gradinaru et al., 2010; Xu and Südhof, 2013) to minimize the possibility of transynaptic retrograde labelling of second-order neurons at the level of the thalamus and the VTA.

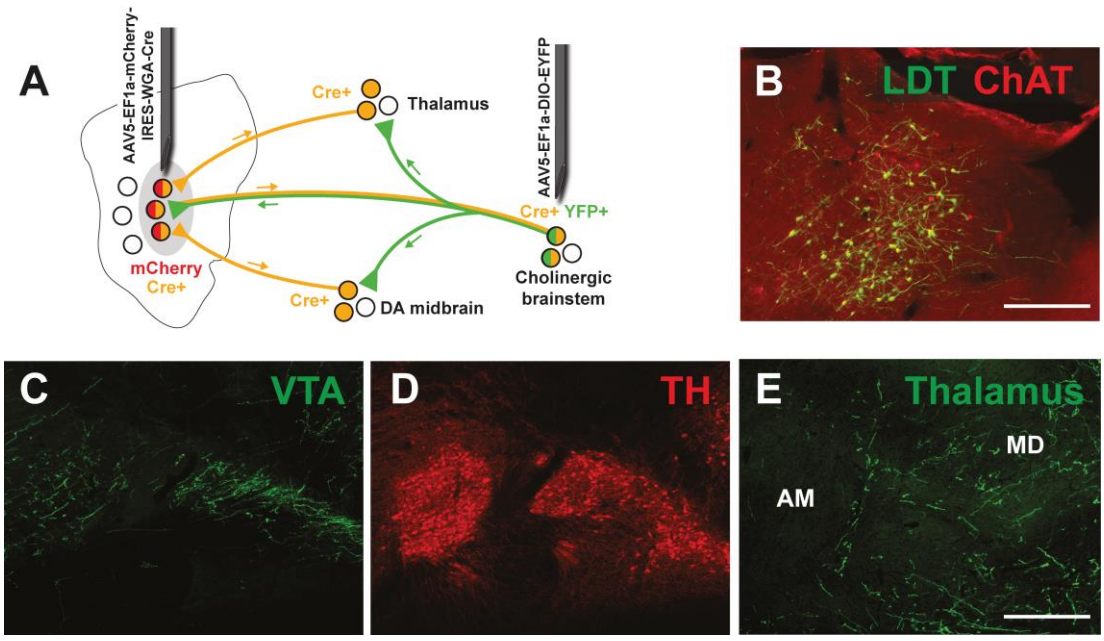

Figure 16: labelling of axon collaterals from striatal-projecting brainstem neurons. A, Using a combination of two viral vectors, one of which possesses transneuronal retrograde capabilities, brainstem neurons that innervate striatal targets were selectively labelled, including their axon collaterals. **B**, Neurons in the LDT that retrogradely transported WGA–Cre from the NA core expressed the YFP after the local injection of a Cre-dependent virus. **C–E**, Axon collaterals expressing YFP were detected in the VTA (**C**), here defined by the border of the TH staining (**D**), and in the mediodorsal (MD) but not in the anteromedial (AM) thalamus (**E**), also in agreement with the study by Holmstrand and Sesack (2011). Scale bars: **B**, 250µm; (in **E**) **C–E**, 500µm.

However, because we cannot rule out this possibility entirely, we performed additional experiments using double retrograde tracer injections into NA core and either VTA (n=2) or the mediodorsal thalamus (n=2). We observed a large number of neurons retrogradely labelled from all three structures, and the majority of them were immunopositive for ChAT (68% for NA, 60% for VTA, and 77% for thalamus). In neurons projecting to the NA, we also detected the presence of the tracers transported from the VTA (in 16,47% of neurons, corresponding to 58 double-labelled neurons of 325 retrogradely labelled neurons from the NA, **Fig. 16F**) and the thalamus (7,16% of neurons, corresponding to 21 double-labelled neurons of 293 retrogradely labelled

neurons from the NA, (**Fig. 16G**), thus indicating the existence of axon collaterals to those targets and validating the results obtained with the WGA-Cre fusion protein. Together, our results suggest that neurons in LDT are able to provide both direct and indirect innervation (via VTA or thalamus) of the NA.

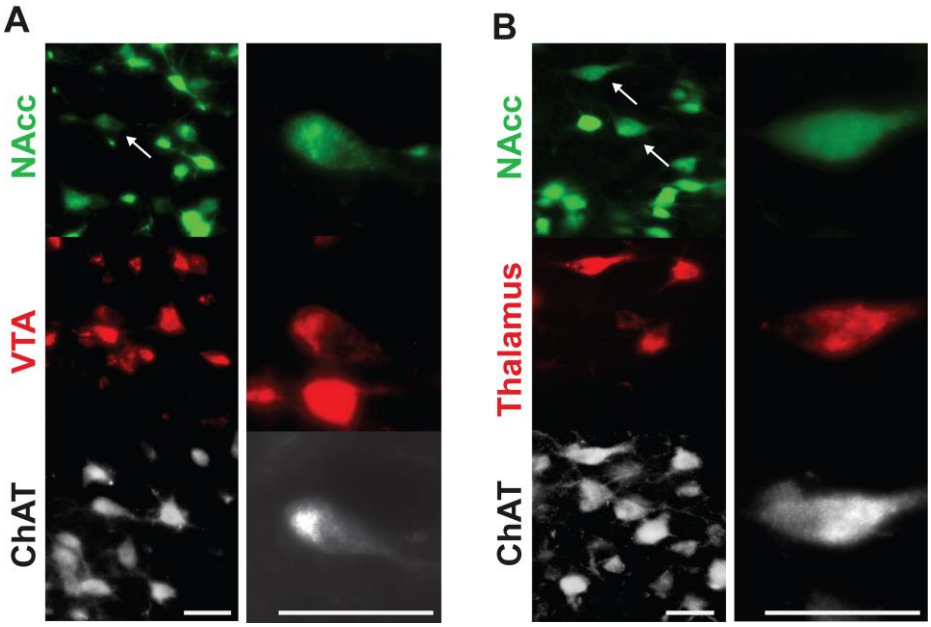

Figure 17: Double-tracer injections. A, **B**, Fluorescent images showing triple immuno labelling for ChAT, CTb, and FG in the LDT after injections in the NA (FG) and the VTA (**A**; CTb) or the thalamus (**B**; CTb). Examples of neurons with triple labelling in the left panels of **A** and **B** (arrows) are shown at higher magnification in the right panels. Scale bars: **A**, **B**, 50µm.

Discussion

The cholinergic complex is a widespread ensemble of nuclei that send inputs to almost the entire CNS. The main findings of the present study are as follows. First, cortex and thalamus, two of the main structures of the BG, received cholinergic inputs from the medial basal forebrain (MS, VDB and HDB) and the brainstem (PPN and LDT). As previously reported, the brainstem is the only source of ACh in the midbrain and the subthalamic nucleus (STN) (Mena-Segovia et al., 2008). Second, brainstem cholinergic neurons located in the PPN and the LDT provide a major innervation of the DS and NA that mainly avoid striosomes. Projections to the Str were not observed in the animals injected in the other cholinergic nuclei, suggesting that the PPN and the LDT were the exclusive external source of ACh in the Str and the Na. Third, the projection is topographically organised such as the rostral PPNr preferentially innervates the DLS, the PPNc equally innervates the DS and the NA, and the LDT preferentially innervates the DMS and NA. Finally, accumbens-projecting cholinergic neurons in the LDT give rise to collaterals that innervate the DA midbrain and the thalamus, suggesting both a direct and indirect influence of the cholinergic brainstem on striatal regions. Our findings thus demonstrate that, in addition to cholinergic interneurons, cholinergic innervation of the Str is derived from neurons in the PPN and the LDT. The differential activity of striatal cholinergic interneurons and cholinergic neurons of the brainstem during reward-related paradigms suggest that the two cholinergic systems play different but complementary roles in the processing of information in the basal ganglia.

Technical considerations

The use of a Cre recombinase rat line together with AAV injections allowed us to

target anatomically-restricted cholinergic groups and map their projections. However, virus injections must reach a representative proportion of cholinergic neurons in each structure can potentially diffuse several hundred μm (Dautan et al., 2014), which becomes problematic for those cholinergic groups that form a continuum (e.g. Ch1, Ch2, Ch3). To overcome this difficulty, preventing the spread of the transduction over contiguous cholinergic groups and restricting the labelling to the defined borders of each structure, the volume of the injections was adjusted for each structure based on our preliminary assessments. Because our data are not used to evaluate the quantitative expression of axons but they are rather based on the qualitative expression, the variations on the virus injection volumes are unlikely to affect the conclusions of this study.

The expression of eYFP can give rise to a low signal to noise ratio in thin axon shafts and small terminals, some of which can be photobleached rapidly and thus become difficult to detect during online analysis. In order to circumvent the possibility of false-negatives due to these factors, we enhanced the YFP signal by immunostaining and performed the analysis offline, thus minimizing the exposure of the tissue to the fluorescent light. Additional validation was obtained by the use of conventional retrograde tracers in wild-type rats. Our results showed that no retrogradely labelled cholinergic cell bodies were observed in any of the cholinergic cell groups whose axons were absent from the striatal complex (i.e. Ch1-Ch4, Ch7 and Ch8), and in contrast, retrogradely labelled cholinergic cell bodies were detected in the Ch5 and Ch6 regions, whose axons spread over the Str and NA.

Cholinergic transmission in the striatum

The effects of ACh in the Str are varied and complex (Sugita et al., 1991; Koós and Tepper, 2002; Goldberg et al., 2012). Muscarinic and nicotinic receptors are present at both the pre- and postsynaptic levels, and thus are able to regulate the activity of cortical, thalamic and DAergic terminals, as well as the activity of striatal projection neurons and different interneurons (Calabresi et al., 1999; Volpicelli-Daley et al., 2003; Bonsi et al., 2011; see review by Lim et al., 2014). Classically, cholinergic interneurons have been considered the only source of ACh in the Str. They produce a vast innervation over the entire striatal complex (Bolam et al., 1984; Phelps et al., 1985; Phelps and Vaughn, 1986) and provide a steady tone of ACh release regulated by an intrinsic tonic level of firing (Bennet and Wilson, 1999; Bennett et al., 2000; Goldberg and Reynolds, 2011). During conditioned reward behaviour, a synchronous pause in the tonic firing of putative cholinergic interneurons has been proposed to signal the probability of obtaining a behavioural outcome (Blazquez et al., 2002) and the outcome delivery (Joshua et al., 2008; for review see Schultz and Reynolds, 2013), a mechanism that has been shown to be dependent on direct thalamic innervation (Ding et al., 2010).

The existence of an additional source of ACh, provided by the brainstem or the basal forebrain, may underlie the functional segregation of ACh receptors. Dissecting the entire cholinergic systems thus becomes critical to fully understand the implications of cholinergic signaling in the Str. Furthermore, because the cholinergic brainstem provides collaterals to the thalamus and the DAergic midbrain, two of the most important afferent systems to the Str, it is likely that their influence on striatal circuits will be highly correlated with the thalamic and midbrain inputs. Such connectivity thus situates the PPN/LDT as an important station for striatal

computations.

The lack of evidence of additional sources of ACh to the Str arising from the basal forebrain, the parabigeminal nucleus or the medial habenula, while not surprising, does emphasize the key role of the cholinergic brainstem for modulating striatal activity and basal ganglia function. Furthermore, because of the involvement of the PPN/LDT in neuropsychiatric disorders that predominantly affect the basal ganglia, and whose pathophysiology is associated with abnormal cholinergic transmission, such as PD (Hirsch et al., 1987; Hall et al., 2014), Huntington's disease (Picconi et al., 2006; Smith et al., 2006), progressive supranuclear palsy (Warren et al., 2005), and dystonia (Sciamanna et al., 2012), the evidence of a direct projection to the Str opens new avenues for the interpretation of these abnormal processes and the challenges they pose.

Brainstem cholinergic projections to the striatum

In contrast to the firing of cholinergic interneurons of the Str, cholinergic neurons in the brainstem follow different dynamics: they typically show phasic increases in their relatively slow firing rate associated with brain state transition (Mena-Segovia et al., 2008), the latter being caused by increased cholinergic transmission in the thalamus (Steriade, 1996; Kezunovic et al., 2012). Furthermore, neurons in the PPN are phasically activated in response to salient stimuli (Pan and Hyland, 2005) and to cues that predict changes in the magnitude of reward outcome (Okada et al., 2009). Thus it is possible that cholinergic terminals in the Str that are derived from the brainstem lead to phasic increases in ACh release associated with different behavioural contexts. Therefore, our data, together with previous data, suggest a dual mode of cholinergic transmission in the Str: the release of ACh by

cholinergic interneurons is tonic and interrupted by behaviourally relevant events, whereas the release of ACh by cholinergic terminals from the brainstem would be phasic and increased during salient events. Additional support for the dual innervation comes from the ultrastructural analysis. Our data revealed that the majority of cholinergic synapses from the brainstem are asymmetric. Previous analyses of ChAT-immunopositive synapses in the striatal complex (Wainer et al., 1984; Phelps et al., 1985; Phelps and Vaughn, 1986; Pickel and Chan, 1990) revealed both asymmetrical and symmetrical synapses, the earlier accounting for ~35% in the Str. This coincides with our data showing that two-thirds of synapses originating from cholinergic interneurons are symmetrical. Together with the fact that cholinergic synapses in other target of the brainstem are asymmetric (Bolam et al., 1991; Bevan and Bolam, 1995; Omelchecko and Sesack, 2006), this suggests that a significant proportion of cholinergic synapses in the Str may arise in the brainstem. Future experiments are necessary to address the impact of the direct brainstem cholinergic transmission and information processing on striatal circuits and the effect on their convergent influence through DA and thalamic afferents.

Non-cholinergic projections from the basal forebrain to the striatal complex

As described before, our data suggest evidence for the direct projections of non-cholinergic neurons to the accumbens arising from the PPN, LDT and nucleus basalis of Meynert. Those Ch4 neurons were small sized and sparse, suggesting a GABAergic nature as previously reported (Zaborsky and Cullinan, 1992). This may reflect a role of GABAergic neurons of the nucleus basalis of Meynert in accumbens functions. Similar observation have been made for non-cholinergic neurons of the PPN/LDT that project to the Str and the Na and represent a small proportion of the

brainstem-Str pathway. Based on the proportions observed in neurochemical studies, those neurons are more likely to be glutamatergic (Wang and Morales, 2009).

Future tracing experiments could use *in situ* hybridization to clarify the nature of these non-ACh neurons projecting to the Str/NA and their role.

Functional organisation of the cholinergic brainstem

Cholinergic neurons in the PPN and the LDT display functional specializations that are related to their connectivity and their positions within different neuronal systems. The PPN is associated with arousal/attentional functions mainly through its projections to the intralaminar thalamic nuclei (Steriade et al., 1988; Parent and Descarries, 2008) and with motor function through projections to structures embedded in different motor circuits (e.g. SNc, subthalamic nucleus, gigantocellular nucleus; Bolam et al., 1991; Kita and Kita, 2011; Martinez-Gonzales et al., 2013). In contrast, the LDT is associated with limbic functions underscored by its connections with limbic structures, including the VTA (Omelchenko and Sesack, 2005), midline thalamic nuclei, and infralimbic and cingulate cortex (Cornwall et al., 1990), and its involvement in motivational behaviour (Lammel et al., 2012). Furthermore, previous studies, as well as this one, observed that PPN and LDT cholinergic neurons selectively target distinct subtypes of DA neurons in the VTA and induce different modes of discharge in their postsynaptic targets (Floresco et al., 2003; Lodge and Grace, 2006; Grace and Onn, 1989). The findings in the present study are in line with such a functional dichotomy of brainstem cholinergic neurons: PPN cholinergic neurons project preferentially to the DS, which is involved in sensorimotor circuits and integrates inputs from the intralaminar thalamus and the SNc, whereas LDT cholinergic neurons preferentially project to the medial Str and NA, both of which

are involved in limbic functions and integrate inputs from the midline thalamus and the VTA. Our results suggest that the motor/limbic specialization observed in dorsal/ventral striatal system is reflected at the level of brainstem cholinergic neurons.

Non-cholinergic projections from the PPN and LDT to the striatal complex

The PPN and LDT are heterogeneous structures composed of glutamatergic and GABAergic neurons (Mena-Segovia et al., 2009; Wang and Morales, 2009), in addition to the cholinergic neurons. These non-cholinergic populations have been shown to project to many of the targets of the cholinergic neurons, including subthalamic nucleus (Bevan and Bolam, 1995; Kita and Kita, 2011; Martinez-Gonzales et al., 2013), the substantia nigra (Futami et al., 1995; Charara et al., 1996), the thalamus (Barroso-Chinea et al., 2011), and gigantocellular nucleus (Martinez-Gonzales et al., 2013), among others. We observed that a proposition of striatal-projecting PPN and LDT neurons were immunonegative for ChAT, suggesting that they may be glutamatergic or GABAergic. This opens the possibility that other brainstem neurotransmitters with distinct dynamics may influence the activity of striatal microcircuits. Furthermore, although the presence of glutamate in cholinergic terminals is only found in <5% of cholinergic neurons (Wang and Morales, 2009), additional studies should address the possibility of cotransmission from brainstem afferents and potential heterogeneous effects on postsynaptic targets.

Conclusions

Following the recent report of a possible extrinsic source of ACh to the Str (Mesulam, 2004; Smith and Parent, 1986) showing positive neurons in cholinergic structures following retrogrades tracer injections in the striatum we evaluated here the possibility that other cholinergic structures across the brain may contribute to the cholinergic transmission in the Str. We targeted all 8 cholinergic groups to induce anterograde conditional labelling in cholinergic axons and observed that, besides the PPN and LDT (Ch5 and Ch6, respectively); no other cholinergic group gave rise to ChAT axons in the Str and NA. A comprehensive mapping of the axon distribution of these structures shows the presence of labelled axons in all of their known targets, thus corroborating the accuracy and specificity of our cholinergic transductions. Furthermore, analysis of retrogradely labelled neurons from the Str and NA shows labelling of cholinergic neurons exclusively in the PPN and LDT. Our results thus confirm that no other sources of ACh for the Str exist besides the local interneurons and the cholinergic brainstem. Our results further suggest that the influence of the cholinergic brainstem on the striatal complex involves direct and indirect projections arising from the same set of neurons, the latter mediated by DA and thalamic neurons. The convergent activity on striatal microcircuits is likely to have varied and complex effects on striatal information processing. These findings have important implications for understanding the role of the brainstem in striatal modulation.

Chapter 3: Dopaminergic and non-dopaminergic neurons of the ventral tegmental area are differentially modulated by brainstem cholinergic pathways.

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DD, AS, MV, TG, PB and JMS designed the experiments, analysed the data and wrote the manuscript. DD and AS performed the surgeries and experiments (28 neurons reported here were recorded by AS, behaviour, elepctrophysiology and anatomy experiments were performed by DD). IHO performed the electron microscopy experiments. IW and KD provided the animals.

Abstract

DAergic and non-DAergic neurons of the VTA are causally related to reward oriented behaviour and reinforcement, and this role critically depends on the modulation by their afferent connections. Cholinergic neurons innervate the VTA and arise from two functionally distinct brainstem structures: the PPN and the LDT, involved in arousal and limbic functions, respectively. Cholinergic transmission is critical for determining the responsiveness and firing mode of DAergic and non-DAergic neurons, but the impact of such functional specialization on the activity of those neurons in the midbrain is likely to be different. To test this directly, we mapped the distribution of PPN and LDT cholinergic axons in the VTA and optogenetically manipulated them to dissect their specific contributions to the activity of individual and confirmed DAergic and non-DAergic neurons *in vivo*. Optogenetic activation of cholinergic axons from both PPN and LDT led to increased firing frequency of DAergic neurons. PPN stimulation appeared to switch the firing mode of DAergic neurons to bursting, whereas LDT stimulation increased the number of spikes within a burst without altering their firing mode. Furthermore, LDT cholinergic axons selectively modulate DAergic neurons that are excited by aversive stimulation while PPN targeted DAergic neurons inhibited by aversive stimulation. To determine the behavioural significance of PPN and LDT cholinergic input to VTA we carried out behavioural experiments in which we substituted optogenetic stimulation of these projections for sugar rewards in an extinction of lever pressing for food paradigm. We also assessed the effect of activating these projections on locomotor activity. We found that optogenetic stimulation of PPN and LDT fibres in VTA increased lever pressing during extinction and also affected locomotion in the open field. LDT stimulation increased overall activity, whereas PPN stimulation increased locomotion transiently during stimulation periods. These results show fundamental differences in the modulation of DAergic neurons by two distinct cholinergic structures. They also demonstrate the behavioural significance of these projections for reward-related behaviour.

Key words: Dopamine, ventral tegmental area, pedunculopontine, laterodorsal tegmental are, locomotion, electrophysiology

Introduction

DAergic neurons of the ventral tegmental area (VTA) are implicated in goal-directed behaviours and reinforcement learning (Wise, 2004). They change their discharge mode from tonic to phasic in response to sensory events that predict a reward outcome (Montague et al., 2004). This phasic activation produces synchronous burst (Joshua et al., 2009) and encodes a prediction error signal which is crucial for reinforcement learning (Schultz et al., 1997; Schultz, 2013). Thus, the variation in the firing mode of DA neurons, triggered by excitatory drive, is critical for the expression of reward oriented behaviour (Tsai et al., 2009).

Half of the GABAergic neurons in the VTA project to the NA (Brown et al., 2010; Van Bockstaele and Pickel, 1995), prefrontal cortex (Carr and Sesack, 2000) or amygdala (Fallon et al., 1984), while the other half is considered as interneurons and making synaptic contact with DA neurons (Olmelchenko and Sesack, 2009; Dobi et al., 2010; Tan et al., 2012). GABAergic VTA neurons are implicated in place preference (Tan et al., 2012) and reinforcement learning (Van Zessen et al., 2012). *In vivo* recording of GABAergic neurons of the VTA showed an increase of their firing activity during reward expectation; which may help to compute the reward prediction error (Cohen et al., 2012).

The VTA receives excitatory inputs from several regions including the prefrontal cortex, amygdala, lateral hypothalamus, subthalamic nucleus and mesopontine tegmentum in the brainstem (Sesack and Grace, 2009; Beier et al., 2015). While all of these afferent systems provide a glutamatergic input, the mesopontine tegmentum, composed of the PPN and LDT, also provides the only cholinergic innervation of DA neurons (Mena-Segovia et al., 2008). Previous reports

have shown the involvement of glutamatergic mechanisms following PPN stimulation that lead to increase in the number of VTA neurons bursting (Floresco et al., 2003; Lokwan et al., 1999). Similarly, activating a predominantly glutamatergic projection from the LDT produces burst firing in DA neurons (Lodge and Grace, 2006) and elicits conditioned real-time place preference in behaving rodents (Lammel et al., 2012). However, less clear is the influence of cholinergic afferents over the activity of DA and non-DA neurons *in vivo.* Nicotinic and muscarinic ACh receptors are widely expressed in the VTA (Clarke and Pert, 1985; Dani and Bertrand, 2007) and their activation in brain slices causes depolarization (Calabressi et al., 1989; Lacey et al., 1990) and burst firing (Zhang et al., 2005) in DA neurons. While *in vivo,* the response of DAergic neurons of the VTA to nicotine administration seems to be more complex (Marinelli, 2007; Funk et al., 2007; Marinelli and White, 2000; Eddine et al., 2015). Previous studies suggest that activation of GABAergic neurons by nicotine may also modulate DAergic neurons activity (Tolu et al., 2012). Furthermore, behavioural experiments have consistently shown a prominent role for VTA ACh receptors in goal-directed behaviour and addiction (Yeomans and Baptista, 1997), presumably through the activation of DA neurons (Miller and Blaha, 2005). It is thus likely that cholinergic afferents, derived from the mesopontine tegmentum, play a role in tuning the activity of DA neurons in the VTA.

The PPN and the LDT share similar connections and neurochemistry, but differ in the functional networks to which they contribute. Whereas PPN is connected to sensorimotor and associative structures (Semba and Fibiger, 1992), as well as those involved in the regulation of arousal (Steriade, 1996), the LDT is connected to limbic systems (Cornwall et al., 1990). Because DAergic neurons are heterogeneous in terms of their firing properties, connectivity and functionality (Lammel et al., 2008;

Matsumoto and Hikosaka, 2009; Brischoux et al., 2009; Roeper, 2013; Ikemoto, 2007; Bromberg-Martin et al., 2010), it is likely that brainstem cholinergic pathways produce different effects within the VTA and differentially affect subpopulation of DA neurons.

The activity of DAergic neurons in the VTA has been reliably associated with reward prediction stimuli (Ljunberg et al., 1992; Mirenowicz and Schultz, 1996) and locomotion (Perez et al., 2008). Also, it has been demonstrated that cholinergic agonist injected in the VTA, or cholinergic specific lesions of the PPN and/or the LDT caused locomotor (Laviolette et al., 2000; Dobbs and Cunningham, 2014; Chintoh et al., 2003) and reward- prediction impairment (Wilson et al., 2009; Alderson et al., 2001; Ikemoto et al., 2003).

VTA is one of the structures showing the highest density of cholinergic receptors and also the largest variability of receptors. PPN and LDT are the only cholinergic inputs to the VTA. It is believed that cholinergic release at the level of the VTA will facilitate glutamatergic and GABAergic release on DA neurons. However, some evidence suggests that PPN and LDT cholinergic projections could have separate functions on the electrophysiology and behaviour functions of DA neurons (refs for this paragraph). In order to address these issues, we characterised the innervation of the VTA by PPN and LDT cholinergic neurons by retrograde and anterograde labelling. Using optogenetic approach in ChAT::cre^{+} rats combined with *in vivo* juxtacellular single cell recording and labelling, microiontophoretic drug delivery, and retrograde labelling, we characterised the effect of the activation of PPN or LDT cholinergic afferents on the spontaneous activity of identified DA and non-DA neurons in the VTA. Further, we identified the targets of a subset of DA

neurons and correlated their responses to cholinergic modulation with the functional circuit in which they participate. Finally, we tested the extent to which PPN and LDT cholinergic projections in the VTA support reward-related behaviour using optogenetic self-stimulation during extinction of lever pressing for food. We also assessed the role of these projections in locomotor activity.

Materials and methods

Animals

Male adult (250-450g) Long Evans (LE) wild-type and ChAT::cre^{+} (Witten et al., 2011) rats were used for all experiments. Rats were maintained on a 12:12 light cycle (lights on 07:00) and had *ad libitum* access to water and food. All procedures were performed in accordance with the Society for Neuroscience policy on the use of animals in neuroscience and the animals (Scientific Procedures) Act, 1986 (UK), under the authority of Project Licence approved by the Home Office and the local ethical committee of the University of Oxford and the University of Leicester for the behaviour.

Stereotaxic Injections

All stereotaxic injections were performed during deep isofluorane anesthesia (2-4% in O2; Isoflo, Schering-Plough, Welwyn Garden City, UK). For the anatomical studies, the rats (n = 4, LE wild-type) were injected with cholera toxin b (CTb 2.5% in water, 100 nl over 10 min; cat. number C9903, Sigma-Aldrich) in the rostral VTA (from bregma in mm, AP: - 5.2; ML: + 0.8; DV: - 7.5 ventral of the dura) and red retrobeads (RB-R, 150 nl over 10 min; Lumafluor, USA) in the caudal VTA (from

bregma in mm, AP: - 5.8; ML: + 1.1; DV: - 7.7 ventral of the dura) to retrogradely label PPN and LDT neurons that innervate the VTA. Another group of rats (n = 6; LE ChAT::cre+) were injected with adeno-associated virus serotypes 2 (AAV2) and 5 (AAV5) carrying fusion genes for channelrhodopsin 2 (ChR2) and yellow fluorescent protein (YFP) or mCherry (University of North Carolina Gene Therapy Centre Virus Vector Core). Thus, we injected either AAV5-EF1a-DIO-YFP in the PPN (500 nl over 10 min; from bregma in mm, AP: - 7.8; ML: + 1.8; DV: - 6.5 ventral of the dura) and AAV2-EF1a-DIO-mCherry in the LDT (300 nl over 10 min; from bregma in mm, AP: - 8.5; ML: + 0.9; DV: - 6.0 ventral of the dura), or AAV5-EF1a-DIO-YFP in the LDT (n = 3), to quantify the transduction rate of cholinergic neurons (YFP in PPN and mCherry in LDT) and anterogradely label the cholinergic axons innervating the VTA (YFP in LDT). The PPN injections led to a roughly circular transduction area of about 1 mm diameter, and LDT injections about 0.5 mm. In each experiment the transduced area was verified to be largely restricted to the PPN or LDT.

For the rats used for the electrophysiological studies, we first injected AAV2-EF1a-DIO-hChR2-YFP into the PPN or LDT of ChAT::cre^{+} rats to transduce cholinergic neurons, as above. Two weeks later, we injected fluorogold (FG; 2.0%, 300 nl over 10 min; Fluorochrome, LLC) or CTb (2.5%, 500 nl over 10 min; Sigma-Aldrich) in the lateral shell of the NA (from bregma in mm, AP: + 1.2; ML: + 2.7; DV: - 6.8 ventral of the dura); the tracers were alternated between animals. For control experiments, ChAT::cre^{+} rats were injected in PPN and LDT in the same coordinates described above using AAV2-EF1a-DIO-mCherry or AAV5-EF1a-DIO-eYFP (i.e., without channelrhodopsin-2). All injections were made using designated 1-µl syringes (SGE Analytical Science) for each vector at a rate of 50 nl/min and a post-injection diffusion time of 5 min. 10-15 days later, juxtacellular recordings/ labelling

of neurons were performed in the VTA.

Juxtacellular recordings

Anesthesia was induced with 4% v/v isofluorane (Schering-Plough) in O^2, and maintained by an injection of urethane (1.3 g/kg, i.p.; ethyl carbamate; Sigma, Poole, UK). Supplemental doses of ketamine (35 mg/kg, i.p.; Ketaset, Willows Francis, Crawley, UK) and xylazine (6 mg/kg, i.p.; Rompun, Bayer, Germany) were administered as required throughout the experiment. Body temperature was maintained at 38°C using a thermistor-controlled heating pad. After local skin anesthesia by a subcutaneous injection of Marcaine (0.25%), the animals were placed in a stereotaxic frame (Kopf). A cutaneous incision was made to expose the skull. Then, craniotomies were made for the electrocorticogram (ECoG; bilaterally, from bregma, AP: + 3.0 mm; ML: ± 2.5 mm; corresponding to the somatic sensorimotor cortex) and its reference (above the right cerebellum), and the optic fibre cannula (from bregma in mm, AP: + 8.8; ML: + 0.8: DV: - 6.2 ventral of the dura, implanted at a 20° angle; aimed at the site where cholinergic axon bundles that originate in the brainstem penetrate the caudal VTA, as evidenced by our anatomical findings). A small craniotomy was made above the VTA (from bregma in mm, AP: -4.0 to -5.3; ML: + 0.4 to + 1.4, to be used at a 5° angle) and the dura mater was gently removed to allow the passage of a glass pipette for single cell recordings; the exposed brain surface was kept moist with sterile saline (0.9% NaCl) throughout the experiment. A supplementary ground for the single cell electrode was placed subcutaneously at the back of the neck.

The ECoG was recorded using 1 mm diameter stainless-steel screws and referenced to a steel screw above the cerebellum. ECoG signals were band-pass filtered at 0.3-

1000 Hz (-3 dB limits), amplified 2000-fold (DPA-2FS filter/amplifier; Scientifica, Harpenden, UK) and digitized online at 2.5 kHz. The ECoG was used to monitor the depth of anesthesia. The anaesthetics used typically produce slow wave activity alternating with episodes of spontaneous cortical activation (Mena-Segovia et al., 2008). Extracellular recordings of action potentials of individual VTA neurons were made using glass micropipettes (15-25 MΩ, measured in the cortex; tip diameter ~1.5 μm) filled with 1.5% w/v neurobiotin (Vector Laboratories Ltd., Peterborough, UK) in 0.5 M NaCl. Signals from the glass micropipettes were band-pass filtered at 0.3-5000 Hz (NL125: Digitimer), amplified 10-fold through the active bridge circuitry of an Axoprobe-1A amplifier (Molecular Devices Corp., Sunnyvale, CA), AC-coupled and amplified a further 100-fold (NL-106 AC-DC Amp: Digitimer Ltd., Welwyn Garden City, UK), and digitized online at 17.5 kHz. Data were acquired and stored using an analog-to-digital converter (Power 1401; Cambridge Electronic Design, Cambridge, UK) connected to a PC running Spike2 (ver. 7; Cambridge Electronic Design).

The whole of the VTA was scanned with the glass micropipettes for spontaneously firing neurons. When action potentials were detected, a minimum of 5 min of basal firings were recorded to establish a mean baseline firing rate and spontaneous discharge pattern. Subsequently, sensory stimulation and induced global activation were elicited by a pinch of the hind paw delivering a standard pressure of 183 g/mm^2 (“aversive stimuli”). Once the firing rate returned to the baseline (approximately 5 min), the activity of putative VTA neurons was recorded during optical stimulation of brainstem cholinergic afferents using different protocols of light stimulation as follows. A multimode fibre optic patch cable (300 μm diameter, 2 m long; M56L01, ThorLabs, Newton, NJ, USA) was connected to one end of the implanted ceramic

cannula (300 μm diameter, 10 mm long; CFMC13L10, ThorLabs) using a mating sleeve, and the other end was connected to a class IIIb blue laser (473 nm; LRS-0473-PFM-00100-05, LaserGlow Technologies, Canada). At the beginning of each experiment, the output power of the laser at the cannula end was measured and adjusted to deliver ~20 mW. The laser in turn was driven by transistor-transistor logic (TTL) pulses originating in the digital output of the Power 1401 (Cambridge Electronic Design) and fed back to its digital input to record the stimulation events. The stimulation protocol was set as follows: pulse durations 50 ms and frequency of 10 Hz. Train pulses were repeated twice at least, with a minimum interval of 30 s between each. At the end of the recording, a microiontophoretic current was applied to the neuron (1–10 nA positive current, 200 ms duration, 50% duty cycle) to label it with the neurobiotin (Pinault, 1996). To achieve reliable labelling, the firing of the neurons had to be robustly modulated by the current injection for a minimum of 1 min. The neurobiotin was allowed to transport along the neuronal processes for 2-4 h. To ensure discrimination between neurons during the histological analysis (see below), a maximum of 4 were recorded per animal with a minimum distance from each other of 400 μm in all axes. Following the diffusion time, the animals were given a lethal dose of ketamine (150 mg/kg) and intracardially perfused with 0.05 M phosphate buffered saline (PBS), pH 7.4, followed by 300 ml of 4% w/v paraformaldehyde in phosphate buffer (0.1 M pH 7.4). Brains were stored in PBS at 4°C until sectioning.

Microiontophoresis

Custom-made double-barrel pipettes (Dondzillo et al., 2013) were prepared by combining a single glass capillary for juxtacellular recording/ labelling (2-μm tip diameter, resistance 15-20 MΩ) and an additional glass capillary at a ± 20˚ angle for

drugs infusion (10-µm tip diameter). Both pipettes tips were glued with cyanoacrylate first at an average distance of 100 µm, and reinforced using epoxy and dental cement. During the experiments, a baseline recording was obtained, during which VTA neurons were stimulated using the parameters described above (80 pulses, 10Hz, 50ms duration). Following a recovery period, a cholinergic antagonist cocktail was applied by iontophoresis (80 nA, 50 ms injection, 1 Hz), consisting of the following: methyllycaconitine (MLA) 20 mM, dihydro-β-erythroidine (DhβE) 40 mM, atropine 40 mM and mecamylamine 100 µM. All drugs were dissolved in 0.9% saline solution. The injection was controlled by adjusting the current and the resistance of the drugs injection pipettes. Current injection was counterbalanced using the recording electrode. Neurons were stimulated with the laser during the drug administration. A minimum of 3 laser trials were delivered with a minimum of 60 seconds between each. After 300 seconds of drugs iontophoresis, the injection current was stopped and a washing period of 1 minute was allowed. Then the laser stimulation was repeated. At the end of the recording and stimulation trials, neurons were labelled with neurobiotin as previously described. Only immunohistochemically-identified neurons were used.

Electrical stimulation

In a subset of animals, a bipolar concentric electrode was implanted into the PPN (tip diameters 100 µm and an impedance of ~10 kΩ). Following the baseline recording of VTA neurons, electrical stimulation (0.5 ms duration, 0.5 to 0.8 mA amplitude) was delivered at 0.5 Hz using a constant-current isolator (A360D, World Precision Instruments). Neurons were subsequently labelled as previously described. Only immunohistochemically-identified neurons were used.

Behaviour

Surgery. Two weeks following virus (AAV2-EF1a-DIO-ChR2-eYFP) injection in ChAT::cre^{+} (LDT n=10, PPN n=12) and WT (n=12) animals, a second surgery was performed to implant an optic fibre above the VTA. Animals were anesthetized and their skull exposed. Six stainless-steel anchoring screws (Morris Co., Southbridge, Massachusetts, USA, part number OX1/8 flat) were fixed onto the skull. A flat-cut fibre stub (200µm diameter multimode, 0.49NA, 8mm long, Thorlabs) connected to a SMA-905 connector was gently lowered 200µm above the right VTA (AP: -6.5mm, ML: +0.9mm, DV: 6.8 mm from the brain surface, relative to the bregma) and kept in position with light curing dental cement (Flowable Composite, Henry Schein, Gillingham, UK). The connector was protected by a dust cap (CPAMM SMA905, Thorlabs, Munich, Germany) which was replaced if missing. Animals received 5 days of a non-steroidal anti-inflammatory analgesic (Carprieve, 5mg/kg; s.c.; Norbrook Laboratories Ltd., Corby, UK) and antibiotic (Baytril, 2.5%, 0.2ml/kg, s.c., Bayer, Leverkusen, Germany) treatment before behavioural testing. Animals were handled daily and were group-housed.

Apparatus. Initial lever training was conducted in 4 operant chambers (Med Associates Inc.), placed in sound-attenuated and ventilated wooden boxes. There was a recessed food magazine and a single operant lever located to one side of the magazine (right or left, randomly distributed). Box illumination was provided by a 2W incandescent light situated on the opposite wall. A magazine LED was located 3 cm above the magazine and illuminated for 8 s on each reward delivery. The house light and fan were turned on at the beginning of each session and turned off 5 min following the end of the training. For optogenetic stimulation, animals were tested in an identical operant box with the top removed to allow attachment of the optic fibre.

Dustless precision sugar pellets (45mg) from Bio-Serv (Frenchtown, NJ, product number: F0021) were used as rewards. Experimental events were controlled and recorded by Med-PC software.

Procedure. Before implantation, the power of the laser output at the tip of the optic fibre was tested. Any optic fibre unable to provide a minimum of 10mW was excluded. During all lever pressing experiments food was restricted to 2 hours daily, which was provided one hour after testing in order to maintain at least 85% of the starting body weight. After two days behavioural training, each animal was exposed, in the home, to sugar pellets. One day before initiation of training, animals were individually placed in operant boxes where 20 pellets were randomly delivered in the food magazine during a 20 min period in order to habituate animals to magazine and food delivery. This step was repeated until the animal ate the 20 pellets within the 20 min period.

For open field testing, animals were placed into a square (50cm×50cm×50cm) black Plexiglass open field box. Illumination was provided by a 60 W red light bulb and the box was thoroughly cleaned between animals. Motor activity was monitored during 30 min with the AnyMaze software (Stoelting, Ilinois, USA) using a high resolution camera (C615, Logitech, Taiwan, Japan) positioned above the box

Operant training. Animals were manually shaped to lever press in a continuous reinforcement (**CRF**) schedule where every single lever press was reinforced with a single sugar pellet (Gerdjikov et al., 2011). This was continued until rats completed 80 presses in a 30 min session for two consecutive days. Next, animals underwent 2 days of a variable interval 15 schedule (VI15, reinforcements follow the first press after an average of 15 s since the last reward, session duration:

30 min), followed by 2 days of VI30 (reinforcements follow the first press after an average of 30 s since the last reward). Following the second session of VI30, animals were transferred to the optogenetic stimulation chamber and connected to a dummy patch cable (Calu et al., 2013) for another VI30 sessions. These VI30-3 sessions were repeated until rats recovered at least 70% of the lever presses prior to attachment. This was followed by 4 extinction days during which reward delivery was replaced by optogenetic stimulation (8 s pulses, 10Hz, 50ms on/off) maintaining the VI30 schedule. For optogenetic stimulation, a blue laser was connected to a 1 meter reinforced optic fibre patch cord (200µm fibre, multimode, 0.48 NA, Thorlabs Inc.) with an intermediary single rotary joint (FRJ, Doric lenses, Quebec, Quebec) positioned 1 meter above the floor. Laser power was adjusted for each animal in order to deliver a power of 5 mW at the tip of the fibre (40 mW/mm^2, Light transmission calculator, Optogenetics Resource Centre, http://www.stanford.edu/group/dlab/cgi-bin/graph/chart.php). 5 min before each session, the fibre was plugged into the implant and remained in place for 5 min after completion of the session. To avoid overlapping two consecutive stimulations, the minimum reinforcement interval was set at 9s. For all sessions, timestamps of lever presses, magazine entries, reward deliveries or laser stimulations were recorded using the Med-PC software (Med Associates Inc.). Following extinction, animals were given free access to regular chow food pellet and were given 2 test-free days.

Open field. Next, open field activity was recorded for 3 consecutive days. Average velocity (m/sec), maximum velocity, total distance travelled and time immobile were recorded in 5 s bins. On each of the 3 days rats underwent either: **1) baseline recording (*nostim*)**, where the implant was connected to the optogenetic patch cord but no stimulation was delivered, or **2) vehicle injection + stimulation**

(*stim*), where they received saline injection (i.p., 0.3ml) 30 min prior to testing, and were given every two minutes intra VTA laser stimulation (13 stimulations total, 8 s pulses, 10Hz, 50ms on/off, controlled by 28V DC to TTL Adapter), or **3) drug injection + stimulation (*drug*),** where they received 30 min before testing a cholinergic cocktail (i.p., 0.3ml final volume; MLA: 6mg/kg; DHBE: 3mg/kg; atropine: 0.5mg/kg; mecamylamine: 1.0mg/kg in saline) and were given every two minutes intra VTA laser stimulation (parameters as above). Distance travelled was aligned with stimulation using recorded time of the first stimulation and normalised by the average of the distance travelled during the immediate 20 s prior to stimulations.

Behaviour data analysis. The α level was set at 0.05 for all analyses performed on behavioural data. Locomotion data were normally distributed. Data were analysed using one way ANOVAs and mixed-ANOVAs with Tukey post hoc tests. Data analyses were performed using SPSS (SPSS statistics software, IBM).

Immunohistochemistry and Image Processing

Sagittal sections of the right hemispheres were cut at 50 µm thickness using a vibratome (VT1000S, Leica). Sections at the approximate levels of the NA, PPN, LDT and VTA were collected in a 24-well plate. They were incubated in a blocking solution consisting of 10% normal donkey serum (NDS) in PBS containing 1% Triton X100 for a minimum of 1 h. The sections containing the retrograde tracers injected in the VTA were first processed to identify the injection sites. Only those cases where the injections were contained within the borders of the rostral or caudal VTA were included in the study (n = 4). Sections containing the PPN and LDT at three different mediolateral levels (two of which contained both nuclei) were

incubated in an antibody against ChAT (raised in goat; 1:500 dilution in 1% NDS, 0.03% Triton X-100 in PBS, Millipore) and an antibody against CTb (raised in mouse; 1:1000 dilution in 1% NDS, 0.03% Triton X-100 in PBS, Abcam ab35988) followed by several washes in PBS and incubation in a CY5-conjugated donkey anti-goat antibody (1:1000, Jackson Immunoresearch Inc.) and an Alexa488-conjugated donkey anti-mouse antibody (1:1000 dilution, Jackson Immunoresearch). Neurons containing RB-R did not require any further processing. The sections were mounted on slides in VectaShield and examined under a fluorescent microscope (ImagerM2, Zeiss, Carl Zeiss AG, Germany) using the following filters (nm): 504 for CTb-Alexa488, 560 for RB-R and 650 for ChAT-CY5. Multi-channel stacks of images were taken in the Z plane using a digital camera (Hamamatsu ORCA-ER digital camera, Hamamatsu Photonics K.K) in combination with the acquisition software, Axiovision 4.8.1 (Carl Zeiss AG, Germany). The brightness and contrast of the images were subsequently adjusted in Photoshop (Adobe Systems). The distribution of labelled neurons was digitized offline using StereoInvestigator (Micro Bright Field, MBF Biosciences) and each neuron was assigned a category depending on the markers expressed. The number of neurons positive for each marker was then quantified as described below.

For the evaluation of the transduction of cholinergic neurons in the PPN/LDT and axons in the VTA, sections were incubated with an antibody against green florescent protein (GFP, 1:1000, raised in rabbit, Invitrogen, A21311) and either an antibody against ChAT (to label PPN/LDT somata; details as above) or against tyrosine hydroxylase (TH; to define the VTA borders; raised in chicken; 1:500 dilution in 1% NDS, 0.03% Triton X-100 in PBS, Abcam). To determine the proportion of cholinergic neurons transduced, multi-channel stacks were acquired (as described

above) and analysed offline using StereoInvestigator.

For detecting the presence of vesicular transporters in PPN/LDT axons within the VTA, fluorescent images were obtained with a confocal microscope (LSM-510, Zeiss) using the following filters: 504 nm for Alexa Fluor-488, 560 nm for CY3 and 650 nm for CY5 (40X, 1.4 numerical aperture oil immersion). Confocal images were processed using Huygens Professional deconvolution software (version 4.1; Scientific Volume Imaging) with a maximum of 40 iterations.

The sections containing juxtacellularly labelled VTA neurons were incubated in CY3-streptavidin solution (1:1000, Gelifsciences) in PBS, containing 0.03% Triton for a minimum of 4 h. They were then examined under a fluorescent microscope using a 560 nm filter and those sections containing labelled neurons were incubated overnight with the chicken anti-TH, followed by several washes in PBS and incubation with a CY5-conjugated donkey anti-chicken antibody (1:1000, Jackson Immunoresearch Inc.). Streptavidin-positive neurons (i.e. neurobiotin-labelled) that were TH-immunopositive were processed further. The presence of FG in labelled neurons was evaluated using a UV filter (450 nm), which revealed a strong signal in the somata of retrogradely labelled neurons. Images of triple-positive neurons (streptavidin-CY3, TH-CY-5 and FG) were captured using a fluorescent microscope and Axiovision software. FG-negative neurons were then incubated overnight with an antibody against CTb (as above) followed by several washes in PBS and incubation with an AMCA-conjugated donkey anti-mouse antibody (1:1000 dilution, Jackson Immunoresearch). Images of triple-positive neurons (streptavidin-CY3, TH-CY-5 and CTb-AMCA) were captured as above. All streptavidin^{+}/TH^{+} neurons were digitized using Neurolucida (Micro Bright Field) and their location within the VTA

recorded in a sagittal 3D map obtained from the rendered outline of the TH-stained boundaries of the VTA across all mediolateral levels. The outlines of individual sections containing labelled neurons were then rotated and aligned to build a map in which all labelled neurons were contained.

In addition to the analysis of VTA sections containing labelled neurons, all brains were also processed to confirm the sites of injection of the retrograde tracer in NA and the transduction sites (in PPN or LDT). Thus, sections were examined under fluorescent microscopy to identify the FG deposit, the CTb deposit (following immunohistochemical processing as described above) and the hChR2-YFP signal. If tracers were off target, the recorded and juxtacellularly labelled neurons were still included in the analysis but the retrograde labelling was not taken into consideration. If the transduction was weak or overlapped between the two cholinergic nuclei, the neurons were discarded.

Analysis of connectivity

Neurons projecting to the rostral and caudal VTA were quantified and their distribution evalued throughout mediolateral and rostrocaudal levels to identify topographical differences. Neurons within the PPN and LDT, as delimited by the ChAT-immunopositive cell bodies, were classified as: 1) $ChAT^{+}/CTb^{+}$, 2) $ChAT^{+}/RB\text{-}R^{+}$, 3) $ChAT^{-}/CTb^{+}$ or 4) $ChAT^{-}/RB\text{-}R^{+}$. Three representative mediolateral levels were selected for the analysis, corresponding to the following levels from the midline: 1.13, 1.55 and 1.9 mm (Paxinos and Franklin, 2007). To determine the rostrocaudal distribution, VTA-projecting neurons were analysed using an adaptation of a method based upon the subdivision of the PPN into equally spaced segments, as described previously (Mena-Segovia et al., 2009). Using the centre of

the substantia nigra pars reticulata (SNr) as a reference point, concentric circles at 300 µm intervals were drawn outwards to cover the entire extent of the PPN; the first two segments covered the SNr and marked the PPNr. From this border, and using the concentric circles, the PPN was divided into three equally sized segments of 900 µm each along its rostrocaudal axis, representing the rostral (S-I; 0.6 to 1.5 mm from the SNr), middle (S-II; 1.5 to 2.4 mm) and caudal (S-III; 2.4 to 3.3 mm) PPN, from lateral to medial sections. LDT neurons were quantified in one single segment. Results are expressed as the total number of neurons in each category, and as the percentage of cholinergic neurons expressing one of the tracers, using the rostrocaudal segments to average across all animals. We used an ANOVA on ranks analysis to compare differences (the data were not normally distributed).

For the analysis of the cholinergic innervation of the VTA, we measured the length of axons of cholinergic neurons transduced in the PPN (n = 3) and LDT (n = 3) as an estimate of density of innervation. In order to obtain a reliable comparison between the innervation of each structure, we analysed the density of axons following transduction using only the AAV5-EF1a-DIO-eYFP, thus avoiding confounding factors such as possible differences in the efficiency of the vector to transduce cholinergic neurons or differences in the strength of the fluorescent signal. In each case, the location of the transduction area was confirmed to be contained within the limits of the PPN or the LDT. Images were acquired using a 5X magnification objective for delimiting the border of the VTA defined by TH-immuno labelling, and 20X for detecting fluorescent axons. For each brain, three sections corresponding to approximately 0.4, 0.9 and 1.4 mm from the midline (Paxinos and Franklin, 2007) were analysed. A grid of 250 μm^2-squares was superimposed and in each square the total length of labelled axons was measured by tracing all fluorescent axons using in-

built Neurolucida functions. The results are expressed as total axonal length in each square (expressed as μm) or as a normalised value for each square relative to the total length in each VTA level (expressed as percentage). Data were then calculated in three dimensions (rostrocaudal, mediolateral and dorsoventral) in order to identify topographical differences within the VTA. To identify differences in the axonal length across rostrocaudal segments, we used an ANOVA on ranks analysis (the data were not normally distributed), and to compare between the axonal length at specific segments between PPN and LDT axons, we used an unpaired t-test. The level of significance for all tests was taken to be $P < 0.05$. Data are expressed as mean ± standard error of the mean (SEM) unless otherwise indicated.

Electrophysiological data analysis

Following histological confirmation of their location and neurochemical nature, the recordings of VTA neurons were analysed to determine their basic electrophysiological properties. Spike trains were digitized and converted into a time series of events using in-built Spike2 functions. The baseline activity, firing patterns and action potential duration were determined from 5 min-long initial recordings. To determine variations in the firing rate following experimental manipulations, 20 sec segments of baseline activity preceding the manipulation were compared to the firing rate during the pinch or laser stimulation and expressed as percentage change.

Neurons' responsiveness was determined by computing a time-resolved average response to light stimulation. For this, 2 to 4 trials for each neuron were obtained and a modified estimation of the mean instantaneous firing rate locked to the onset of the laser was computed (Blejec, 2005). By considering each spike train $j = 1,...,k$ as a sequence of discrete events with occurring times $\{t_{i,j}\}, i = 1,2,...,n_j$, a cumulative

distribution function (CDF) at each time $t_{i,j}$ can be defined. The number of events prior and up to $t_{i,j}$ is a strictly increasing step function changing at $t_{i,j}$ in unit steps. The slope of CDF divided by the number of trials represents the mean density of events across the k trials and is equal to the mean density of events per unit time or the trial-averaged firing rate. Then a local linear regression based on $N_{i,j} = 6 \times k$ neighbouring events may be used to estimate the CDF slope for each spike time and the regression slope estimates the instantaneous firing rate at each reference time $t_{i,j}$ while considering the response of the k trials. Finally, an evenly sampled time series $FR(t)$ was obtained by smoothing the resulting (unevenly-sampled) time series with a 1 sec Gaussian kernel. The distribution $FR(t)$ of spontaneous activity prior to the stimulus was then used to compare the activity during laser application with the basal activity. Percentiles 5 (C_5) and 95 (C_{95}) were selected as thresholds to assess inhibition and excitation in the post-stimulus interval respectively. Significance of response periods was assessed by means of a cluster-based permutation tests (n = 200 permutations, $P < 0.05$) on the duration of the intervals. If a neuron showed a post-laser period with $FR(t)$ above percentile 95 or below percentile 5 for a longer period than any other within the baseline (and not less than 3 s), then the neuron was considered to respond to cholinergic axonal activation either by activation or inhibition. If these criteria were not met then a neuron was considered as non-responding.

Neurons that were classified as regular (only for descriptive purposes) showed more than three spaced peaks in their autocorrelogram. To determine bursting activity, typical parameters for DAergic neurons were used where the burst onset was defined by a minimum of two events with an interval of 80 ms or less, and the burst

termination was determined if the interval between events was larger than 160 ms (see Grace and Bunney, 1984). The number and characteristics of the bursts were analysed using Spike2 scripts (Cambridge Electronic Design), including the number and percentage of spikes inside bursts, the number and percentage of inter-burst spikes and the burst probability (normalised burst count difference between basal and laser stimulation). Student's t-test or the Mann-Whitney Rank Sum Test were used to compare data between groups. Linear regressions were used to compare the firing rate changes between different modalities of experimental manipulations (i.e. laser v pinch), calculating the Studentized Deleted Residuals to detect outliers (SigmaPlot 12.0, Systat Software, Germany). The significance level for all tests was taken to be $P < 0.05$. Data are expressed as mean ± SEM.

Results

Electronic microscopy data were collected by IHC and were reported here to aid interpretation.

Cholinergic neurons modulate the activity of DA and non-DA neurons of the VTA

In order to define topographical relationships between cholinergic neurons of the brainstem and the VTA we first made deposits of two retrograde tracers in the VTA (rostral and caudal) to define the topographical organisation of the brainstem cholinergic innervation of the VTA.

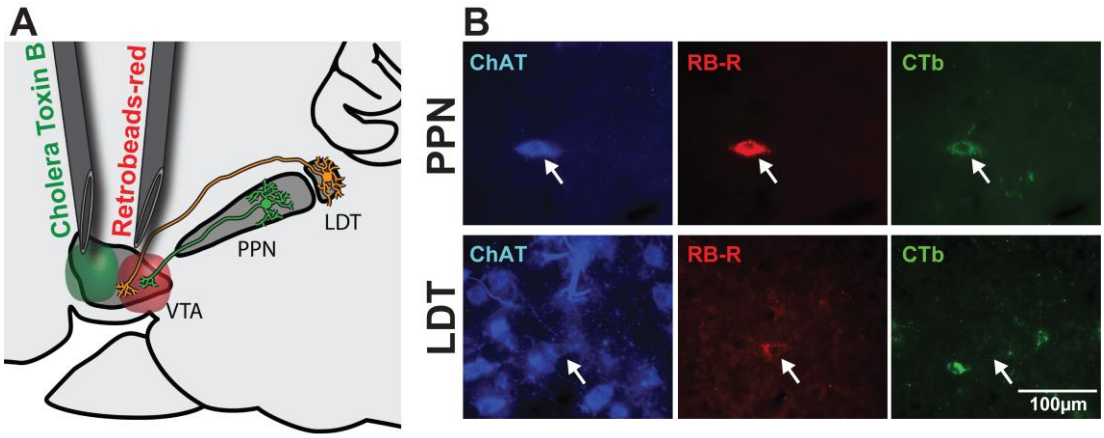

Figure 18: Cholinergic projections to the VTA. (A) Schematic showing the sites of retrograde tracer injections in the VTA: cholera toxin b (CTb) was injected in the rostral VTA and red retrobeads (RB-R) in the caudal VTA to identify the topography of the projections from the PPN and LDT. **(B)** ChAT-immunopositive neurons contained CTb and/or RB-R in both PPN and LDT (arrows; the PPN neuron is triple-labelled and the LDT neuron in double-labelled).

We observed that cholinergic neurons of the PPNc and LDT innervate both rostral and caudal regions of the VTA, arising from similar numbers of cholinergic neurons in the PPN and LDT (**Figs. 18 and 19**). Next we used a transgenic rat line that expresses Cre recombinase under the ChAT promoter (ChAT::cre^{+}; Witten et al.,

2011). We stereotaxically injected into the PPN or LDT an AAV vector that incorporated a transgene coding for a fluorescent reporter (enhanced yellow fluorescent protein, eYFP) and a light-activated ion channel (channelrhodopsin-2, ChR2) (AAV2-EF1a-DIO-hChR2-YFP; **Fig. 21a**).

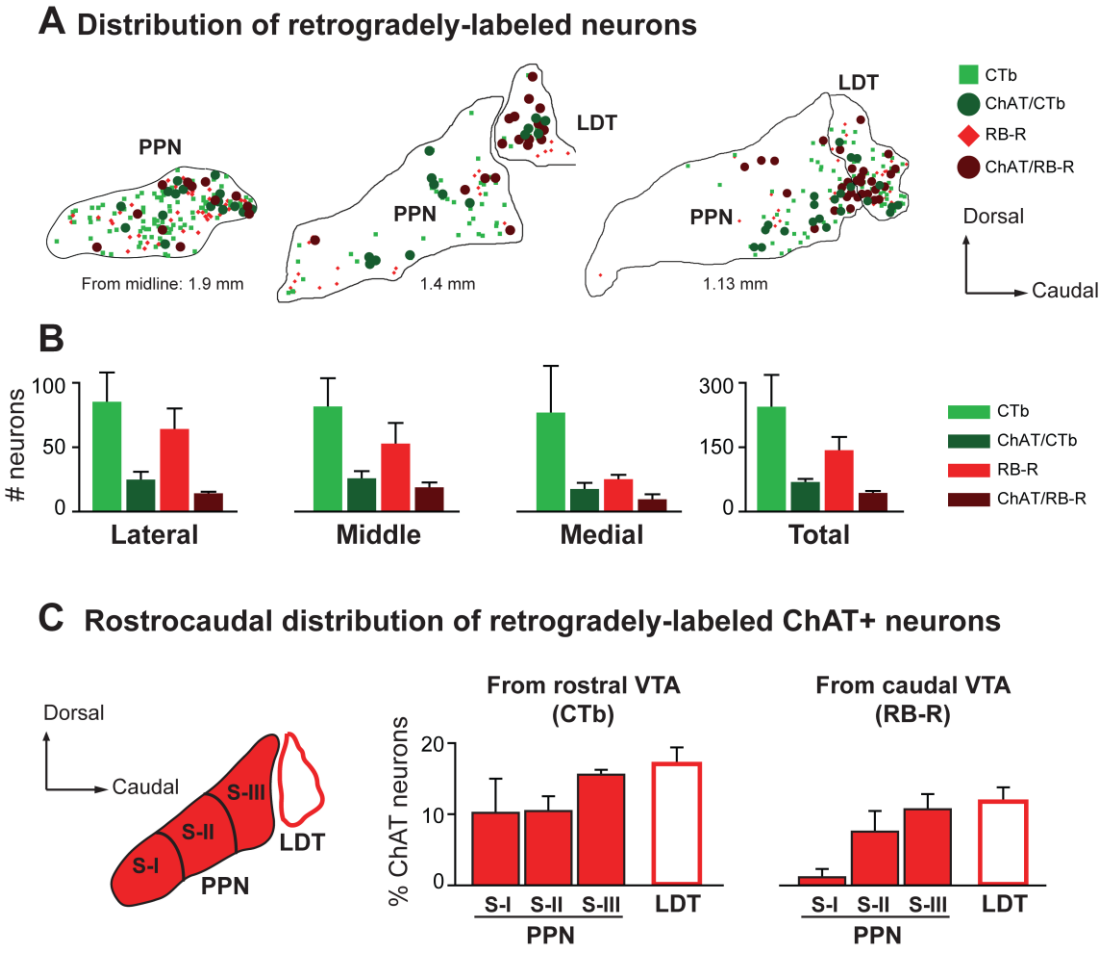

Figure 19: Cholinergic neurons projecting to the VTA are concentrated in the caudal mesopontine region. (A) Pattern of retrograde labelling in relation to the expression of ChAT at three mediolateral levels of the PPN/LDT in a representative animal. **(B)** Similar numbers of VTA-projecting cholinergic neurons were detected at different mediolateral levels. A larger proportion of non-cholinergic VTA-projecting neurons (albeit not significant) was observed in the most lateral sections of the PPN. **(C)** The number of cholinergic neurons containing one of the tracers was normalised against the total number of cholinergic neurons and expressed as a percentage of the total. This was quantified at three rostrocaudal 900 µm-segments of the PPN and in the LDT. The number of cholinergic neurons projecting to the VTA is higher in the PPNc segments and the LDT. This contrasts with the low number of $ChAT^{+}/RB\text{-}R^{+}$ neurons projecting to the caudal VTA located in the PPNr. Bars represent SEM (n = 4).

Appropriate controls were used to verify the specificity of the vector for cholinergic neurons both in the brainstem and other brain regions using a vector that encodes only the fluorescent reporter (**Fig. 20 and 21**).

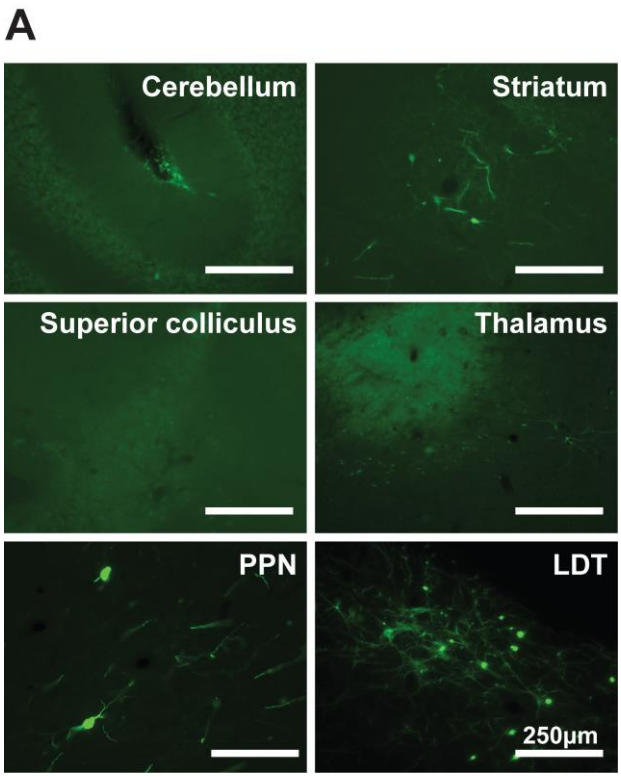

Figure 20: Specificity of transduction for cholinergic neurons in different brain areas. We tested the specificity of the transduction by injecting AAV5-EF1a-DIO-YFP in other brain regions in a different set of animals, including the cerebellum, ventromedial thalamus, superior colliculus and Str as well as the PPN and LDT. Positive (i.e. YFP-expressing) somata were only observed in the Str, the PPN and LDT, and no retrograde transduction of cholinergic neurons was detected.

In both PPN- and LDT-injected rats, we detected fluorescently-labelled axons in the VTA, the borders of which were defined by the distribution of tyrosine hydroxylase-immunopositive (TH^+) neurons (**Fig. 22, 23b** and **25b**).

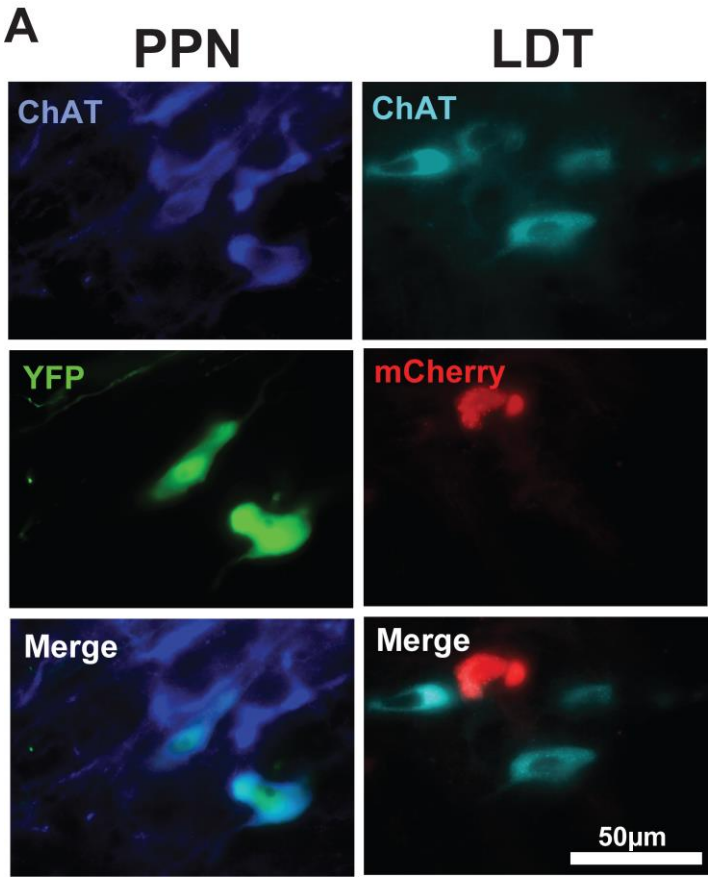

Figure 21: Virus expression was visible only in ChAT neurons. The presence of two fluorescent markers in the cholinergic neurons of the PPN (AAV5-EF1a-DIO-YFP) and LDT (AAV2-EF1a-DIO-mCherry) of ChAT::Cre^{+} rats was used to evaluate the specificity for the transduction, in combination with immunofluorescent detection of ChAT.

Labelled axons were mapped across the full extent of the VTA and showed a relatively homogeneous distribution whether derived from the PPN or LDT, although LDT axons had a greater overall length than PPN axons (albeit not significant; **Fig. 22**) and with an area of higher density in the dorsal part of the parabrachial pigmented area (n = 6; **Fig. 23c and 25c**).

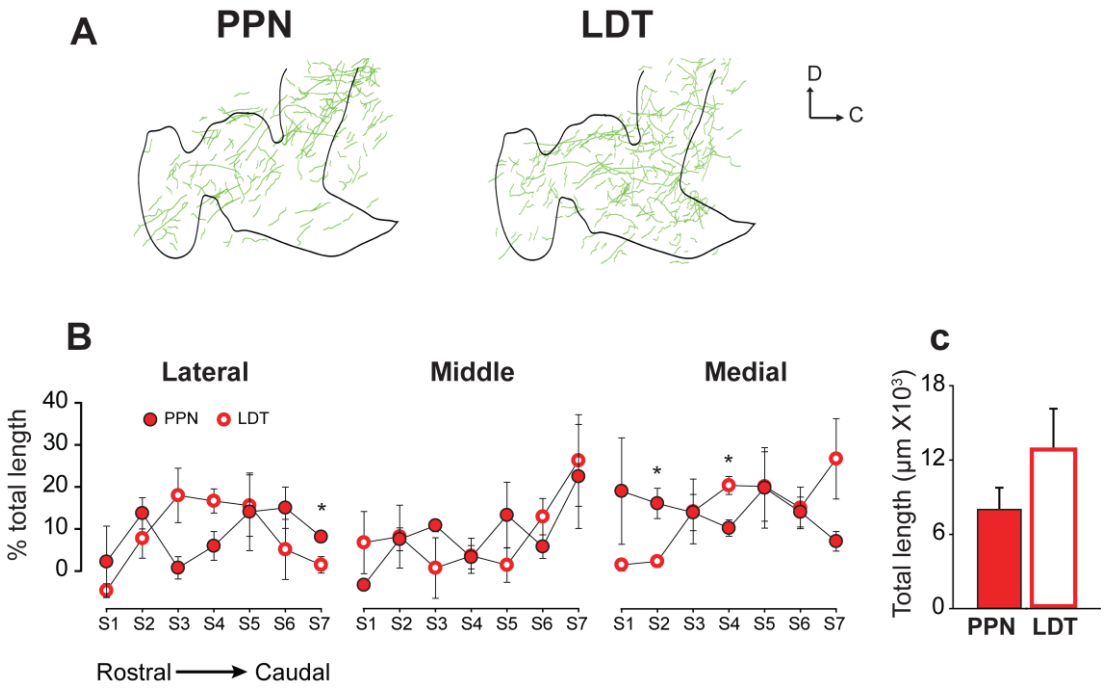

Figure 22: Mediolateral distribution of cholinergic axons in the VTA. (A) The axons used in the analyses in Fig. **24c** and **26c** were traced and digitized, as shown in this representative sections. **(B)** The axonal density was normalised to detect areas of preferential innervation from either the PPN or the LDT. Whereas in the lateral and middle VTA we observed a similar pattern, in the medial VTA some differences were observed: PPN tends to innervate the most rostral regions of the VTA, where LDT innervation is low, but then drops in the most caudal regions, where LDT innervation is higher. This coincides with the low numbers of retrogradely labelled cholinergic neurons in the PPN when the injection was located in the caudal VTA. Asterisks represent $P < 0.05$. **(c)** Quantification of the total length cholinergic axons in the PPN and the LDT ($t = -1.29$, $P = 0.266$). Bars represent SEM.

Consecutive sections were processed to reveal the YFP by a permanent peroxidase reaction product and processed for electron microscopy to characterise the synapses formed by YFP-expressing, cholinergic axons. Gray's Type 1 synapses (asymmetrical) were formed by PPN cholinergic axons with DA dendrites, whereas LDT cholinergic axons formed asymmetrical synapses with both DA and non-DA dendrites (40/60%, respectively).

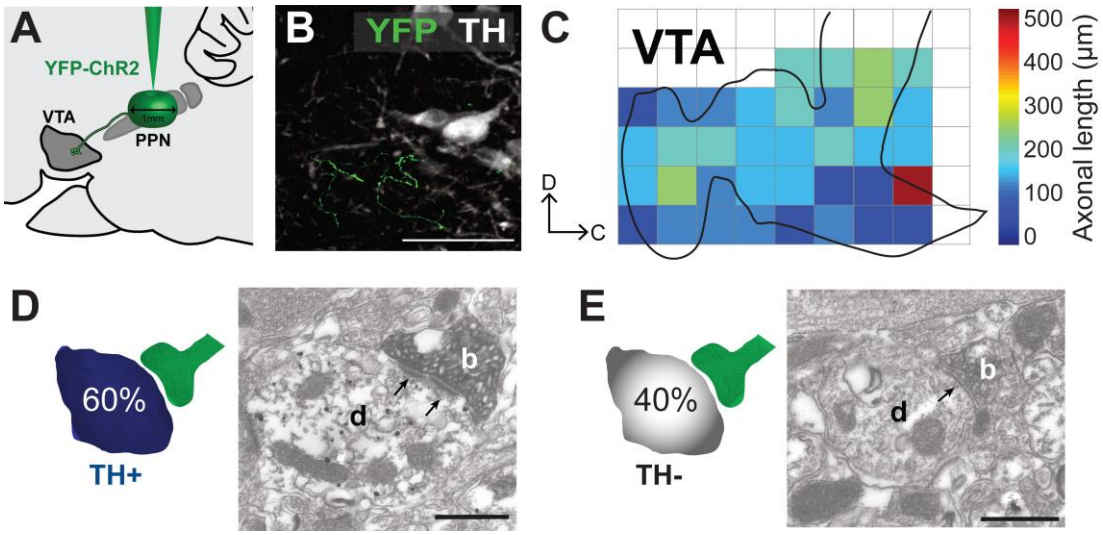

Figure 23: PPN cholinergic projection to DA and non-DA neurons of the VTA. **(A)** An AAV vector (AAV2-DIO-E1Fa-YFP-ChR2) was injected into the PPNc of ChAT::Cre^{+} rats. **(B)** YFP-positive axons were detected in the VTA and **(C)** mapped using 250-µm^{2} grids. **(D, E)** PPN cholinergic axons (b, bouton) make synaptic contacts (arrows) with both TH^{+} and TH^{-} dendrites (d). Scale bars (in µm): **b**, 70; **d** and **e**, 0.5.

In contrast, Gray's Type 2 synapses (symmetrical) from both structures were formed predominantly in non-DA structures (80% for PPN and 100% for LDT). Furthermore, the synapses derived from the PPN made contacts with a larger proportion of DA processes (**Fig. 23d and 25d**), whereas those derived from the LDT showed a greater preference for non-DA processes (**Fig. 23e and 25e**). The combined results from the anatomical characterisation thus reveal that cholinergic axons originating in the PPN and LDT are intermingled within the VTA and contact both DA and non-DA neurons. In addition, while the number of VTA-projecting cholinergic neurons is similar between PPN and LDT, there is an indication from the axonal mapping that the latter has a higher level of collateralization that gives rise to a larger number of synaptic contacts with non-DA neurons.

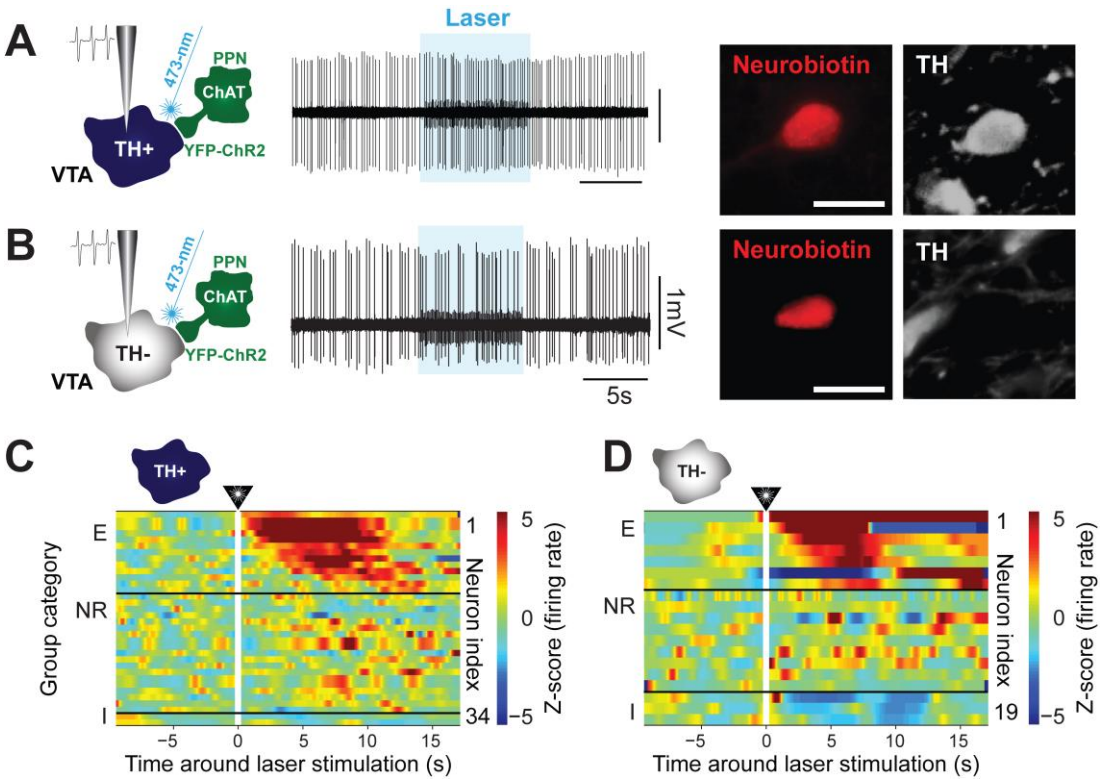

Figure 24: Optogenetic activation of PPN cholinergic axons modulates DA and non-DA neurons of the VTA. (A) Individual TH^+ neurons were recorded in vivo during optogenetic stimulation of PPN axons (n = 34) and were subsequently labelled with neurobiotin. **(B)** The same protocol was followed for TH- neurons (n = 19). **(C)** Normalised firing rate (z-score along the whole trial period) for each TH^+ neuron around the laser stimulation in the VTA showing three categories of neurons: excited (E, 38%), non-responsive (NR, 56%), and inhibited (I, 6%). **(D)** Normalised firing rates of TH- neurons show similar proportions in each group in relation to their response (E, 37%; NR, 47%; I, 16%). Scale bars (in µm): **f** and **g**, 50.

Next we tested the effect of optogenetic stimulation of the brainstem cholinergic axons on neurochemically identified VTA neurons recorded *in vivo* and juxtacellularly labelled. Following the *post hoc* histological identification of the recorded neurons, we classified them as DA (TH^+; *n* = 60) or non-DA (TH^-; *n* = 36) (**Fig. 24Af, 24B, 26A and 26G**). The firing rate and pattern of each neuron during the light stimulation (8 s, 10 Hz, 50 ms pulses) was compared to their firing characteristics during the preceding baseline period (10 s). Neurons whose firing rate during the light stimulation was within 10% of their basal firing rate were considered

as non-responsive (n = 16 for PPN, n = 13 for LDT; overall firing rate increase including non-responsive neurons: 11.8 % for PPN and 10.6 % for LDT). Thus, in responsive neurons, we observed a 31.3 ± 5.04 % increase during PPN axon activation (basal firing rate: 3.3 ± 0.57 Hz to laser stimulated: 4.18 ± 0.66 Hz; P = 0.002) and a 25.8 ± 3.43 % increase during LDT axon activation (basal firing rate: 3.35 ± 0.52 Hz to laser stimulated: 4.23 ± 0.7 Hz; P = 0.008). There was no significant difference between the response to PPN and the response to LDT axon stimulation. In order to compare the latency of the activation, we carried out z-score analysis. Two to four trials were carried out for each neuron and combined to calculate the average response (**Fig. 27**; see Methods). If a neuron increased (above 95th percentile of firing rate before laser stimulation) or decreased (below 5th percentile) its firing rate for a period of time that is significantly longer (cluster-based permutation test, $P < 0.05$; 200 permutations) than any increase or decrease in firing observed during the baseline period (and not less than 3 s), then the neuron was considered to respond to cholinergic axon stimulation either by activation or inhibition, respectively. If these criteria were not met then a neuron was considered as non-responding. Following light stimulation of cholinergic axons from either the PPN or LDT we observed both excitatory and inhibitory responses in both DA and non-DA neurons, although the proportions varied slightly.

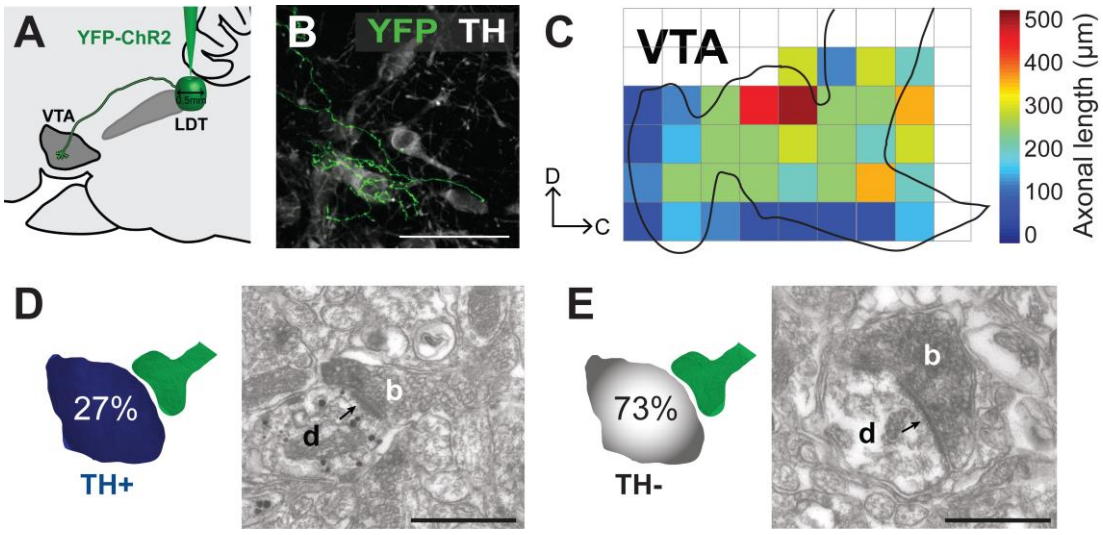

Figure 25: LDT cholinergic projection to DA and non-DA neurons of the VTA. **(A)** An AAV vector (AAV2-DIO-E1Fa-YFP-ChR2) was injected into the LDT of ChAT::Cre^{+} rats. **(B)** YFP-positive axons were detected in the VTA and **(C)** mapped using 250-µm^{2} grids. **(D, E)** LDT cholinergic axons (b, bouton) make synaptic contacts (arrows) with both TH^{+} and TH^{-} dendrites (d). Scale bars (in µm): **b**, 70; **d** and **e**, 0.5.

Stimulation of axons from the PPN produced predominantly excitation in responding DA neurons (**Fig. 24C**), while only a small fraction showed inhibition. This activation was maintained throughout the period of the laser stimulation. The excitation of responding non-DA neurons followed similar patterns of activation during the stimulation period, however, a larger proportion of non-DA neurons were inhibited (**Fig. 24D**).

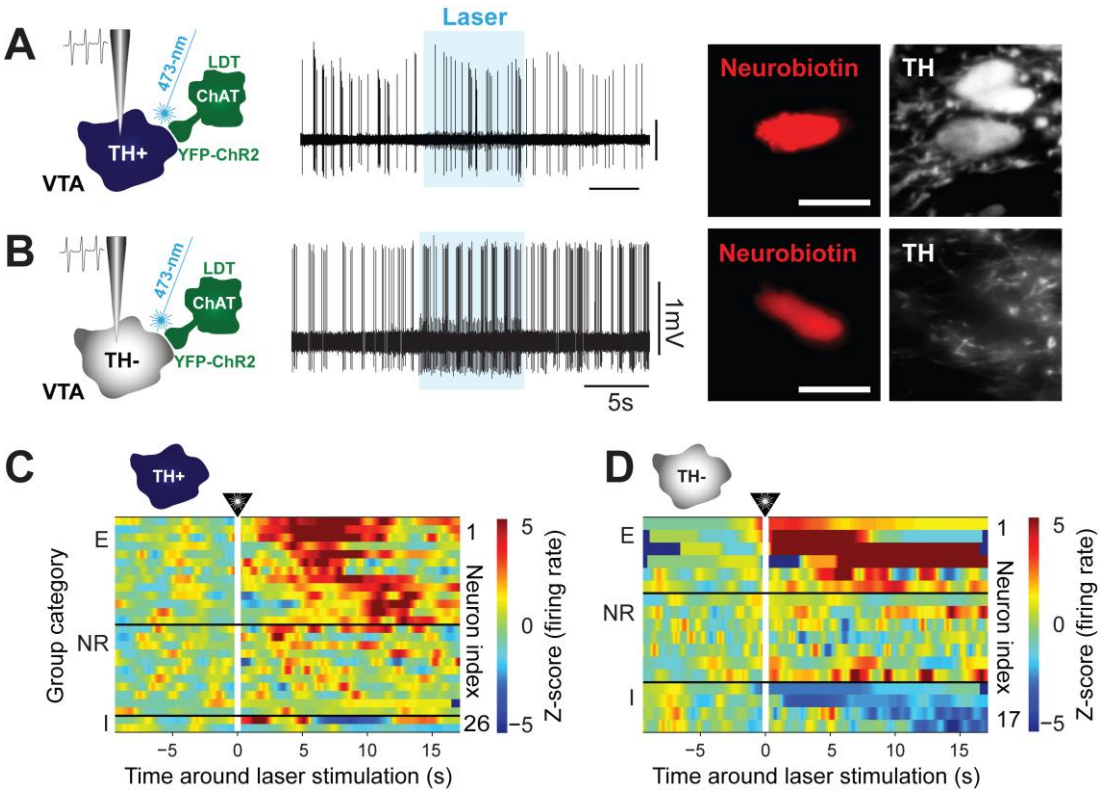

Figure 26: Optogenetic activation of LDT cholinergic axons modulates DA and non-DA neurons of the VTA. (A, B) Protocol for recording, stimulation and labelling of TH^+ (n = 26) and TH^- (n = 17) neurons. **(C)** Neurons were separated into three categories according to their responses to the laser stimulation: excited (E, 50%), non-responsive (NR, 42%), and inhibited (I, 8%). **(i)** There was more variability in the responses of TH^- neurons to the LDT stimulation (E, 35%; NR, 41%; I, 24%). Scale bars (in µm): **f** and **g**, 50.

The stimulation of LDT cholinergic axons also produced predominantly excitation in responding DA neurons, but the proportion of responding neurons was larger than following PPN axon stimulation (50% vs 38%; **Fig. 26C**). In contrast to PPN axon stimulation, we observed a proportion of 'late-responding' neurons, whose firing rate increased once the light stimulation stopped. The effects of stimulation of LDT cholinergic axons on non-DA neurons were more variable: the excitation was not as prominent as for PPN axon stimulation and a slightly larger proportion of non-DA neurons were inhibited (**Fig. 26D**).

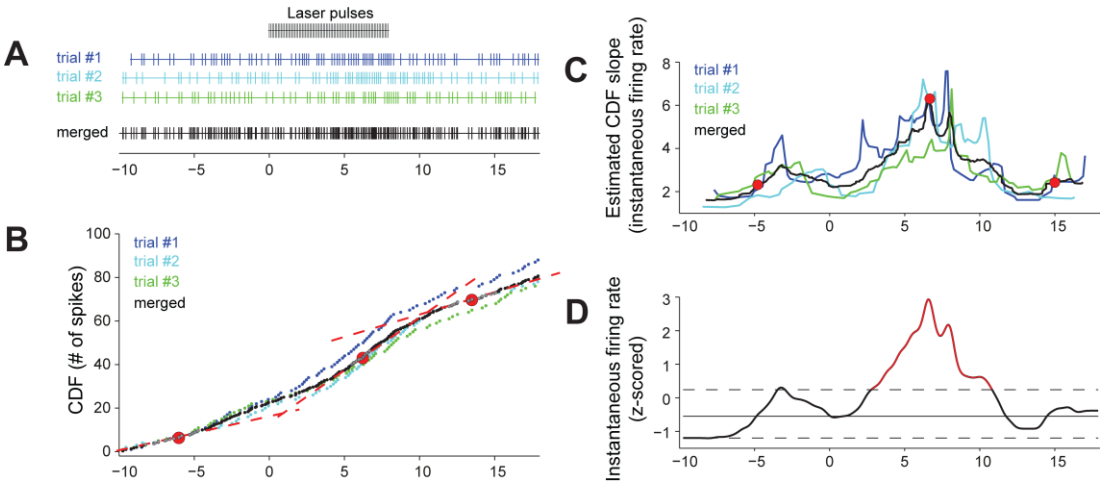

Figure 27: Analysis of the response of a representative neuron to the laser stimulation. (a) Three responses to the laser were obtained for this neuron. Spike events were extracted and merged into a single spike train (black trace). **(b)** Cumulative distribution function (CDF) of the three individual spike trains together with that of the merged spike train (black dots) were computed by increasing the CDF one unit each time a spike occurred (note that in order to maintain the same scale, the CDF of the merged spike train must be divided by the number of spike trains merged). Regression slope of the CDF was computed at each spike time by using a local (18 neighbouring spikes) linear regression analysis (see gray dots on the black trace and dashed red lines for an example at three different time points, red dots). **(c)** Estimated instantaneous firing rate (regression slope) for the three individual trains and for the merged spike train where red dots correspond to the regression points marked in panel **b**. **(d)** Smoothed version and z-scored version of the instantaneous firing rate shown in panel **c** (for merged trace only). Horizontal lines correspond to the mean (full) and 5^{th} and 95^{th} percentile (dashed) values of the firing rate during pre-stimulus period (-10 to 0 s). Red trace demarks the response period of the neuron (i.e., a significant $P < 0.05$ increase in the firing rate).

The activation of DA neurons showed a slow response that increased as a function of the number of pulses and reached its maximum toward the end of the light stimulation period (**Fig. 28**). These slow dynamics were observed following both PPN- (**Fig. 28a**) and LDT cholinergic axon stimulation (**Fig. 28b**), but the magnitude of the response was slightly greater for the PPN axon stimulation, albeit not significant (cluster-based permutation test, $P = 0.715$; 200 permutations).

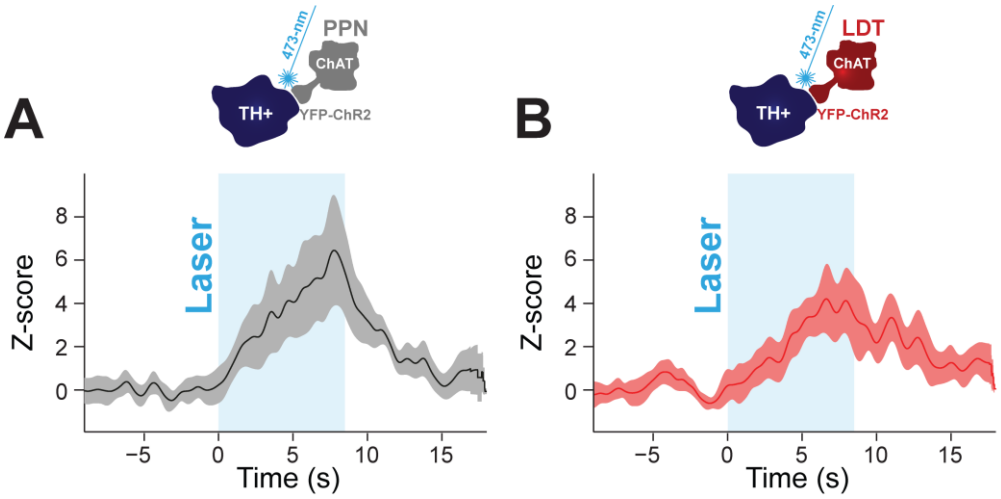

Figure 28: Activation of cholinergic axons produces a slow and robust excitation of DA neurons. The normalised firing rate of all TH^+ neurons that were excited by the laser stimulation show a similar slow modulation when cholinergic axons of either PPN **(a;** n = 15**)** or LDT **(b;** n = 15**)** were stimulated. The responses following PPN cholinergic axon stimulation were greater in magnitude, no significant differences were observed.

This contrasts with the short-latency responses observed in nigral neurons following electrical stimulation of the PPN output (Lokwan et al., 1999; Scarnati et al., 1986; Futami et al., 1995). In a separate set of experiments we tested the effects of *in vivo* electrical stimulation of the PPN/LDT region on identified VTA DA and non-DA neurons. In line with the above reports, we observed short-latency excitatory and inhibitory responses in DA and non-DA neurons in the VTA (*n* = 16; **Fig. 29**). This suggests that electrically stimulating the output of the PPN/LDT leads to a combined response mediated by glutamatergic, GABAergic and cholinergic transmission, whereas the optogenetic manipulation dissects out the cholinergic effects.

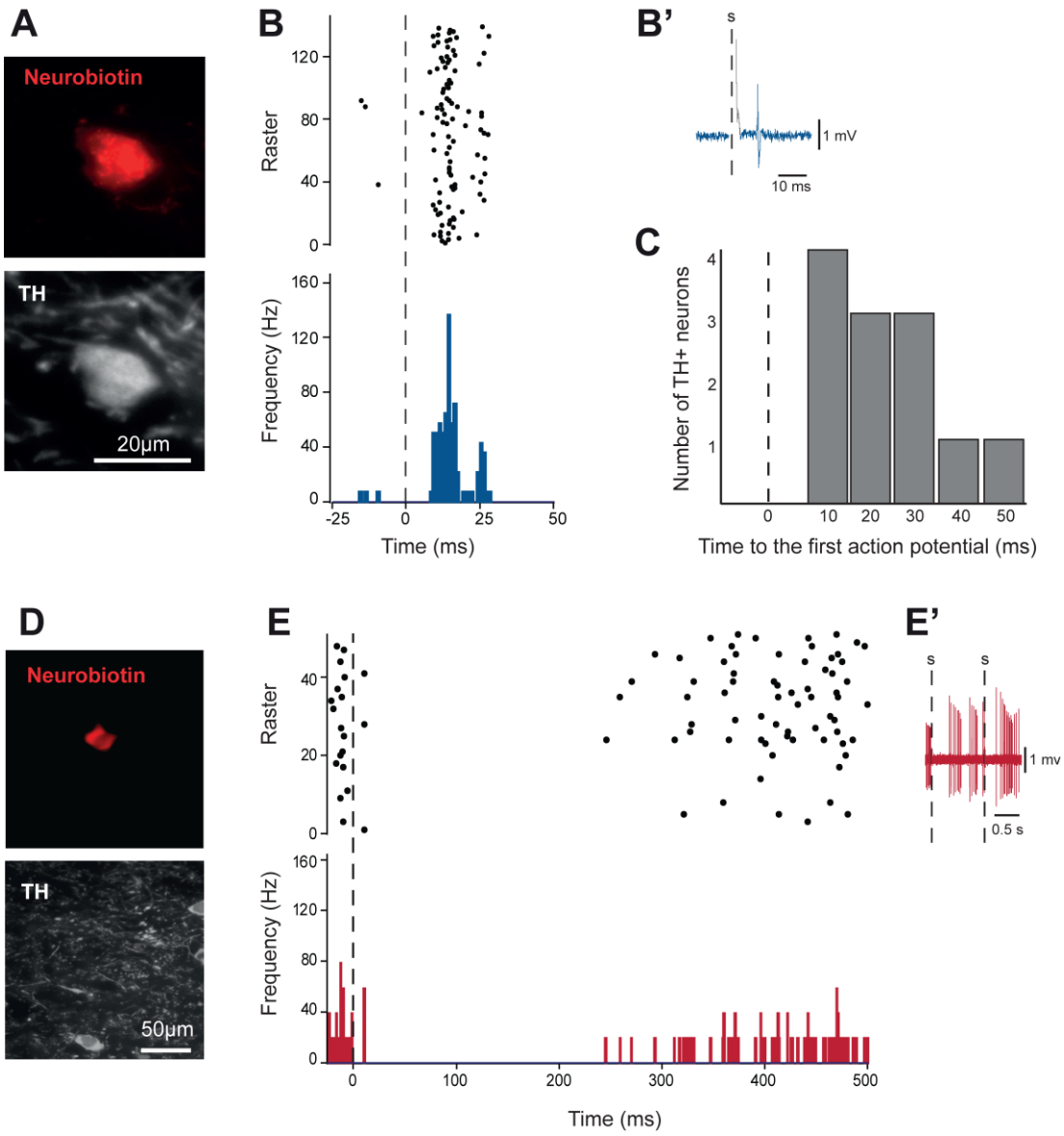

Figure 29: Electrical stimulation of the PPN produces short-latency responses in DA neurons. (a) A neuron that was recorded and labelled by the juxtacellular method and identified as DAergic by the expression of immunoreactivity for TH. **(b)** Raster plot and peri-stimulus time histogram (PSTH) of the neuron shown in **a,** following electrical stimulation of the PPN (bin size 1 ms). **(b')** A representative spike following the stimulus (s; 0.5 ms duration, 0.5-0.8 mA, 0.5 Hz). Consistent with previous reports, short-latency action potentials were elicited in the DA neurons within a few milliseconds of the stimulus being delivered in the PPN; this contrasts with the slow modulatory effect of the optogenetic activation of PPN cholinergic afferents. **(c)** Short-latency responses were consistent across the DA neuronal population (n = 12). **(d, e, e')** Non-DA neurons (i.e. TH-immunonegative, **d**) were also sampled (n = 4). PSTH (bin size 2 ms) showing a long-lasting inhibition (average time of inhibition: 30ms ± 8.6ms, **e**). Responses in non-DA neurons were more heterogeneous.

In order to ensure that the responses to light stimulation were mediated by released ACh, in a separate set of experiments we attached a glass pipette for the microiontophoresis of drugs to the juxtacellular recording electrode (**Fig. 30a**). This allowed us to test *in vivo* the local effects of ACh receptor antagonists on the responses of individual VTA neurons to the stimulation of cholinergic axons from either the PPN or LDT. Neurons were subsequently identified as DA (*n* = 14; **Fig. 30b**) or non-DA (*n* = 12).

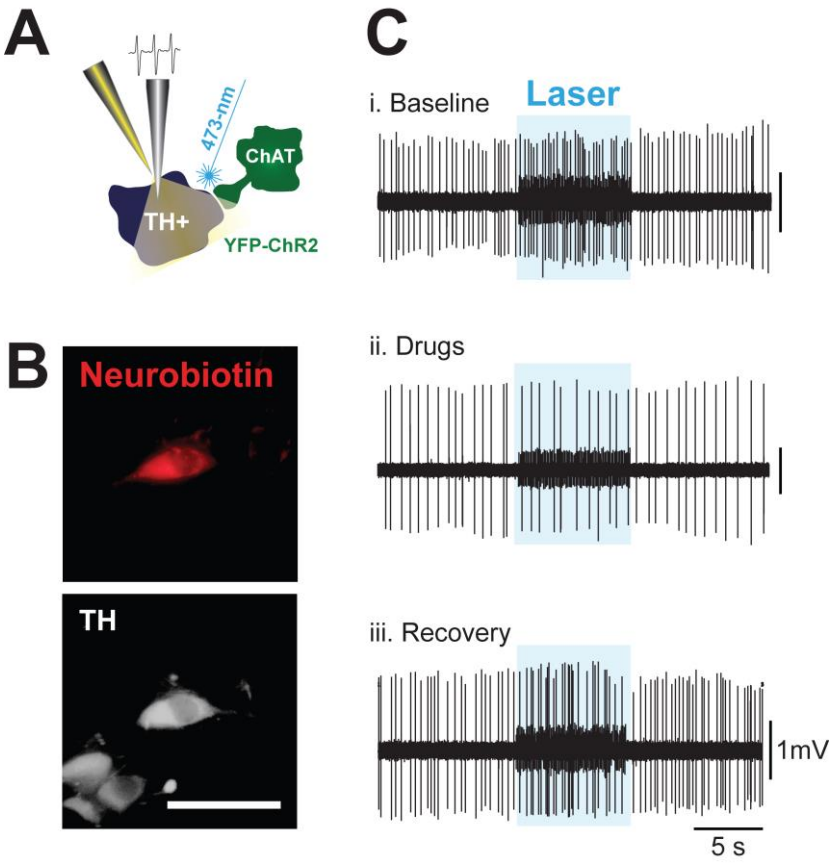

Figure 30: Cholinergic antagonists block the response to laser stimulation in DA neurons. (a) Individual TH+ and TH- neurons were recorded in vivo during optogenetic stimulation of PPN axons and local microiontophoretic administration of nicotinic and muscarinic antagonists (methyllycaconitine 20 mM, dihydro-β-erythroidine 40 mM, atropine 40 mM and mecamylamine 100 µM). **(b)** Neurons were subsequently labelled with neurobiotin and their neurochemical profile identified. **(c,** i**)** Example of a neuron that was recorded during a baseline response to the optogenetic activation of LDT cholinergic axons. (ii) Following the iontophoretic application of the ACh antagonist cocktail, the same laser stimulation failed to produce a response, but (iii) the responsiveness to the laser stimulation recovered following drug wash-out (2 min after). Scale bar in **b**: 50 µm.

The excitatory responses to the laser in both DA and non-DA neurons following the stimulation of either PPN and LDT cholinergic axons were abolished during the iontophoretic administration of nicotinic and muscarinic antagonists in the vicinity of the recorded neurons (TH+: *P* = 0.0006; TH-: *P* = 0.001; **Fig. 30c, 31A and 31B**), and were quickly reversed within a few minutes of stopping the microiontophoretic current for the drug delivery. No significant difference in the firing activity was observed following drug delivery.

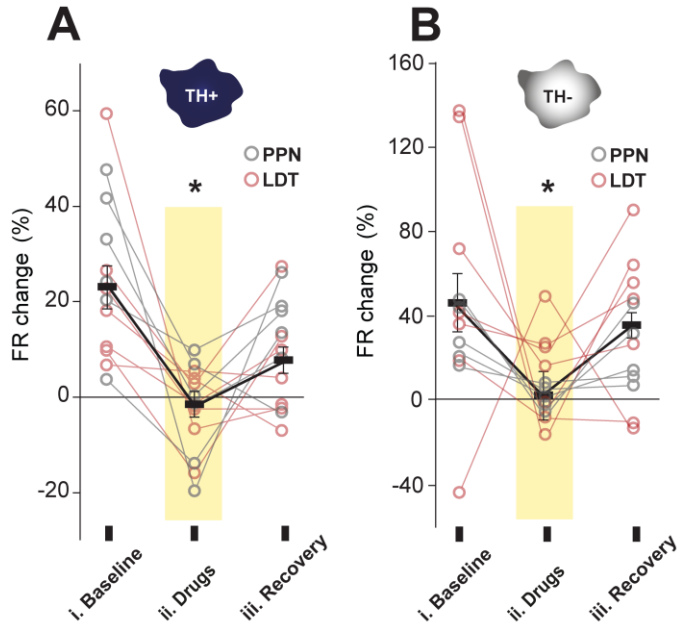

Figure 31: Cholinergic antagonists block the response to laser stimulation in DA and non-DA neurons. (a, b) Excitatory responses to laser stimulation of both PPN and LDT cholinergic axons were blocked in DA (n = 14; $F_{1, 12}$ = 21.3, P = 0.0006, 2-way mixed ANOVA) and non-DA (n = 12; $F_{1, 9}$ = 20.26, P = 0.001, 2-way mixed ANOVA) neurons following the administration of ACh antagonist cocktail and recovered following wash-out. No significant effects in the axon source (PPN/LDT) factor or in the interaction (stimulation x source) were observed. Bars represent mean ± SEM.

These results thus demonstrate that the observed effects on VTA neurons following stimulation of the cholinergic axons are a consequence of the release of ACh and not the release of glutamate or GABA. The specificity of the viral expression for

cholinergic neurons is further supported by the absence of glutamate vesicular transporter-2 in PPN/LDT axons expressing the fluorescent reporter within the VTA (**Fig. 32**). Thus, in contrast to the short-latency excitatory (presumably glutamatergic) effects of the electrical stimulation, our findings demonstrate a slow cholinergic modulation of VTA DA neurons arising from both PPN and LDT.

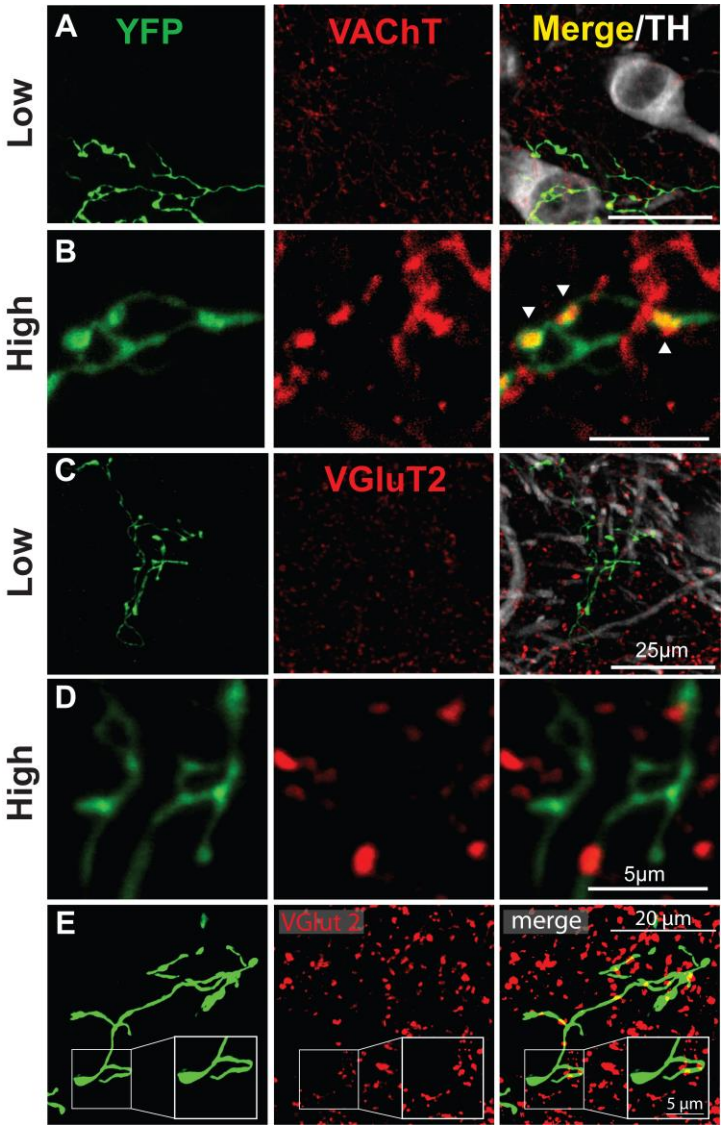

Figure 32: Expression VAChT in YFP-labelled axons. Axons expressing YFP in the VTA from both PPN- and LDT-transduced animals were tested for the presence of vesicular transporters by immunofluorescence. VAChT immunofluorescence was detected in the majority of axonal varicosities expressing YFP (arrowheads), as has also been reported in the striatum (Dautan et al., 2014). In contrast, the vesicular glutamate transporter 2 (VGluT2) was never observed to co-localise with YFP in axonal varicosities, even though we detected large numbers of VGluT2-positive varicosities within the VTA. Thus, in agreement with in situ hybridization data (Wang and Morales, 2009), the axons of cholinergic neurons from the brainstem lack VGluT2.

Bursting activity is enhanced by LTD stimulation

In anesthetized rats, DA neurons fire in regular, irregular or bursting mode. Here we

analysed the spike trains of DA neurons that showed bursting activity during the baseline and/or during optogenetic stimulation of PPN and LDT cholinergic axons. We detected a switch from non-bursting to bursting mode and *vice versa* only following PPN stimulation. Thus, 27% of neurons that showed bursting activity during the stimulation did not have any bursts during the baseline (**Fig. 33a, left**). On the other hand, 18% of neurons that were spontaneously bursting during the baseline stopped bursting during the stimulation. This contrasts with LDT axon stimulation that did not elicit any switch to or from bursting activity. Nevertheless, following LDT axon stimulation, neurons that were already firing in bursts during baseline increased their bursting activity (**Fig. 33a, right**), detected as an increase in the number of bursts episodes (P = 0.02 for comparisons to both baseline and PPN stimulation; **Fig. 33b**) and a higher burst probability (P = 0.039 between PPN and LDT; **Fig. 33c**). Accordingly, we observed a tendency for an increase in the proportion of spikes within bursts only after LDT axon stimulation (n.s., P = 0.06 compared to its baseline; P = 0.09 compared to PPN axon stimulation; **Fig. 33d**). In addition, stimulation of LDT axons led to a decrease in the number of inter-burst spikes (T = 132, Mann-Whitney; P = 0.027; PPN n = 13; LDT n = 13). Further differences between PPN and LDT effects were observed: whereas LDT axon stimulation decreased the ratio of spikes outside bursts to spikes inside bursts (decreasing the burst entropy) in the majority of cases, PPN stimulation tended to produce the opposite effect, disrupting the burst organisation (P = 0.006 between PPN and LDT; **Fig. 33e**). These data suggest that the effect of PPN cholinergic axon activation is heterogeneous, such that it is able to switch the activity of some DA neurons to bursting mode while disrupting the burst organisation in DA neurons that were already bursting. On the other hand, LDT cholinergic axon stimulation

reorganises the spiking into bursts. These data show that cholinergic neurons in the PPN and LDT modulate the activity of DA neurons in the VTA by different mechanisms.

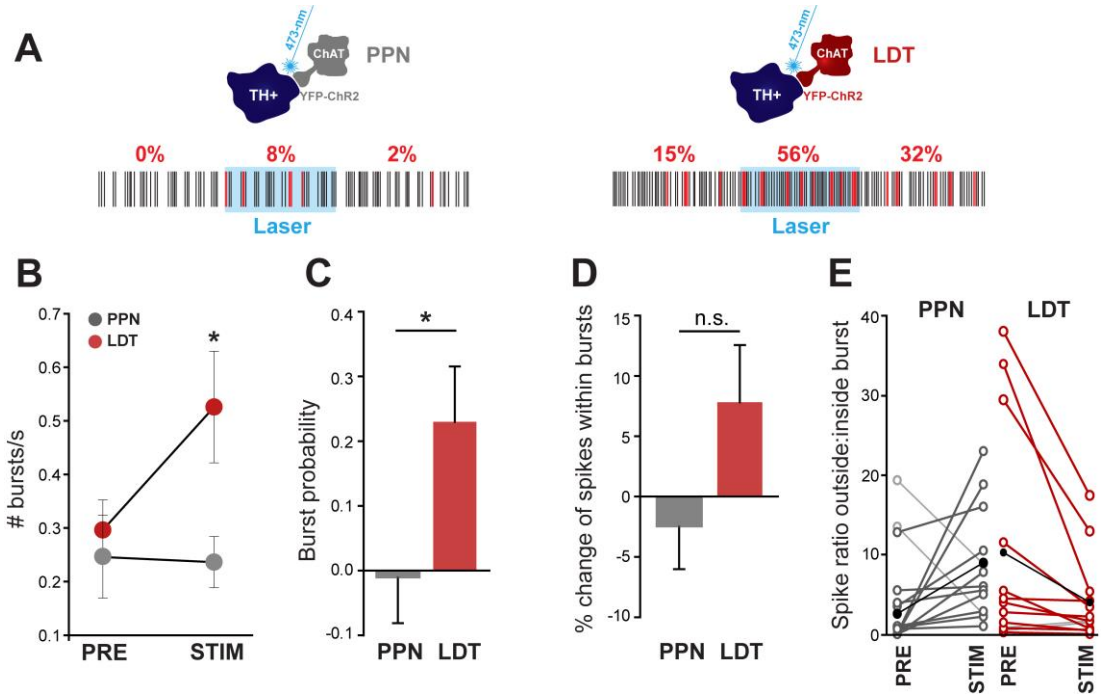

Figure 33: Laser stimulation of cholinergic axons modifies the bursting activity of DA neurons. (a) DA neurons modified their bursting activity following optogenetic activation of PPN or LDT cholinergic axons. Red numbers represent the percentage of spikes within a burst before, during and after laser stimulation. While PPN stimulation tended to switch the pattern of activity of DA neurons, LDT axon stimulation did not change the bursting regime but increased the number of spikes within bursts (red) of already bursting neurons. **(b)** LDT axon stimulation significantly increased the number of bursts in those neurons already bursting when compared to the baseline ($F_{1, 12}$ = 7.18, P = 0.02, 1-way RM ANOVA, n = 13) and to PPN axon stimulation (T = 238.5, P = 0.02, Mann-Whitney, n = 15). **(c)** Increased burst probability during LDT axon stimulation (t = 2.18, P = 0.039). **(d)** LDT axon stimulation produced more spikes within bursts during the stimulation whereas PPN stimulation resulted in fewer spikes within bursts (t = 1.76; P = 0.09). **(e)** Ratio of spikes outside:inside bursts during baseline and laser stimulation. During PPN axon stimulation, in all but 2 cases (light gray), there was a disruption in the bursting activity characterised by a larger number of spikes outside bursts. In contrast, during LDT axon stimulation, in all but 2 cases (gray) there was an increase in the concentration of spikes within bursts. This change in the ratio was significantly different between PPN (n = 16) and LDT (n = 14; t = 2.98; P = 0.006). Group means are depicted in black.

Mesolimbic DA neurons are selectively activated by LDT cholinergic axons

Since cholinergic neurons of the PPN and LDT are components of functionally distinct forebrain circuits, we tested the hypothesis that they may innervate functionally distinct subsets of VTA neurons. We injected a tracer into the NA shell to retrogradely label the so-called mesolimbic neurons in the VTA (**Fig. 34a**) and in the prefrontal cortex for the mesocortical neurons in the VTA (**Fig. 35A**). These injections led to labelling mesolimbic neurons predominantly in the dorsal half of the VTA (**Fig. 34b**) and included both DA and non-DA neurons, while mesocortical neurons were located in the ventral part (**Fig. 35B**). We obtained a sample of 17 DA and 6 non-DA mesolimbic neurons that were recorded and labelled using the juxtacellular method and in which the retrograde tracer was also detected (**Fig. 36A** and **36B**). We were not able to find any DAergic mesocortical neurons responding to optogenetic activation of either PPN or LDT cholinergic axons.

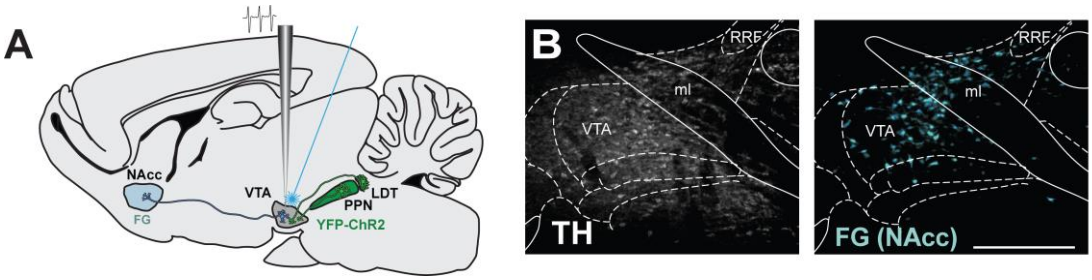

Figure 34: Retrograde tracers revealed mesolimbic neurons. (a) Schematic of the experimental design. Fluorogold (FG) was injected into the NA of ChAT::Cre+ rats that also received a virus injection into the PPN or LDT. **(b)** FG-labelled neurons were observed throughout the VTA, most prominently in the dorsal regions. Scale bars (in µm): **b**, 500.

The basal firing rate and action potential duration of mesolimbic neurons was not significantly different to that of neurons that did not contain the tracer (TH+, n = 43; TH-, n = 30; basal firing rate: TH+, U = 322, P = 0.854; TH-, U = 138, P = 0.495; action potential duration: TH+, U = 397.5, P = 0.649; TH-, U = 165.5, P = 0.171, Mann-Whitney). We observed that mesolimbic DA neurons had a greater excitatory response following LDT axon stimulation than following PPN axon stimulation (P = 0.04; **Fig. 37A** and **37B**).

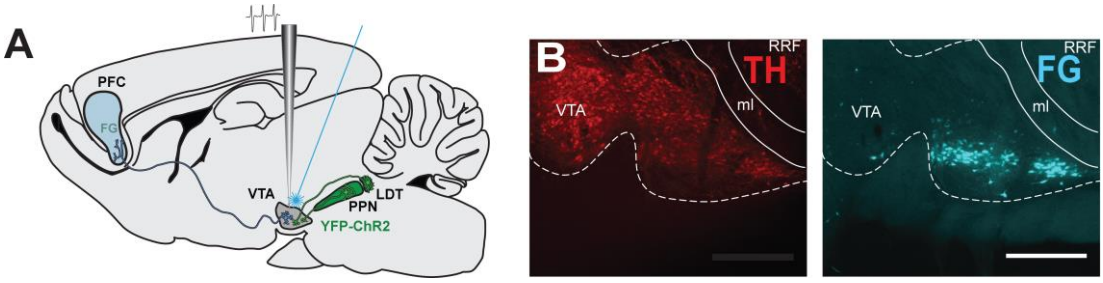

Figure 35: Retrograde tracers revealed mesocortical neurons. (a) Schematic of the experimental design. Fluorogold (FG) was injected into the prefrontal cortex (mPFC) of ChAT::Cre+ rats that also received a virus injection into the PPN or LDT. **(b)** FG-labelled neurons were observed throughout the VTA, most prominently in the ventral regions. Scale bars (in µm): **b**, 500.

Those DA neurons that increased their firing rate following PPN ChAT axon stimulation lacked the retrograde tracer (**Fig. 37C**), suggesting that they innervate a subset of neurons that project to other targets of VTA. In contrast, mesolimbic non-DA neurons were inhibited by LDT, but not PPN, ChAT axon stimulation (P = 0.003; **Fig. 37C**).

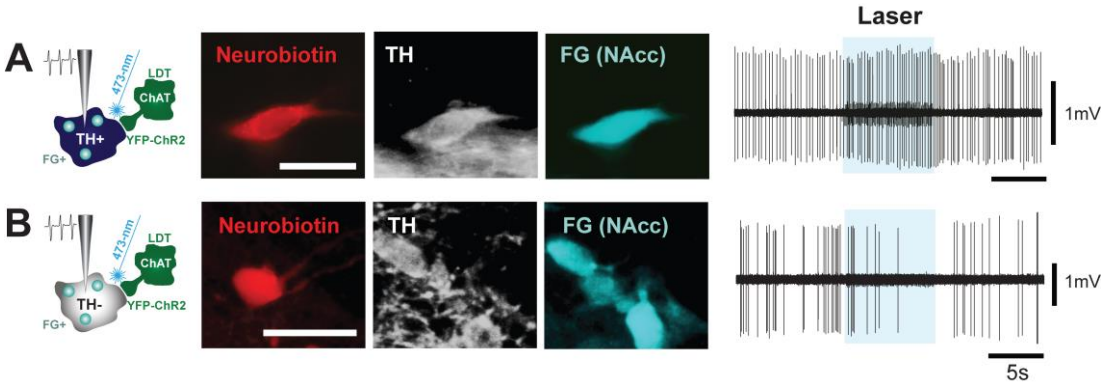

Figure 36: LDT cholinergic modulate mesolimbic DA and non-DA VTA neurons activity. (A) Example of a FG+/TH+ neuron that was excited by LDT axon stimulation. **(b)** Example of a FG+/TH- neuron that was inhibited by LDT axon stimulation. Scale bars (in µm): **a** and **b**, 30.

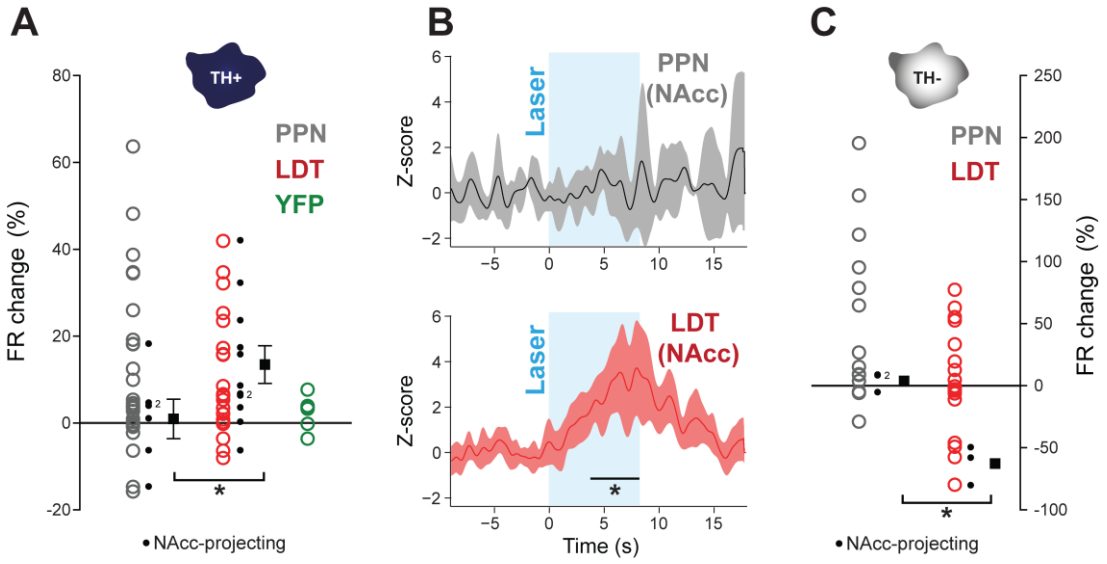

Figure 37: LDT cholinergic axons preferentially target mesolimbic DA and non-DA VTA neurons. (A) DA neurons that project to the NA were preferentially excited by the optogenetic stimulation of LDT cholinergic axons (n = 11). In contrast, PPN axon stimulation did not activate NA-projecting neurons (n = 6; t = -1.84, one-tailed t-test, P = 0.04 between PPN and LDT for NA-projecting neurons). Control experiments, in which animals were transduced with YFP only (no ChR2), did not show a response to the laser. **(B)** Normalised firing rate of all TH+/NA-projecting neurons following PPN or LDT cholinergic axon stimulation. Black line in the bottom panel represents the time points during which response to LDT stimulation was significantly greater than PPN (cluster-based permutation test; P = 0.02, 200 permutations). **(C)** Non-DA neurons that project to the NA were inhibited by LDT axon stimulation but not by PPN axon stimulation (t = 6.5, P = 0.003, although this is a small n). Black boxes represent means ± SEM of NA-projecting neurons in **A**, or only means in **C**.

Cholinergic axons differentiate between DA neurons involved in distinct functional circuits

Midbrain DA and non-DA neurons have been classically associated with reward mechanisms (Wise, 2004; Schultz, 2013). While the majority of DA neurons increase their firing in response to motivating stimuli and are consequently inhibited by aversive stimuli, a proportion of them are excited by noxious (aversive) stimuli (Mantz et al., 1989; Coizet et al., 2006). This can be emulated in the anesthetized rat by a hind paw pinch (Ungless et al., 2004) or foot-shock (Brischoux et al., 2009). Such differences in responses have been proposed to be associated with the functional pathways in which DAergic neurons are integrated (Lammel et al., 2012; Matsumoto and Hikosaka, 2009). We therefore correlated the responses of DA and non-DA neurons to the hind paw pinch with their responses to the optogenetic activation of the cholinergic axons arising in either the PPN or the LDT (**Fig 38**).

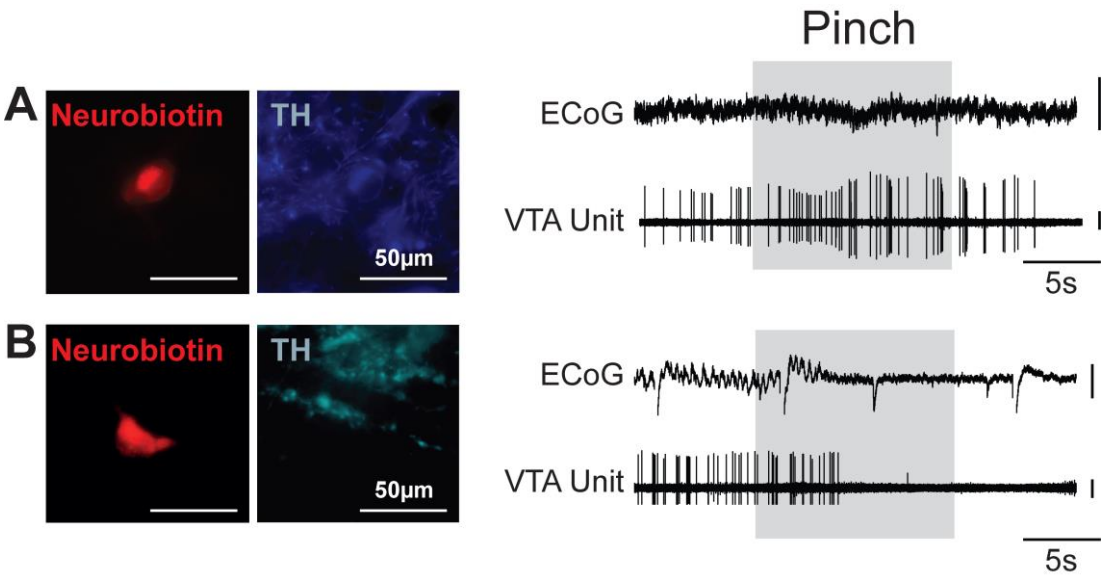

Figure 38: DA and non-DA neurons are responding to paw pinch. (A) Example of a TH+ neuron that was excited by paw pinch. **(b)** Example of a TH- neuron that was inhibited by paw pinch.

We observed an opposite trend in the effect of PPN and LDT drive: whereas PPN axon stimulation tended to modulate more consistently those neurons that showed a greater inhibition to the pinch (**Fig. 39a**), LDT axon stimulation more robustly modulated the neurons that were excited by the pinch (**Fig. 39b**). Indeed, 75% (6/8) of aversive stimuli-excited DA neurons increased the number of spikes within the bursts during LDT axon stimulation; in contrast 85% of aversive stimuli-excited DA neurons (6/7) decreased their number of spikes within bursts or switched to non-bursting mode during PPN axon stimulation. These results further support the notion that subsets of DA neurons that receive cholinergic afferents from the PPN and LDT are organised into functionally distinct pathways arising in the VTA.

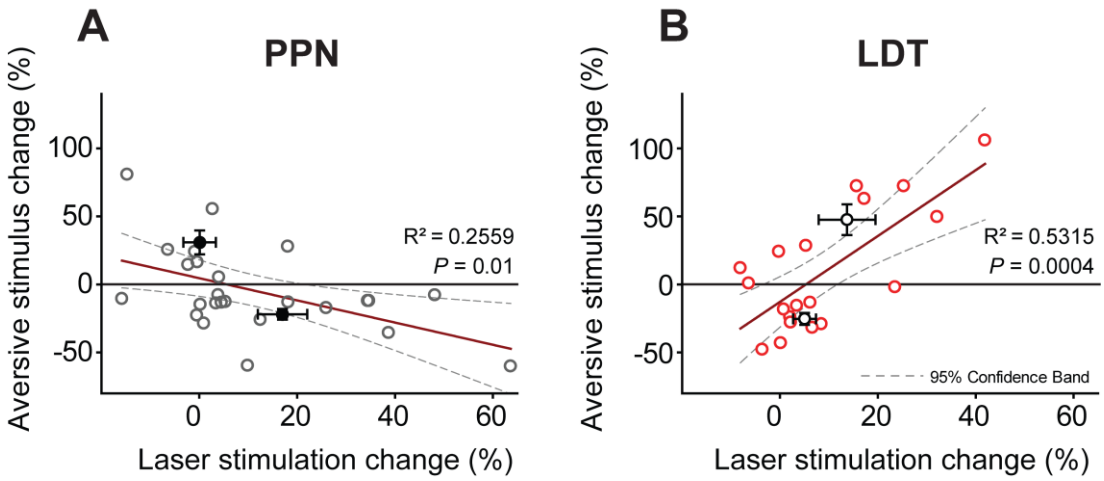

Figure 39: Cholinergic axon stimulation differentially modulates functionally distinct DA neurons. (a, b) Significant correlations were observed between the change in the firing rate of DA neurons during the hindpaw pinch (aversive stimulus) and their responses to the laser activation of PPN **(a;** n = 25**)** and LDT **(b;** n = 19**)** axons. Thus, DA neurons that are more inhibited by the pinch tend to respond more to PPN stimulation, whereas DA neurons that were excited by the pinch are more strongly modulated by the LDT. Means and SEM for positive or negative values in the change to the aversive stimulus are indicated by black (PPN) and white (LDT) circles with error bars.

Cholinergic axons stimulation is sufficient to slow down lever press extinction

Here, I examined whether optogenetic stimulations of cholinergic terminals in the VTA were necessary to maintain lever pressing previously associated with reward delivery. Two weeks following virus injection (AAV2-ChR2-eYFP, Fig. 40A) in the PPN or the LDT, we implanted an optic fibre above the VTA (Fig. 40B and C, 42A). Animals were first trained to press operant levers for sugar pellets on a variable interval schedule. On extinction, we replaced sugar pellet delivery with laser stimulation in a VI30 schedule (8 s, 10Hz, 50ms On/Off, 20mW, **Fig. 41A**).

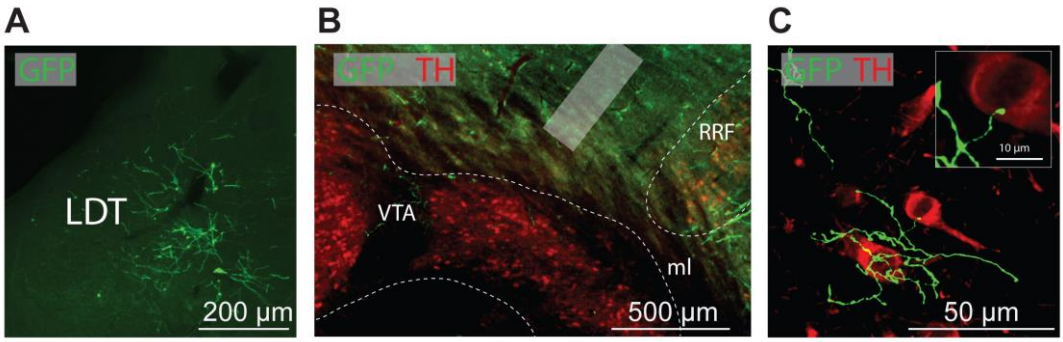

Figure 40: Optogenetic activation of cholinergic axons in the VTA in behaving rats. (**A**) Confocal images of transfected cholinergic axons in the LDT following injection of AAV2-ChR2-eYFP. (**B**) Confocal images of the position of the optic fibre during behaviour experiments. The white line represents the location of the optic fibre, DAergic neurons were stained in red and cholinergic axons from the brainstem in green. (**C**) Confocal images of DAergic neurons (TH; red) below the fibre track, and expression of AAV-ChR2-eYFP (cholinergic axons, green).

During the first 5 days of training, we saw a robust acquisition of lever pressing for sugar pellet (day effect) but no difference in lever pressing (**Fig. 41B**) between the PPN (n=12), LDT (n=10) and WT (n=10) groups (group effect). This was supported by a mixed day × group ANOVA (number of lever presses: day effect: $F(4,26)=4,254$, $p=0.009$; group effect: $F(2,29)=0.184$, $p=0.833$, interaction:

F(8,116)=1.362, p=0.221).

After acquisition we transferred the animals to a different but identical chamber used for optogenetic stimulation (see methods). We attached the head implant to a dummy patch cord and gave rats one last VI30 sugar pellet reinforced training session before extinction. We saw a small decrease in the number of lever presses between days but no group effect. This was supported by a significant day × group mixed ANOVA which produced significant effect of day (last day in old box vs new box, $F(1,29)=14.779$, $p<0.001$) but no effect of group ($F(2,29)=0.740$, $p=0.486$) or interaction ($F(2,29)=0.549$, $p=0.583$).

As expected, during extinction sessions in which the sugar pellets were replaced by optogenetic stimulation, we saw a reduction in lever presses across days in WT animals in which laser stimulation is expected to have no behavioural effect. However, this extinction effect was reduced in both LDT and PPN groups (**Fig. 41D**). During the 4 extinction sessions, we observed a significant decrease in lever pressing in all groups as confirmed by a day × group mixed ANOVA which produced a significant effect of day ($F(3,27)=15.747$, $p<0.001$), a significant effect of group ($F(2,29)=13.781$, $p<0.001$) and no interaction ($F(6,87)=0.997$, $p=0.433$).

We followed up the group effect with a Tukey posthoc test which showed that both PPN and LDT had a higher number of lever presses than the WT group (WT vs PPN: $p<0.001$, WT vs LDT: $p<0.001$). However the PPN and LDT groups did not differ significantly from each other (PPN vs LDT: $p=0.839$).

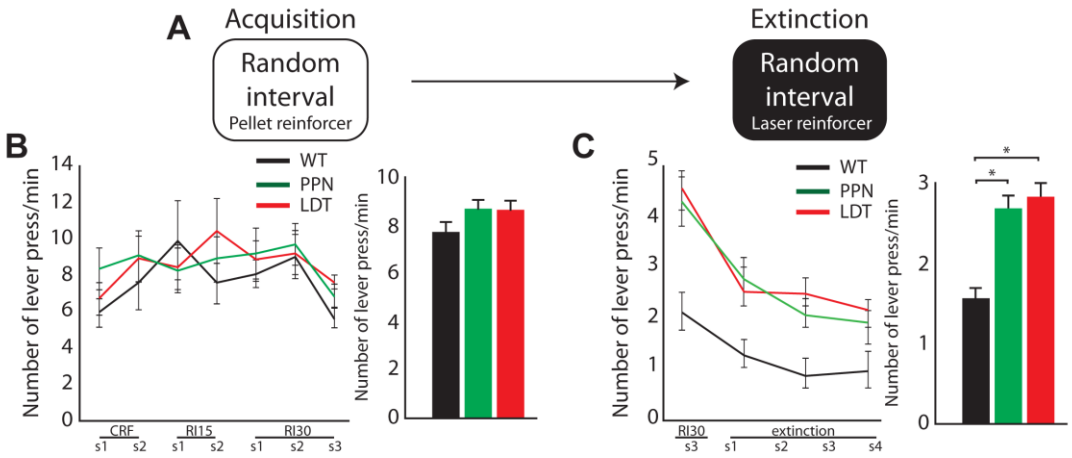

Figure 41: Behaving shift between sugar-outcome and laser stimulation-outcome. (**A**) Schematic of the within-subject behavioural design. Each rat was trained in a random interval schedule. On extinction, sugar pellets were replaced by optogenetic activation of PPN and LDT cholinergic axons in the VTA. (**B**) Number of lever presses per minutes during acquisition for WT (black), PPN (green) and LDT (red) animals. No differences were observed in the average number of lever presses (bar graph). (**C**) Number of lever presses per minutes during extinction for the three groups. PPN and LDT were pressing more the lever than WT during extinction sessions (histogram). *: significant difference from WT ($p<0.05$), #: significant difference between PPN and LDT groups ($p<0.05$). Values are shown as mean ± SEM.

Locomotion is differentially modulated by activation of PPN or LDT cholinergic terminals in VTA

Locomotion was assessed on three consecutive days for 30 min in ChAT::cre animals injected in the PPN (n=12), in the LDT (n=10) and WT animals (n=10). Rats were placed in a small holding cage for 5 min after being connected to the patch cord to reduce the effect of handling, after which they were transferred to the open field. Rats underwent 3 locomotion sessions (counterbalanced across rats): a) baseline: during this session, the animals were connected to the laser but did not receive stimulation; b) stimulation + vehicle: animals received a saline injection 30 min before testing, then received stimulation and c) stimulation + cholinergic antagonist: animals received a cholinergic antagonist cocktail 30 min before stimulation (see

methods).

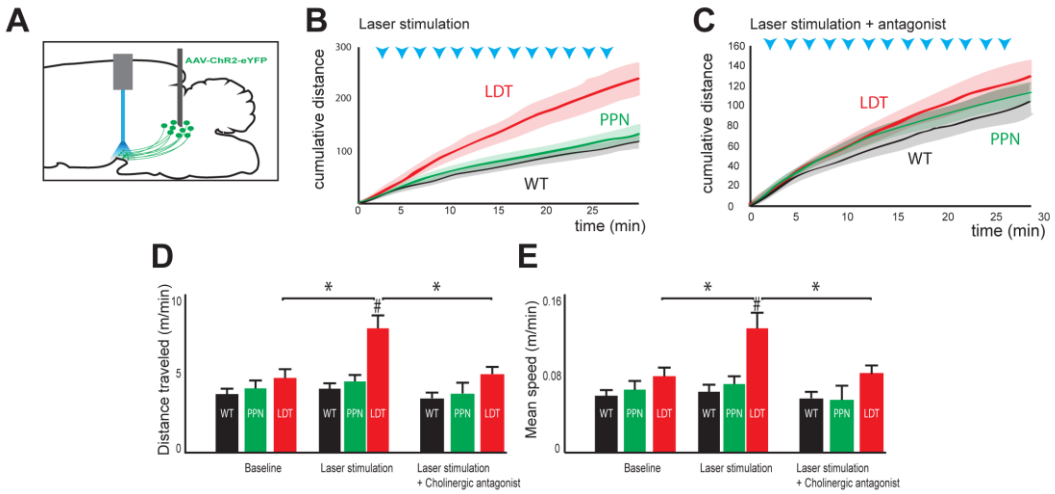

Figure 42: Optogenetic stimulation of LDT cholinergic axons in the VTA increases overall locomotion. (**A**) Schematic of the position of the optic fibre. (**B**) Cumulative distance travelled during laser stimulation of cholinergic axons in WT (black), PPN (green) or LDT (red). Blue arrows represent the laser stimuli. (**C**) Cumulative distance travelled during the 30 min interval after injection of cholinergic antagonist followed by optogenetic stimulation of cholinergic axons in WT (black), PPN (green) and LDT (red). Blue arrows represent laser stimuli. (**D**) Average distance travelled and (**E**) average speed during the three sessions. * is used for significant difference to baseline and drugs conditions ($p<0.05$), # is used for significant difference with WT group ($p<0.05$). Values are shown as mean ± SEM.

First, I analysed the effect of group and day on the distance travelled using a treatment (stimulation vs non-stimulation) × group (PPN, LDT, WT) mixed ANOVA. This ANOVA produced a significant effect of treatment ($F(1,29)=9.005$, $p=0.005$), a significant effect of group ($F(2,29)=6.027$, $p=0.006$) and interaction ($F(2,29)=6.594$, $p=0.004$). The group effect was followed up with a Tukey posthoc test which showed that the LDT group present significantly higher locomotion during stimulation + vehicle than both baseline and stimulation + cholinergic antagonist (WT vs PPN: $p=0.843$, WT vs LDT: $p=0.009$, PPN vs LDT: $p=0.024$).

Then I analysed the effect of group and treatment on the mean speed using a treatment × group ANOVA. This ANOVA produced a significant effect of treatment

(F(1,29)=5.702, p=0.038) and a significant effect of group (F(2,29)=5.702, p=0.008). This was followed up with Tukey post hoc test which showed that the LDT group present significantly higher mean speed during stimulation + vehicle than both baseline and stimulation + cholinergic antagonist (WT vs PPN: p=0.839, WT vs LDT: p=0.007, PPN vs LDT: p=0.018) (**Fig. 42**).

To confirm the cholinergic mechanism of the observed increase in locomotion, we tested whether selective blockade of cholinergic receptors in the VTA will disrupt the observed locomotor effect in the LDT group. Cholinergic blockade indeed reduced the effect of optogenetic stimulation (**Fig. 42C**). These results were confirmed by the non-significant treatment × group effect mixed ANOVA which produced a non-significant effect of treatment (baseline vs stimulation + cholinergic antagonist) (distance per minutes: F(1,19)=0.769, p=0.391; mean speed: F(1,19)= 1.043, p=0.320), no significant group effect (distance per minutes : F(2,19)=0.703, p=0.508; mean speed: F(2,19)=0.746, p=0.487) and no interaction (distance per minutes : F(2,19)= 1.405, p=0.270; mean speed: F(2,19)=1.746, p=0.201).

Thus, the LDT group showed increased locomotion in both distance travelled and mean speed. Next, we analysed the phasic effects of optogenetic stimulation by measuring activity in 5 sec bins over 20 sec before and 20 sec after stimulation.

First, for each of the 3 groups (PPN, LDT and WT) I compared the phasic locomotor response during stimulation normalised to the baseline (20 sec period immediately before each stimulation train). The effect of the stimulation was significant in both the PPN and LDT groups, but not in the WT group (distance normalised to the baseline; F(2,29)=364.306, p=0.006). This was due to a significant increase in locomotion in the PPN group, a significant decrease in the LDT group and no

significant change in the WT group (Tukey: WT vs LDT: p=0.044; WT vs PPN: p=0.029; PPN vs LDT p<0.001) (**Fig 43 A, C, E**).

Next I compared the phasic locomotor responses during stimulation on both vehicle and cholinergic antagonist sessions. The stimulation effects observed in LDT and PPN were absent during stimulation + cholinergic antagonist period. These results were confirmed by non-ignificant session × group (prior stimulation vs during stimulation) mixed ANOVA (distance normalised to baseline: $F(2,19)=1.412$ p=0.268,). The non-significant effect between groups was also confirmed by the post hoc Tukey test (WT vs PPN: p=0.465, WT vs LDT p=0.271, PPN vs LDT p=0.881) (**Fig. 43 B, D and E**), confirming the impression that both the PPN produced increase in locomotion and the LDT-produced decrease in locomotion were reversed by cholinergic blockade, confirming the cholinergic mechanism of the observed effects.

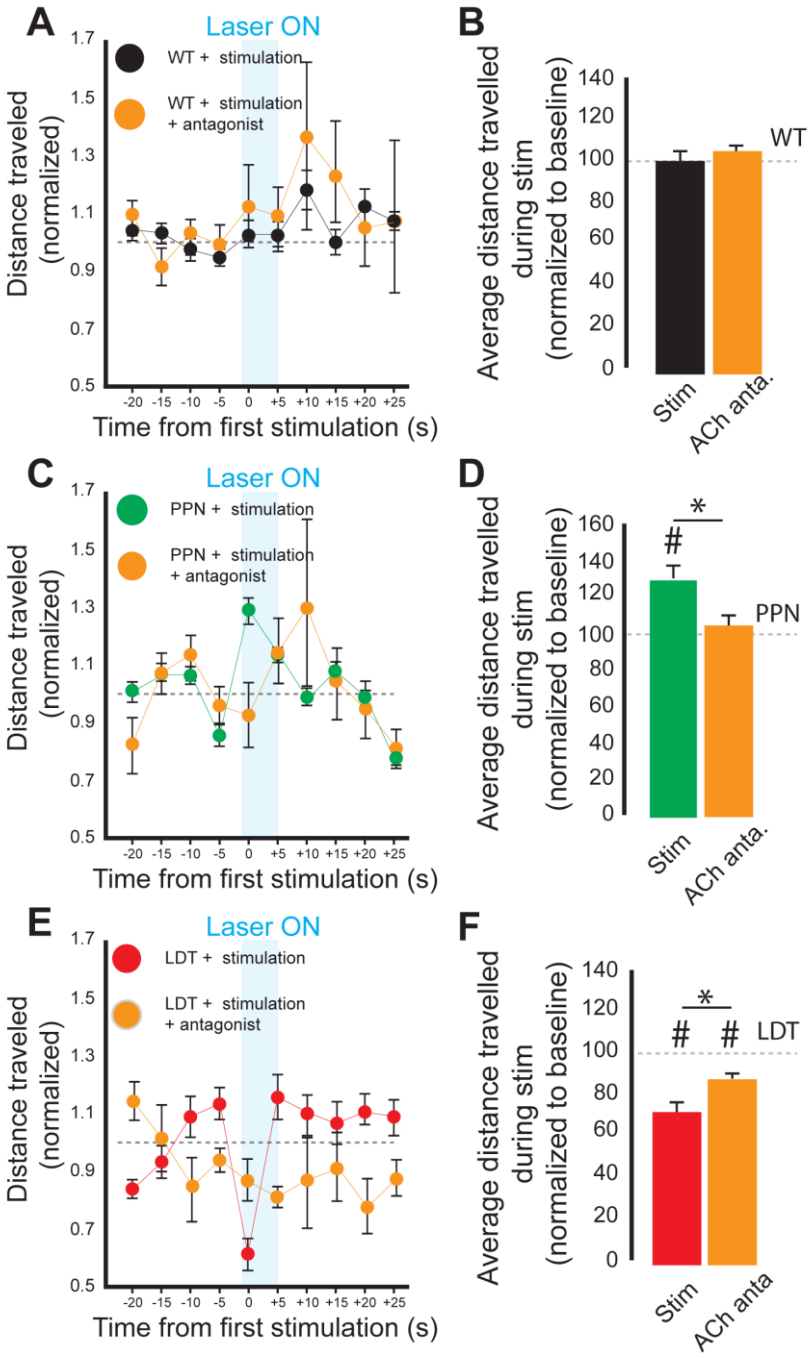

Figure 43: Phasic effects of optogenetic stimulation of PPN and LDT on locomotion. (**A**,**C** and **E**) Distance travelled (5 sec bins) (normalised over 20s before stimulations) in WT (**A**; black), PPN (**C**; green) and LDT (**E**; red) time-locked to laser stimulations (blue square). Distance travelled following injections of cholinergic antagonist in WT (**A**; orange); PPN (**C**, orange) and LDT (**E**; orange) and time-locked to laser stimulations (blue square). (**B**, **D** and **E**) Average distance travelled during laser stimulations fold over baseline in WT (**B**, black), PPN (**D**, green) and LDT (**F**, red) or following cholinergic antagonist injections in WT (**B**, orange), PPN (**D**, orange) and LDT (**F**, orange). * is used for significant difference to cholinergic antagonist condition ($p<0.05$), # is used for significant difference with distance travelled during baseline ($p<0.05$). Values are shown as mean ± SEM.

Conclusion

In this study, we demonstrate that two brainstem cholinergic nuclei, one typically associated with motor/arousal functions and the other with reward, have differential effects on subsets of neurons in the VTA. First, we observed that cholinergic neurons of the PPN and LDT project extensively throughout most of VTA. Second, stimulation of either of the two cholinergic pathways produces a slow modulation of the firing rate of DA and non-DA neurons; effects that are mediated by ACh. Third, cholinergic modulation of DA neurons takes on two different forms depending on the source of innervation: PPN axon stimulation switches the firing pattern to bursting mode in a proportion of DA neurons and increases the level of entropy in the spike train of most neurons (increases burst probability), whereas LDT axon stimulation enhances the organisation in bursting spike trains. Fourth, LDT axons predominantly target mesolimbic DA neurons that are excited by aversive stimulation, whereas PPN axons target a distinct subset of DA neurons which are predominantly inhibited by aversive stimulation. Finally, we observed that PPN and LDT axon stimulation are able to reduce the action-outcome-extinction by a rewarding effect and to differentially modulate locomotion. Our findings thus suggest that functional segregation of brainstem cholinergic neurons is maintained at the level of the VTA and is underpinned by differential modulation of subpopulations of DA and non-DA neurons.

Functional microcircuits in the VTA

DAergic neurons receive excitatory afferents from several regions of the brain, including the prefrontal cortex, the lateral hypothalamus and lateral preoptic area, and the brainstem (Sesack and Grace, 2009; Ikemoto, 2007). The projections

originating in the PPN and LDT are heterogeneous and consist of cholinergic, glutamatergic and GABAergic components (Bevan and Bolam, 1995; Charara et al., 1996; Omelchenko and Sesack, 2005). Although the PPN and the LDT are structurally and neurochemically similar (Wang and Morales, 2009), and indeed share some of their afferent and efferent connections (Woold and Butcher, 1986), they differ in the functional circuits to which they contribute. For example, the PPN is connected to structures involved in motor (including most regions of the basal ganglia; Mena-Segovia et al., 2004) and arousal functions (Steriade, 1996). On the other hand, LDT is connected to cortical and thalamic regions associated with the limbic system (Semba and Fibiger, 1992; Cornwall et al., 1990; Bolton et al., 1993). The VTA, in turn, is heterogeneous, with distinct distributions of DAergic neurons that project to different targets involved in different functional pathways (Lammel et al., 2012; Ikemoto, 2007). We thus hypothesized that the two cholinergic pathways would have different effects in the VTA and possibly differentially target subpopulations of DA neurons. The anatomical data shows that the projections from both the PPN and LDT innervate most of the VTA and indeed, DA and non-DA neurons that were modulated by PPN axons were intermingled in the same VTA regions as those modulated by LDT axons. Nevertheless, LDT cholinergic axons selectively targeted mesolimbic DA neurons, whereas PPN axons had little influence on them. In contrast, PPN axons primarily modulated DA neurons that are components of different circuits and whose targets are yet to be determined (e.g. amygdala, hippocampus). This suggests that neighbouring DA neurons can be differentially modulated by cholinergic afferents that encode either motor or limbic signals. The motor - limbic segregation is maintained in other brainstem cholinergic targets, most notably the Str: cholinergic LDT neurons that innervate the NA send

collaterals that innervate the midline thalamus and the VTA, both of which in turn also project to the NA (Dautan et al., 2014). This suggests that the cholinergic LDT neurons that modulate mesolimbic DA neurons also target postsynaptic structures in the NA and potentially converge with the axons of the same mesolimbic DA neurons that they modulate within the VTA.

We also observed that cholinergic LDT axons show a higher degree of collateralization in the VTA and contact a higher proportion of non-DA neurons than PPN cholinergic axons. Thus, LDT cholinergic neurons may have a greater influence on non-DA neurons than PPN cholinergic neurons. This suggestion may be consistent with some of our observations in the LDT optogenetic experiments: first, the 'late-responding' DA neurons (**Fig. 2h**) may represent a rebound excitation following the excitation of GABAergic interneurons, and second, mesolimbic non-DA neurons (putative GABAergic) that are inhibited by cholinergic activation (**Fig. 6d**) may act in coordination with DA neurons to reinforce DA transmission in the NA (e.g. by inhibiting cholinergic neurons in NA; Brown et al., 2012). Overall, our data demonstrate multiple functional mechanisms by which the cholinergic brainstem neurons may influence the activity of limbic circuits.

The involvement of cholinergic brainstem neurons in salience and reward

The two types of behaviour tested here (locomotion and extinction of lever pressing) are known to be modulated by DA transmission (Wise et al., 2004; Wise et al., 1987; Spyraki et al., 1982). The present results provide evidence that activation of brainstem cholinergic terminals in the VTA retarded extinction of lever pressing for food. These results confirm that activation of this system has rewarding properties.

We also showed that activation of cholinergic axons in the VTA results in modulation

of locomotion, with optogenetic stimulation of LDT cholinergic axons increasing overall locomotion but reducing phasic locomotion during single stimuli. On the other hand, PPN axon stimulation did not produce cumulative effect, but phasically increased activity. We showed that these effects were disrupted by cholinergic blockade.

The cumulative effect of LDT stimulation observed here is consistent with previous work showing that LDT lesions show disruption in locomotion produced by psychostimulant drugs (cocaine, nicotine) (Dobbs and Cunningham, 2014; Dobbs and Mark., 2012; Shabanni et al., 2010; Laviolette et al., 2000; Alderson et al., 2005; see review: Kohlmeier et al., 2013) suggesting that the mechanisms of LDT-produced increase in locomotion may implicate reward-related limbic circuitry (Clarke, 1990; Clarke, 1991; Baker et al., 2013). The phasic decrease in locomotion by stimulation of LDT afferents observed here is consistent with work suggesting that tonic stimulation of mesoaccumbens neurons decreased the locomotion during the stimulation only (Song et al., 2014; Hwan Kin et al., 2015). It is notable that the majority of neurons responding to LDT stimulations were projecting to the NA.

The phasic increase in locomotion by stimulation of PPN cholinergic terminals in the VTA observed here is consistent with previous studies showing that optogenetic burst-stimulation (>20Hz) of DAergic neurons in the VTA increase the locomotion during stimulation (Tye et al., 2013; Qi et al., 2014).

Cholinergic neurons of the brainstem are an essential component of the reticular activating system. Sensory stimulation triggers the activation of PPN cholinergic neurons (Mena-Segovia et al., 2008) that, in turn, activate neurons in their targets and, through the induction of fast frequency oscillations (Steriade et al.,

1991), increase the responsiveness of their target neurons enabling them to bind other modalities of stimuli (Munk et al., 1996). This suggests a role in salience, where cholinergic neurons signal the presence of potentially relevant cues that in turn increase the level of behavioural arousal (Pan and Hyland, 2005). Recent evidence, however, points to a more specific role of the PPN in coding reward and reward prediction error (Okada et al., 2009; Norton et al., 2011; Hong and Hikosaka, 2009), suggesting that PPN neurons also encode motivational value. Nevertheless, cholinergic neurons only constitute a fraction of the PPN, and indeed glutamatergic neurons may also modulate the activity of DA neurons, either directly or indirectly through cholinergic neurons (Good and Lupica, 2009). Glutamatergic neurons, however, have different connectivity and dynamic properties to cholinergic neurons (Mena-Segovia et al., 2008; Boucetta et al., 2014), suggesting that they are likely to have different, if not complementary, effects on VTA neurons. In view of the similarities between LDT with PPN (see above), it is quite possible that LDT neurons are also involved in the encoding of motivational value, but further evidence is necessary, including the unequivocal identification of the different cell types contributing to system physiology and behaviour.

Our findings also demonstrate that activation of brainstem cholinergic axons changes the bursting behaviour of DA neurons. Activation of PPN axons produced bursting in some neurons while increasing the ratio of spikes outside bursts in the majority of responding DA neurons. In contrast, LDT afferents reorganised the spike train into bursts. These differences may give clues to the specific functions of these cholinergic neurons. By switching the discharge mode of DA neurons, PPN afferents may be triggering a state change where neurons disengage from their preceding activity and increase their responsiveness to other inputs, in line with the notion of an

arousal system that generates orienting or attentional responses. In contrast, by increasing the number of spikes within bursts, the LDT increases the amount of information contained within each burst, equivalent to increasing the value associated with a reward prediction. Thus, the differences observed here may underlie the neuronal basis of saliency and reward at the level of the cholinergic brainstem and VTA neurons (see also Hong and Hikosaka, 2014).

In summary, cholinergic neurons of the brainstem provide a functionally segregated modulation of DA and non-DA neurons of the VTA, consistent with their connectivity with other structures within motor and limbic circuits in the basal ganglia and thalamus. Our findings thus demonstrate the importance of the cholinergic inputs for the modulation of DA neuron function. It remains to be established how brainstem cholinergic neurons work in concert with brainstem glutamatergic neurons at the level of both the midbrain and the Str to shape behaviour and to determine an organism's response to reward-related stimuli.

Chapter 4: Cholinergic circuits and modulation of striatal functions

Modulation of striatal neurons by cholinergic interneurons and cholinergic brainstem projections: an electrophysiological and behavioural study.

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DD, MV, TG, PB and JMS designed the experiments, analysed the data and wrote the manuscript. DD performed the surgeries and experiments. IHO performed the electron microscopy experiments. MC recorded 8 neurons reported below.

Abstract

ACh powerfully modulates neuronal computations in the striatum, contributing to its key role in action selection and associative learning. Despite their low number within the striatum, cholinergic interneurons (CINs) form large dendritic networks that extend across the whole striatum. Originally thought to arise exclusively from the CINs, ACh is also provided via the brainstem (Chapter 2). These cholinergic projections from the pedunculopontine (PPN) and the laterodorsal tegmental (LDT) nucleus topographically target subdivisions of striatum involved in behaviours. Using simultaneous local optogenetic activation of cholinergic terminals from PPN, LDT and CINs combined with juxtacellular recording, we showed that PPN and LDT inputs modulate different activities in the striatum by inhibiting MSNs activity and activating CINs firing. Pharmacogenetic inhibition of the ACh release in the DMS and DLS disrupts specifically goal-directed and habitual learning. All these results suggest a similar role of ACh arising from CINs and brainstem

Key words: Striatum, cholinergic interneurons, acetylcholine, DREADd, devaluation

Introduction

Abnormal functioning of the Str is implicated in a wide variety of psychiatric and neurological disorders, involving memory, locomotion or motivation (Kobb, 1992; Chudasama and Robbins, 2006; Berardelli et al., 1998; Delmaire et al., 2005). While up to 95% of striatal neurons have been described as medium spiny neurons (MSNs), the remaining 5% have been described as GABAergic and cholinergic interneurons (CINs). Str receives important thalamic, cortical and DA midbrain inputs that regulate the activity of MSN; however, interneurons also significantly modulate MSNs activity (Ebihara et al., 2013). In addition, different behavioural roles have been attributed to the Str and demonstrated a dissociation between dorsomedial (DMS) and dorsolateral (DLS) regions (Balleine, 2007; Ragozzino et al., 2002; Murray et al., 2012). The DLS is predominantly described as a sensorimotor structure and receives DAergic inputs from the *substantia nigra pars compacta* (SNc). The DMS plays a role in associative learning, and receives DAergic inputs from the SNc and the VTA (Ikemoto, 2007).

There is evidence that CINs significantly modulate striatal functions, and impaired cholinergic activity in the striatum has been proposed to play a role in the pathophysiology of PD, Huntington's disease, Alzheimer's disease or schizophrenia (Ding et al., 2010; Pisani et al., 2003; Holley et al., 2015; Albin et al., 1995). CINs receive inputs from local GABAergic interneurons, MSNs and GABAergic neurons from the midbrain, glutamatergic inputs from thalamostriatal, corticostriatal pathway, DAergic inputs from the nigrostriatal pathway, cholinergic inputs from other CINs, serotoninergic inputs from the dorsal raphe and noradrenergic inputs from the locus coeruleus (see Chapter 1 and review: Lim et al., 2014). CINs possess a very extensive axonal field and present a widespread dendritic tree that allows CINs to

integrate inputs from different regions and to project to a wide area in the Str (Gerfen et al., 1987; Berndse and Groenewegen, 1990; Lapper and Bolam, 1992; Rodriguez and Gonzalez-Hernandez, 1999; Ding et al., 2010; Brown et al., 2012; see review: Lim et al., 2014).

ACh released in the Str was traditionally thought to arise from a single source, the cholinergic interneurons (Wang et al., 2006; Ding et al., 2010). However, our recent studies demonstrated that the PPN and the LDT provide an extrinsic source of acetylcholine to the Str (Dautan et al., 2014; Dautan et al., 2015). These cholinergic axons make synaptic contact with spines and shafts, suggesting that MSNs and interneurons are receiving direct cholinergic inputs from the brainstem. Thus, as explained in Chapter 2, PPN cholinergic neurons target mostly the DLS and LDT the DMS and NA. These direct projections may constitute supplementary level of cholinergic modulation of the striatum activity in addition to the brainstem-midbrain-Str and the brainstem-thalamus-Str pathways (Dautan et al., 2015).

In vitro, optogenetic/pharmacology activation of CINs in the Str has been found to modulate DA release (Threlfell et al., 2012; Cachope et al., 2012) and MSNs firing activity (Koss and Tepper, 2002; Witten et al., 2010). CINs synchronisation is thought to arise from sensory thalamic afferents in response to salient stimuli with short-latency (200 msec) (Ding et al., 2010; Threlfell et al., 2012; Adler et al., 2013; Bertran-Gonzalez et al., 2012; Matsumoto et al., 2001). PPN and LDT neurons show short-latency responses to salient stimuli (<70 msec) (Kobayashi and Okada, 2007; Pan and Hyland, 2005) and also project densely to the thalamus (Mena-Segovia et al., 2008; Saper and Loewy, 1982; Hallanger et al., 1988; Parent and Descarries, 2008). The fast response of PPN and LDT cholinergic neurons to

salient stimuli and their projections to the thalamus suggest that PPN/LDT synchronise CINs activity indirectly in response to salient stimuli and this response can be mediated by the direct cholinergic projections in the striatum.

It has been suggested that PPN and LDT cholinergic projections to the striatum have varied and complex effects on striatal information processing. The role of PPN and LDT cholinergic projections, respectively, to the DLS and the DMS can be complementary to the role of local ACh in the activity of striatal neurons and also in the behavioural function of CINs located in the DMS and DLS.

We recently described direct cholinergic inputs to the striatum arising from the brainstem. The function of this pathway is unknown and can be associated with the function of CINs. Here, using a novel combination of optogenetic activation of cholinergic axons in the Str with *in vivo* single cell juxtacellular recording and labelling, we characterised the effect of ACh inputs activation on MSNs and striatal interneurons. Finally, we used a pharmacogenetic (designer receptors exclusively activated by designer drugs, DREADd) inhibitory approach to identify the role of striatal ACh arising in the brainstem or local interneurons on motivational and associative behaviour.

Materials and methods

Results

PPN and LDT cholinergic axons modulate the firing rate of MSNs and interneurons *in vivo*

We used an optogenetic approach to determine the effects of PPN and LDT cholinergic axon stimulation in the Str. In ChAT::cre^{+} rats (see Chapter 2-3) (**Fig. 44**), we stereotaxically injected in PPNr and LDT a Cre-inducible AAV vector carrying the excitatory opsin ChR2 and a sequence coding for the fluorescent protein eYFP. As previously reported (Dautan et al., 2014), eYFP expression was specific to cholinergic neurons and diffusion was limited to the PPN/LDT boundaries (**Fig 45**).

In vivo, PPN and LDT cholinergic neurons, respectively, fire phasically (Pan and Hyland, 2005; Petzold et al., 2015) or tonically (Lodge and Grace, 2006; Koyama et al., 1999) and are thought to target MSNs and interneurons in the Str (Dautan et al., 2014). First, we recorded single neuron activity with a micropipette in the DS, and the neuronal type was confirmed by juxtacellular labelling and immunohistochemistry. We recorded MSNs (as confirmed by the presence of spiny dendrites and cell body immunostaining for Ctip2) (PPNr: n=30, LDT: n=19, **Fig. 46 A and B**), CINs (as confirmed by the presence of large cell bodies and aspiny dendrites, and immunostaining for ChAT) (PPNr: n=13, LDT: n=8, Fig 47 A and B) and PV (as confirmed by small cell body size and aspiny dendrites and immunostaining for PV) (PPNr: n=8, LDT: n=9, **Fig 48 A and B**).

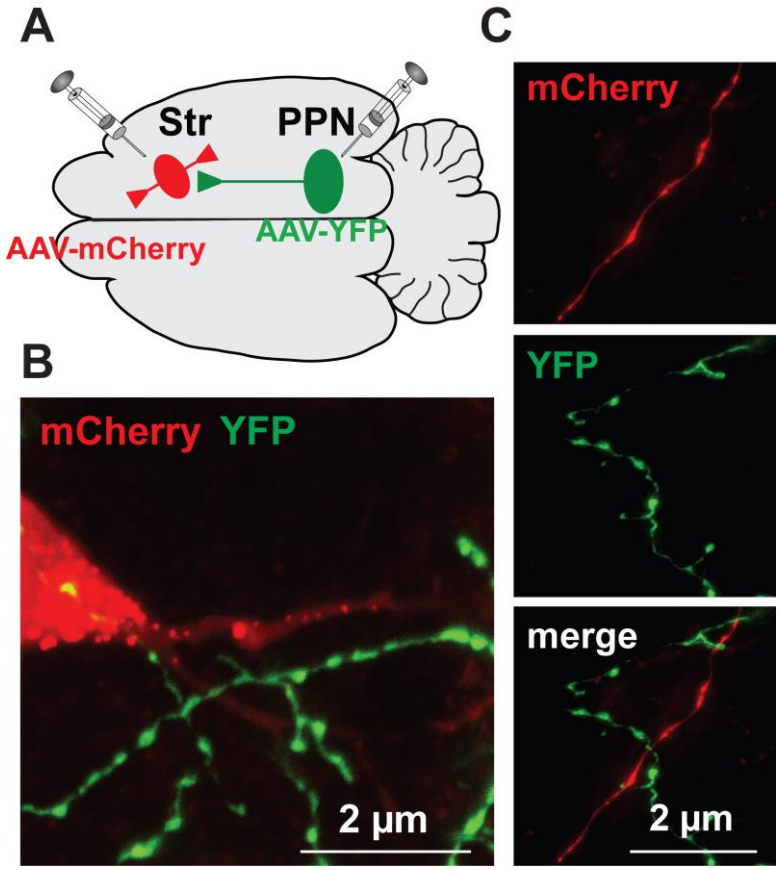

Figure 44: Brainstem cholinergic projections suggest contact with cholinergic interneurons. (**A**) Illustration of the double virus injections. AAV-mCherry was injected in dorsal Str in order to transfect cholinergic interneurons. AAV-eYFP was injected in the brainstem to transfect cholinergic terminals in the Str (**B** and **C**). Confocal images of putative contacts between cholinergic brainstem terminals in the Str (green) and cholinergic interneurons (red). Contacts appears to be proximal (**B**) and distal (**C**) to the cell body.

We found that all neurons responding to the stimulation were surrounded by YFP-positive terminals (**Figs. 46C, 47C, 48C**) (68 MSNs recorded, 49 were surrounded by terminals; 27 PV recorded, 21 were surrounded by terminals; 33 CINs neurons recorded, 17 were surrounded by terminals). As PPNr and LDT project to the dorsolateral and the DMS, respectively, neurons responding to the optical stimulation were topographically distributed, in PPNr experiments responding neurons were found in the DLS while in LDT experiments responding neurons were found in the DMS (**Figs. 46E, 47E, 48E**).

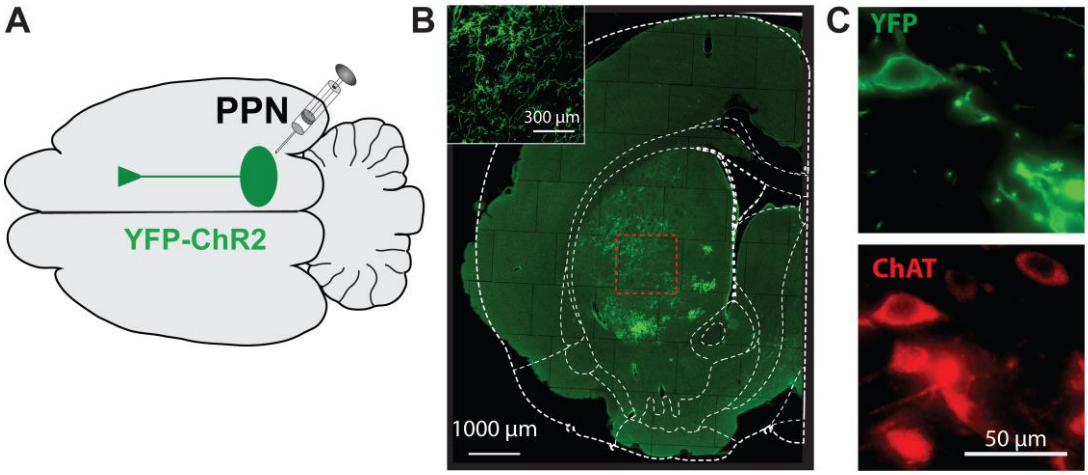

Figure 45: Cholinergic brainstem projection to the striatum. (**A**) Illustration of the virus injection in brainstem. (**B**) Virus injected in ChAT::cre rats brainstem showed dense eYFP positive projections in the Str that formed terminal patches (insert) and showed specific expression in $ChAT^{+}$ neurons (**C**).

Cholinergic brainstem modulation of MSNs

We first analysed the response of MSNs to optical activation of PPN and LDT cholinergic axons in the Str. We were not able to find any difference in the baseline activity (20s immediately before laser stimulation) of MSNs recorded in PPNr (DLS) and LDT (DMS) experiments (t-test: t(37)=0.230, p=0.820). During optogenetic stimulation (8 s, 80 pulses, 10 Hz, 50 ms ON/OFF) of PPN and LDT cholinergic axons, we observed a significant decrease of the firing activity of MSNs compared to the baseline. This was confirmed by a significant stimulation effect on one way ANOVA (PPN: F(1,38)=2.097 p=0.043; LDT: F(1,37)=2.21 p=0.033). We did not observe any differences in the responses of MSNs during PPN and LDT stimulation (t-test: t(37)=0.696 p=0.491). Following optogenetic stimulation, the MSNs activity increased slowly and returned to baseline (after up to 300s); no significant differences were apparent during the post-stimulation (30s following stimulation) period between LDT and PPN, as showed by the interaction effect (stimulation/post-

stimulation vs group: ANOVA: F(1,37)=0.023; p=0.982) (**Fig 46 D**).

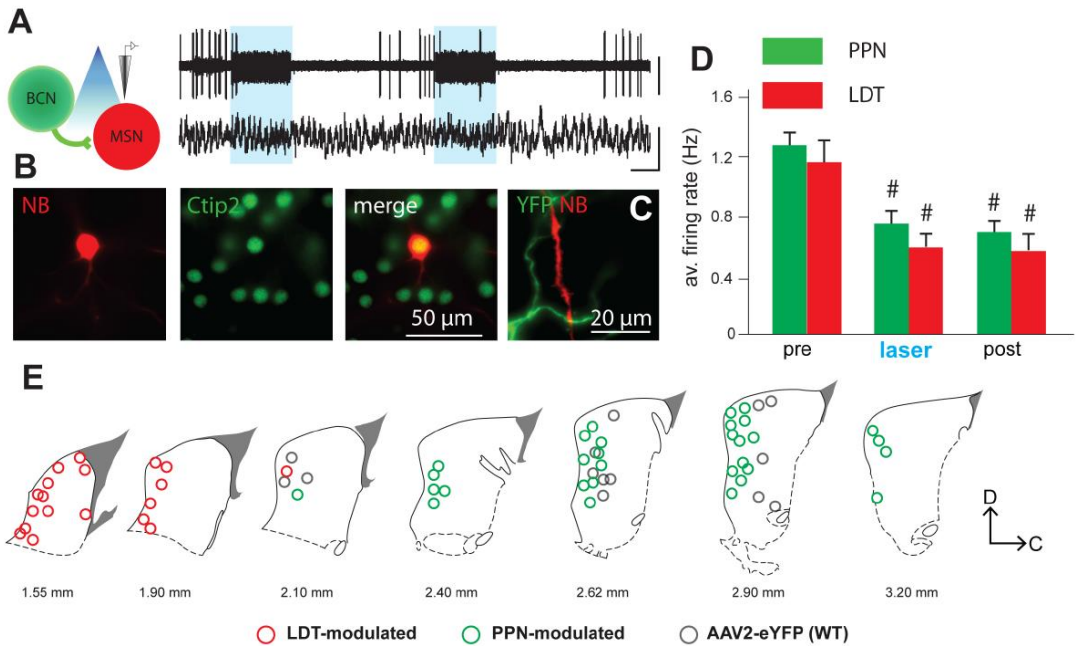

Figure 46: Cholinergic brainstem projections in the striatum inhibit MSN activity. (**A**) Brainstem cholinergic neuron (BCN) axons in the Str were activated with blue light (470 nm) *in vivo*. MSNs activity and ECoG were recorded during laser stimulation (blue square). Horizontal scale bars: 5 s; Vertical scale bars: 1mV. (**B**) Following recording, putative MSNs were labelled with neurobiotin (NB, red) and confirmed as MSN with Ctip2 staining (green). (**C**) Confocal images showing the proximity between identified MSN dendrites (red; see spines) and cholinergic axons from PPN (green). (**D**) Average firing frequency of identified MSNs before, during and after optogenetic activation of surrounding cholinergic axons arising from the PPNr (green) and the LDT (red). (E) Localisation of identified MSNs responding to PPN (green) and LDT (red) stimulation in the Str. Identified MSNs recorded in WT animals were represented in black. #: significant difference from baseline ($p<0.05$). Values are shown as mean ± SEM.

Cholinergic brainstem modulation of CINs

Next, I analysed the response of cholinergic interneurons to optogenetic activation of PPN and LDT cholinergic axons in the Str. No differences were observed in baseline activity (20s immediate before laser stimulation) of CINs recorded between experiments (t-test: t(19)=0.397, p=0.696). During optogenetic stimulation, most of the CINs showed a fast increase in firing rate during optogenetic stimulation of both

PPN and LDT cholinergic terminals in Str (PPN: ANOVA: $F(1,12)=23.97$ $p<0.001$, LDT: ANOVA : $F(1,7)=9.779$ $p=0.017$). No differences were found in the response to optogenetic stimulation between PPN and LDT (t-test: $t(19)=0.07$ $p=0.945$). Following stimulation, firing rates returned quickly to baseline. No differences were found in the post-stim firing activity (20s following stimulation) between PPN and LDT experiments (ANOVA: PPN: $F(1,12)=0.426$ $p=0.526$; LDT: $F(1,7)=0.006$ $p=0.940$) (**Fig .47D**)

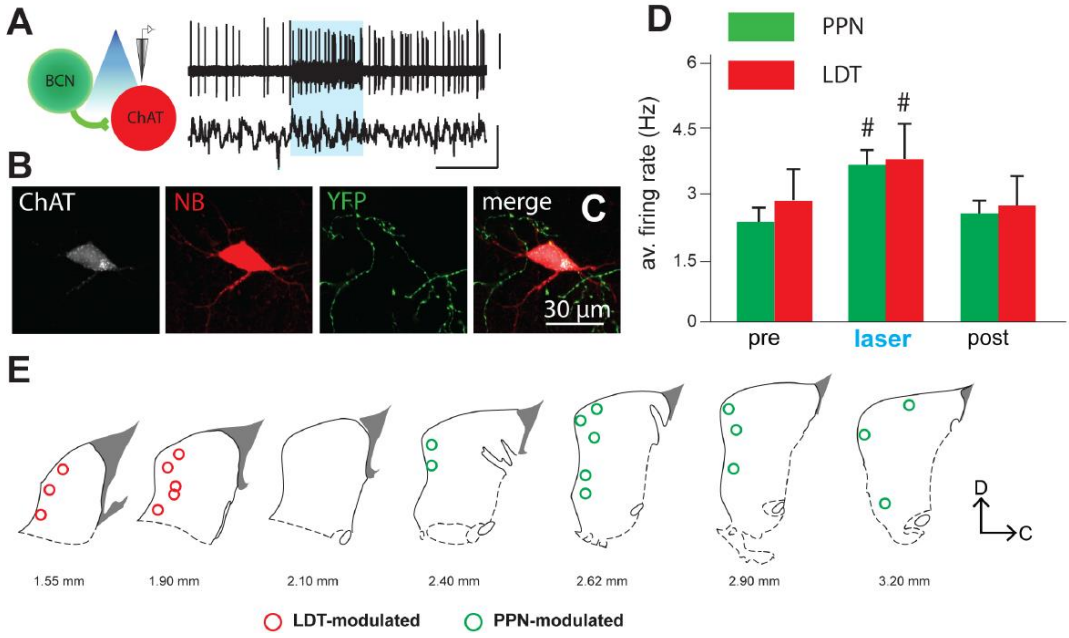

Figure 47: Cholinergic brainstem projections in the striatum activate cholinergic interneurons. (**A**) Brainstem cholinergic neuron (BCN) axons in Str were activated with blue light (470 nm) *in vivo*. CINs activity and ECoG were recorded during optogenetic stimulation (blue square). Horizontal scale bars: 5 s; Vertical scale bars: 1mV. (**B**) Following recording, putative CINs were labelled with neurobiotin (NB, red) and confirmed as CINs with ChAT staining (white); (**C**) Confocal images showing the proximity between identified CINs dendrites (red) and cholinergic axons from PPN (green). (**D**) Average firing frequency of identified CINs before, during and after optogenetic activation of surrounding cholinergic axons arising from the PPNr (green) and the LDT (red). (E) Localisation of identified CINs responding to PPN (green) and LDT (red) stimulation in the Str. #: significant difference from baseline ($p<0.05$). Values are shown as mean ± SEM.

Cholinergic brainstem does not modulate PV interneurons

Next, we analysed the response of FSI expressing the PV marker. No differences

were observed in the baseline firing activity (20s immediate before laser stimulation) of PV interneurons recorded during PPN or LDT experiments (t-test: t(15)=0.743, p=0.469). During optogenetic stimulation most PV^+ neurons showed no response to the optogenetic stimulation of PPN or LDT axons (PPN: ANOVA: F(1,7)=0.363 p=0.566; LDT: ANOVA: F(1,8)=5.32 p= 0.05) . We did not observe any difference in the response between PPN and LDT (t(15)=1.083, t-test: p= 0.296). No late response to the laser was observed by comparing post-stim (20s following stimulation) to baseline in PPN (mixed ANOVA: F(1,7)=0.315, p=0.592) or LDT experiments (mixed ANOVA: F(1,8)=3.72, p=0.09) (**Fig. 48D**).

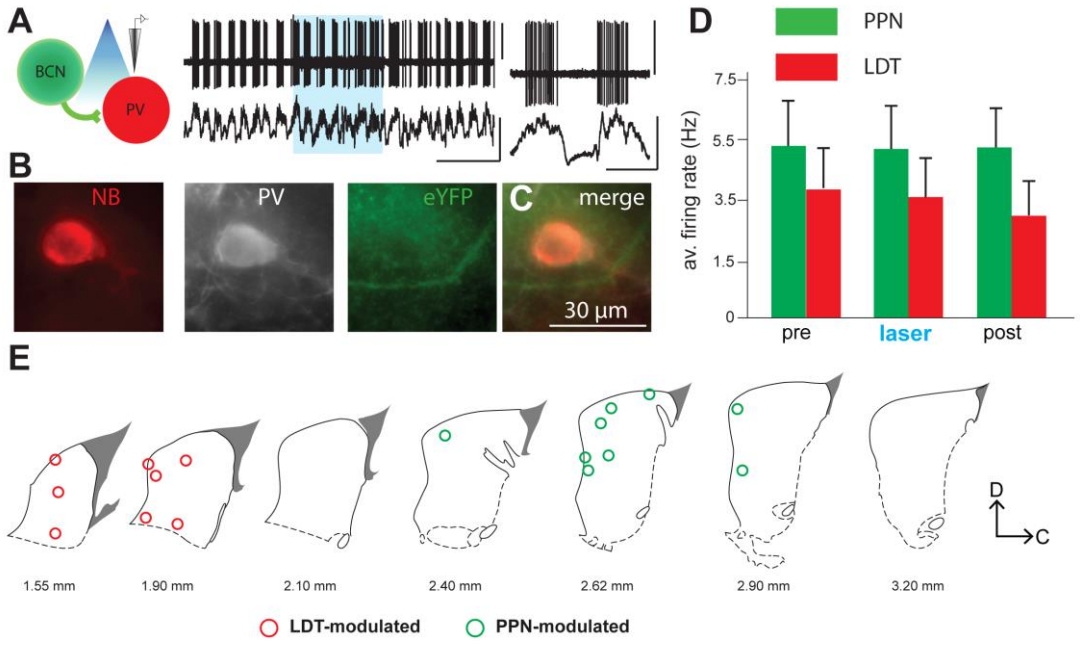

Figure 48: Cholinergic brainstem projections in the striatum do not change PV activity. (**A**) Brainstem cholinergic neurons (BCN) axons in the Str were activated with blue light (470 nm) *in vivo*. PV interneurons activity and ECoG were recorded during laser stimulation (blue square). Horizontal scale bars: 5 s; Vertical scale bars: 1mV. (**B**) Following recording, putative PV interneurons were labelled with neurobiotin (NB, red) confirmed as PV with parvalbumin staining (PV, white) (**C**) Confocal images showing the proximity between identified PV interneurons (red) and cholinergic axons from PPN (green). (**D**) Average firing frequency of identified PV interneurons before, during and after optogenetic activation of surrounding cholinergic axons arising from the PPNr (green) and the LDT (red). (E) Localisation of identified PV surrounded by PPN (green) and LDT (red) cholinergic axons in the striatum. Values are shown as mean ± SEM.

Optogenetic activation of CINs decreases MSNs and CINs activity

As previously reported, cholinergic brainstem terminals and CINs show different synaptic contacts in the Str; PPN synapses were mostly type 1 (asymmetric) while CINs synapses were mostly type 2 (symmetric) (Dautan et al., 2014). These synaptic contacts suggested an opposite modulation of Str neurons by CINs and PPN terminals. Indeed, type 1 synapses are mostly on dendritic shafts while type 2 synapses are mostly on cell bodies. It has been shown *in vitro* that CINs activation results in the inhibition of surrounding CINs (English et al., 2012; Sullivan et al., 2008; Witten et al., 2011). To determine whether CINs stimulation has a different effect on MSNs activity than PPN/LDT cholinergic axon stimulation, we stereotaxically injected in the Str the same virus used for brainstem experiments, and performed juxtacellular recording and labelling (**Fig. 51**).

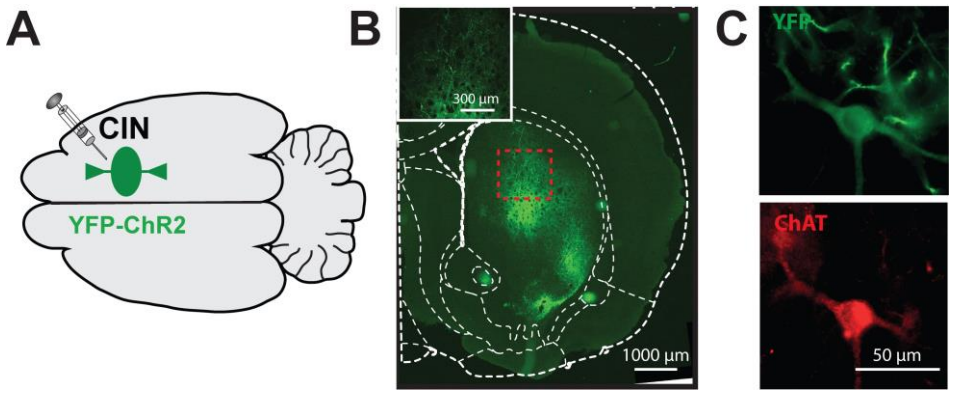

Figure 49: Cholinergic interneuron projections in the striatum. (**A**) Illustration of the virus injection in Str. (**B**) Virus injected in the striatum of ChAT::cre rats showed dense eYFP positive projections in the Str that formed dense distribution (insert) and showed specific expression in $ChAT^{+}$ neurons (C).

CINs modulation of MSNs

We first analysed the response of MSNs to optogenetic activation of CINs axons in

the Str. MSNs showed a significant decrease in their firing rate during stimulation compare to baseline (20s before stimulation). First, I analysed the effect of stimulation and group on the average firing rates using a mixed ANOVA. This ANOVA produced a significant stimulation effect ($F(1,37)=11.372$, $p<0.001$) and a non-significant group effect ($F(1,37)=6.032$, $p=0.713$) and no interaction. (**Fig. 52**).

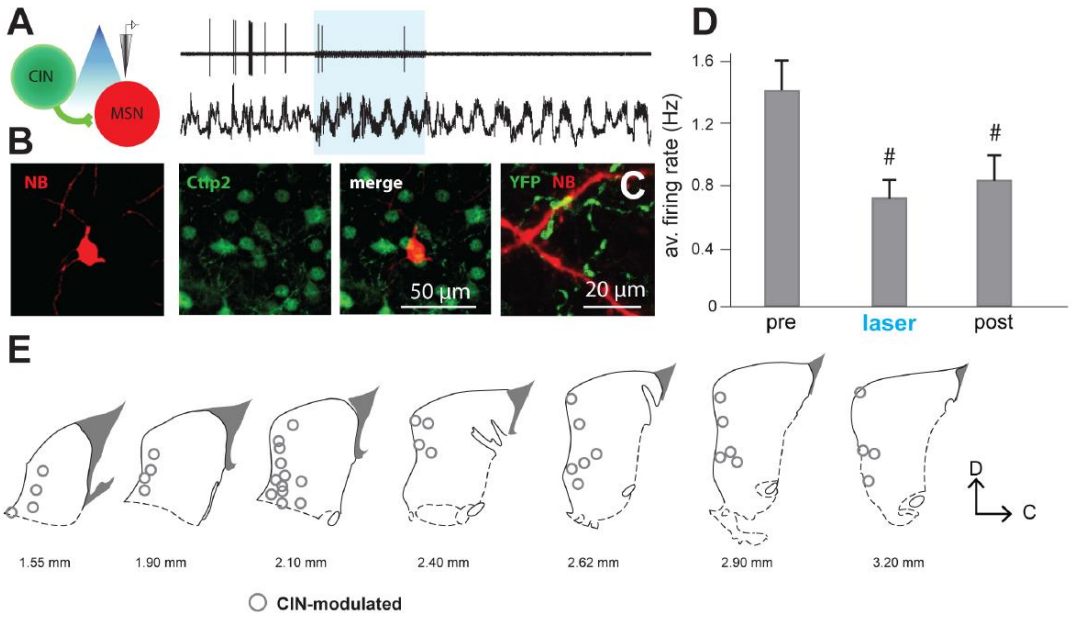

Figure 50: Cholinergic interneuron activation reduces MSNs firing. (**A**) Cholinergic interneurons (CINs) axons were activated with blue light (470 nm) *in vivo*. MSNs activity and ECoG were recorded during laser stimulation (blue square). Horizontal scale bars: 5 s; Vertical scale bars: 1mV. (**B**) Following recording, putative MSNs were labelled with neurobiotin (NB, red) and confirmed as MSNs with Ctip2 staining (green). (**C**) Confocal images showing the proximity between identified MSNs dendrites (red; see spines) and CINs axons (green). (**D**) Average firing frequency of identified MSN neurons before, during and after optogenetic activation of surrounding CINs axons. (**E**) Localisation of identified MSNs responding to CINs stimulation. #: significant difference from baseline ($p<0.05$). Values are shown as mean ± SEM.

CINs modulation of neighbouring CINs

In order to test the effect of CINs on other CINs, we analysed the response of CINs that are expressing the viral construct (GFP^+) and the response of those who are not expressing it (GFP-), during optogenetic activation of CINs. We observed no

difference in the baseline activity between CINs/GFP$^+$ (n=10) and CINs/GFP- (n=8). During optogenetic stimulation, CINs/GFP$^+$ neurons showed a significant increase by 54.7 ± 10.8 % of their firing activity compare to baseline, due to activation of the ChR2. In contrast, CINs/GFP- showed a significant decrease of their firing activity by 30 ±3.2% compare to baseline, due to autoinhibition of CINs. This was shown by the two-way ANOVA (response × cell type) which produced a non-significant response ($F(2,18)=1.17$, $p=0.132$), a significant cell type effect ($F(1,18)=9.65$, $p=0.001$) and an interaction effect ($F(2,18)= 5.503$, $p=0.003$). The cell type effect was followed up with a Tukey post hoc test which showed that CINs-eYFP$^+$ and CINs-eYFP$^-$ responses were significantly different ($p=0.002$). (**Fig. 53**).

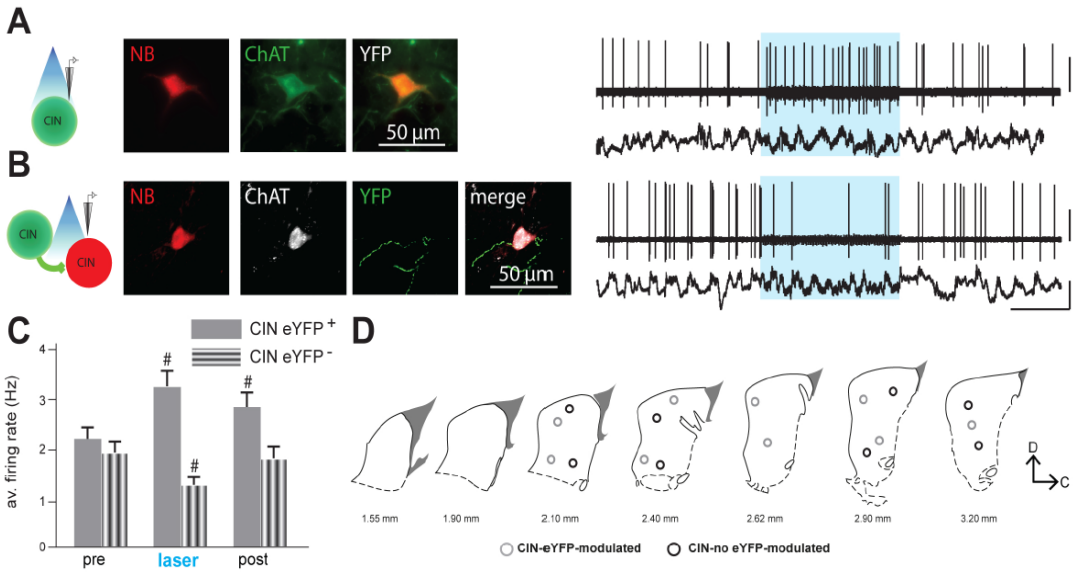

Figure 51: Cholinergic interneurons inhibit other striatal cholinergic interneurons. (**A**) Cholinergic interneurons (CINs) axons were activated with blue light (470 nm) *in vivo*. CINs/YFP$^+$ activity and ECoG were recorded during laser stimulation (blue square). (**B**) CINs/YFP$^-$ activity and ECoG were recorded during laser stimulation (blue square). Horizontal scale bars: 5 s; Vertical scale bars: 1mV. (**A** and **B** panel 3) Following recording, putative CINs, neurons were labelled with neurobiotin (NB, red) and confirmed as CINs with ChAT staining (white) and YFP+ or YFP- with GFP staining. (**C**) Average firing frequency of identified CINs/YFP$^+$ (gray) and CINs/YFP$^-$ (vertical line) before, during and after optogenetic activation of surrounding CINs axons. (**D**) Localisation of identified CINs/YFP$^+$ (gray) and CINs/YFP$^-$ (black) responding to CINs stimulation. #: significant difference from baseline ($p<0.05$). Values are shown as mean ± SEM.

CINs do not modulate PV interneurons

We then analysed the response of PV expressing neurons during the optogenetic stimulation of CINs and saw no significant modulation of their firing. We observed variation of the firing activity during optical stimulation compared to baseline. This was shown by the mixed ANOVA (stimulation × group) which produced a non-significant stimulation, group or interaction effect ($p>0.05$) (**Fig. 54**).

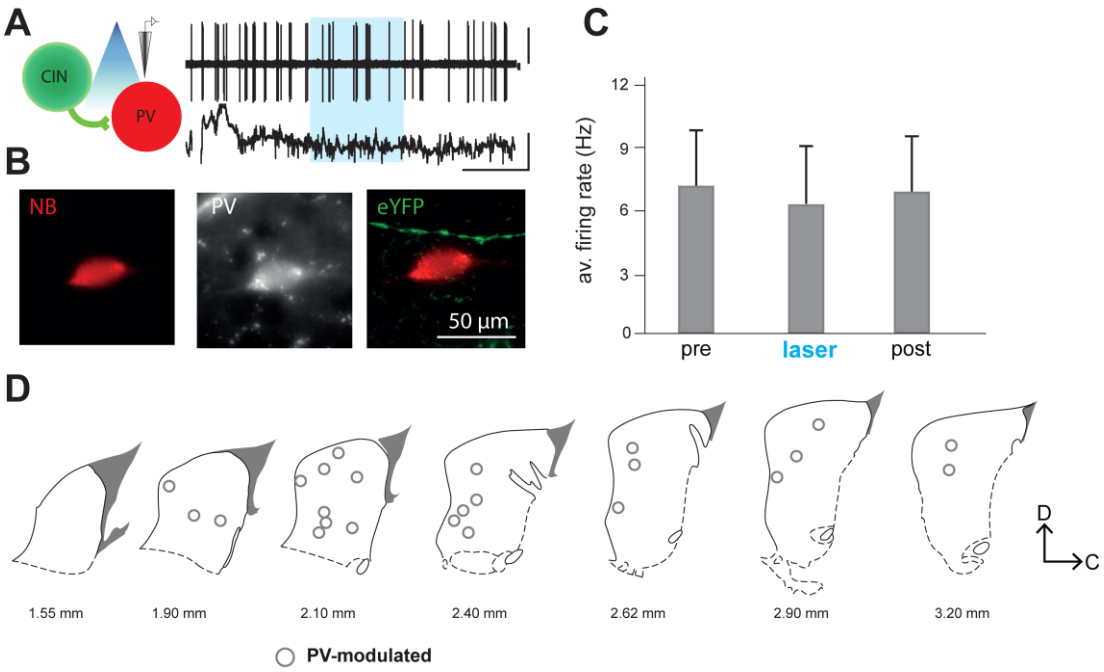

Figure 52: Cholinergic interneurons activation do not change PV interneurons activity. (**A**) Cholinergic interneurons (CINs) axons were activated with blue light (470 nm) *in vivo*. PV interneurons activity and ECoG were recorded during laser stimulation (blue square). Horizontal scale bars: 5 s; Vertical scale bars: 1mV. (**B**) Following recording, putative PV neurons were labelled with neurobiotin (NB, red) and confirmed as PV with parvalbumin staining (PV, white). (**C**) Confocal images of proximity between identified PV (red) and cholinergic axons from CINs (green). (**D**) Average firing frequency of identified PV neurons before, during and after optogenetic activation of surrounding CINs axons. (**E**) Localisation of identified PV+ neurons surrounded by cholinergic terminals (**B**). Values are shown as mean ± SEM.

We also compared the latency of the response of MSNs to optogenetic stimulation of

PPN and CINs cholinergic axons using z-score (see Methods Chapter 3). As PPN and LDT showed no significant difference in their responses, no analyses were performed for MSNs responding to LDT stimulation. We did not observe a significant difference in the baseline activity of MSNs recorded in PPN or CINs experiments, as shown by the non-significant one way ANOVA (group) effect ($F(2,76)=1.627$, $p=0.547$). We compared the firing activity of MSNs before, during and after optogenetic stimulation PPN, LDT and CINs (**Fig. 53A and B**). We observed a decrease in the MSNs activity during and after stimulation as shown by a significant mixed ANOVA stimulation effect ($F(1,76)=35.746$, $p<0.001$) but no group effect ($F(2,76)=0.344$, $p=0.710$) or interaction effect ($F(2,76)= 1.051$, $p=0.355$). No significant difference was found in the laser response latency, the firing rate change or the duration of the inhibition between PPN and CINs experiments (**Fig 55 C, D and E**).

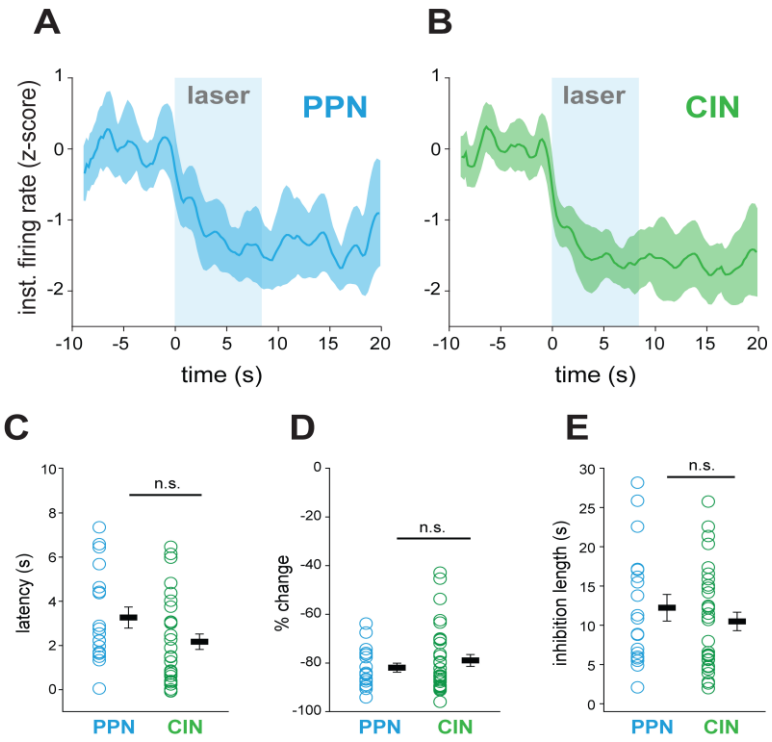

Figure 53: No difference on MSNs response was apparent between CINs or brainstem cholinergic stimulation. Normalised firing rate (z-score along the whole

trial period, see Methods Chapter 3) for each identified MSNs. Following PPN cholinergic axon (**A**) or CINs (**B**) stimulation MSNs showed similar decrease in firing. No significant differences were observed in the response latency (**C**), variation change (**D**) and inhibition length (**E**) between PPN (blue) and CINs (green) stimulation. Values are shown as mean ± SEM.

PPN, LDT and CINs are involved in action-outcome association

Electrophysiology data suggested that optogenetic stimulation of PPN, LDT and CINs cholinergic axon reduces MSN firing. Several studies showed an important role of striatal MSNs activity in operant behaviour (Dickinson, 1994; Gremel and Costa, 2013; Shan et al., 2014; Bradfield et al., 2013). The literature supports dissociation between DLS and DMS such that DLS mediates operant responding which is habit-driven and DMS mediates goal-directed behaviour. This can be demonstrated with outcome devaluation tasks where devaluting a reward (e.g. through satiety) reduces goal-directed but not habit-driven responding (Shiflett et al., 2010; Yin et al., 2005a; Yin et al., 2005b; Balleine and O'Doherty, 2009). Indeed, a shift in DMS MSNs activity has been observed during goal-directed training and the behaviour appears to be independent of the strength of this change. In addition, MSNs located in the DLS increase their synchronisation during habit-driven behaviour but not during goal-directed tasks. The switch between goal-directed and habit learning is believed to be associated with a switch in the MSNs synchronisation from DMS to DLS (Gremel and Costa, 2013).

Here we tested if the PPN, LDT or CINs activity in Str mediates goal-directed vs habit-driven responding. Optogenetic manipulation is not suitable for stimulating a structure such as the Str. In addition, prolonged inhibition by optogenetics is managed by continuous stimulation which increases the temperature at the tip of the optic fibre (Liske et al., 2013). For these reasons we decided to use pharmacogenetic

inhibition that allowed us to inhibit neurons spread over a large area for several hours (Stachniak et al., 2014) (**Fig. 56**).

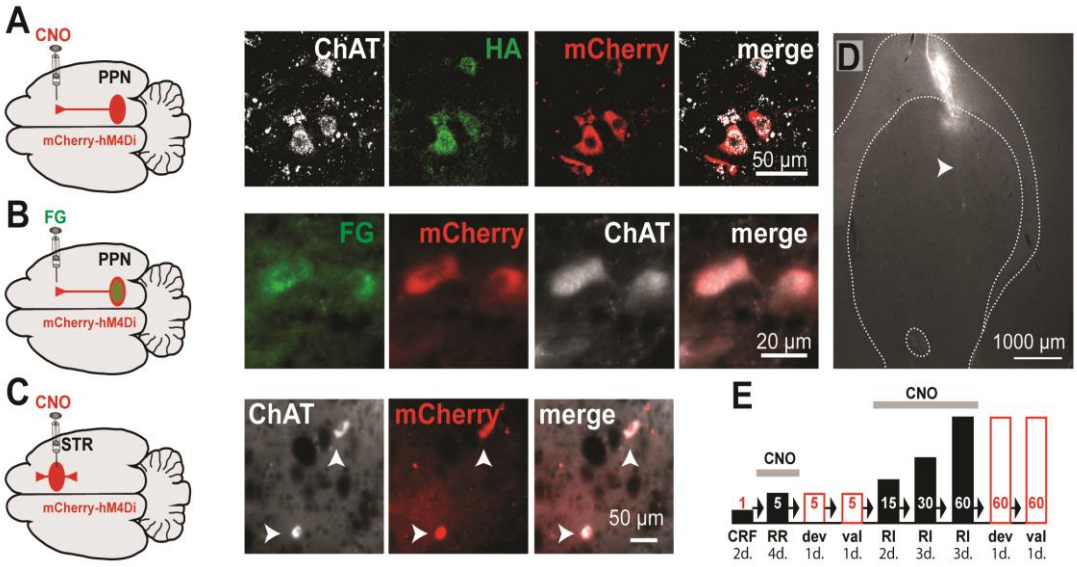

Figure 54: Pharmacogenetic inhibition of cholinergic activity in the striatum in behaving animals. (**A**) Injection of AAV2-hSyn-DIO-hM4Di-mCherry in PPN showed specific expression of mCherry (red) and the virus markers influenza hemagglutinin (HA; green) in cholinergic neurons (gray). (**B**) Following behavioural experiments, ChAT::cre animals injected in PPN and LDT received injection of the retrograde tracer fluorogold (FG) through the Str implanted cannula. Projections of virus positive (red) neurons to the Str were revealed by merging with ChAT (gray) and FG (green). (**C**) Injection of AAV2-hSyn-DIO-hM4Di-mCherry in Str showed specific expression (arrows) of mCherry (red) in cholinergic neurons (gray). (**D**) Location of intracranial cannula and injection site (arrow). ChAT::cre and WT animals were injected in DMS, DLS, PPN or LDT and implanted in dorsomedial (DMS and LDT group) or the dorsolateral (DLS and PPN group) for intracranial injection of CNO. (**E**) Schematic of the within-subject behaviour design. Animals underwent training to press a lever for sugar pellet during random ratio (RR) followed by a random intervals (RI) schedule. Between both schedules and at the end of the RI schedule, animals were tested on two consecutive days for devalued (dev) or valued (val) outcome as presented before (Gremel and Costa, 2013).

Goal-directed

Following CRF training, WT animals were tested for 4 days in a RR-5 schedule; animals received daily injection of CNO 1h before each session (**Fig. 54E**). During

RR-5 training, no significant differences in the number of presses were observed between WT animals that received viral injections in different nuclei (PPN, LDT, DLS or DMS) or implanted in different position (DMS or DLS). This was supported by a significant day effect ($F(3,16)=23.370$, $p<0.001$), a non-significant mixed ANOVA group effect ($F(3,16)=0.340$, $p=0.797$), and a significant mixed ANOVA group × day (4×4) interaction ($F(9,16)=2.090$, $p=0.049$) (**Fig 55A and B**).

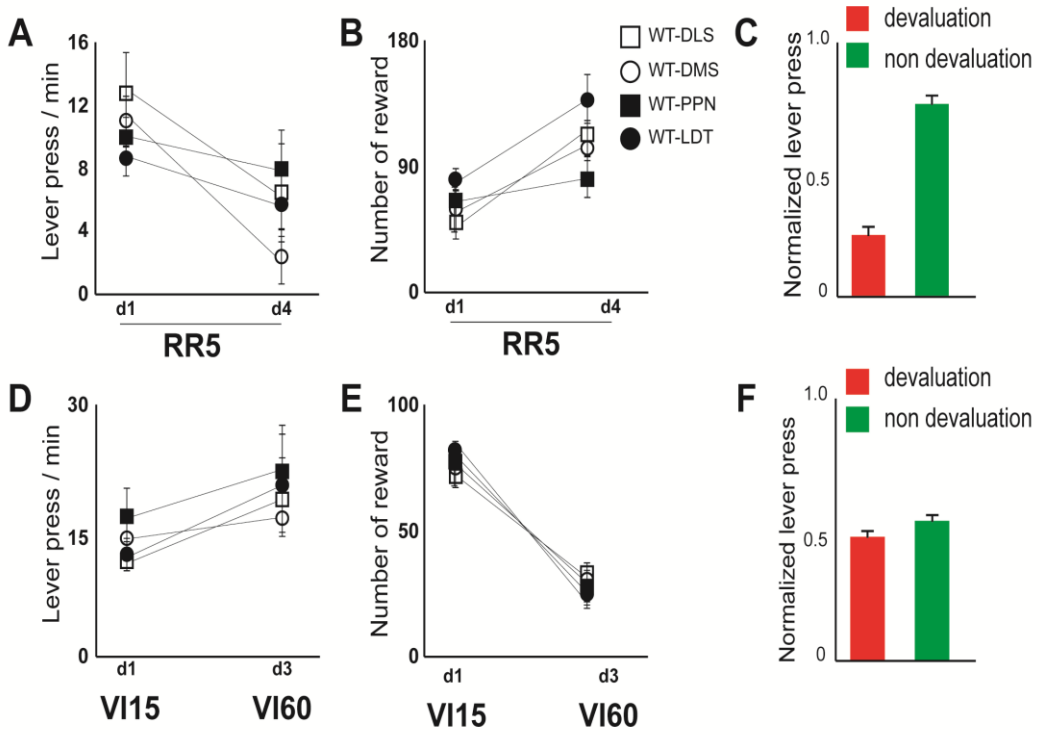

Figure 55: No differences were found in different groups of WT animals during acquisition and outcome devaluation. During random ratio training, no difference was observed in WT animals (group) in the number of lever presses (**A**) and the number of rewards (**B**). Following outcome-extinction, the normalised number of presses showed a difference between valued and devalued condition (**C**) suggesting a goal-directed behaviour. During random interval training, no difference was observed in WT animals in the number of lever presses (**D**) and the number of rewards (**E**). Following outcome-extinction, the normalised number of presses showed no difference between valued and devalued condition (**F**) suggesting a habit-driven behaviour. DMS n=5, DLS n=5, PPN n=5, LDT n=5. Values are shown as mean ± SEM.

We carried out outcome devaluation tests to assess the effect of training schedule on goal-direct vs habit-driven behaviours (**Fig 55 C**). We measured the number of non-reinforced lever presses in "valued" and "devalued" day; if a significant difference is observed, the training is considered as goal-directed (action directed by the outcome value); otherwise the training is considered as habit learning (action is not dependant of the outcome value). Following RR-5 training, WT animals showed a significant difference in the normalised number of lever presses. This was tested by a condition (valued vs devalued) × group (PPN-WT, LDT-WT, DMS-WT, DLS-WT) mixed ANOVA. This ANOVA produced a significant condition effect ($F(1,41)=21.124$, $p<0.001$) but no significant group or interaction effect (stats). The difference in lever presses suggests that following RR training WT animals showed sensitivity to devaluation, and therefore executed the action in a goal-directed manner (**Fig 55C**).

We did not observe any significant difference in the number of lever presses per minutes during training in ChAT::cre animals injected in the PPN (CRE-PPN, **Fig 56G**), LDT (CRE-LDT, **Fig. 56E**), DMS (CRE-LDT, **Fig 56C**) or DLS (CRE-DLS, **Fig. 56A**) as confirmed by the day × group mixed ANOVA effect (day effect: $F(3,41)=60.637$, $p<0.001$; group effect: $F(4,41)= 278.017$, $p=0.568$; day × group interaction $F(12,41)=36.51$, $p=0.218$). Analysis of the normalised lever presses during "valued" and "devalued" day in ChAT::cre animals showed a significant mixed ANOVA condition (valued vs devalued) effect in DLS, in DMS and in PPN. However, a non-significant one way ANOVA condition effect was found for LDT (DLS: $F(1,41)=19.946$, $p<0.001$, **Fig 56B** , DMS: $F(1,41)=12.9$, $p<0.001$, **Fig 56D,** PPN: $F(1,41)=4.512$, $p<0.001$, , **Fig 56F** , LDT: $F(1,41)=0.843$, $p=0.416$, **Fig 56H**). Analysis of the total number of presses showed no significant effect between groups as showed by the one way ANOVA group effect ($F(4,45)=2.110$, $p=0.097$). These

results suggest that LDT cholinergic axons in the DMS are needed for goal-directed learning, but not PPN, DLS or DMS.

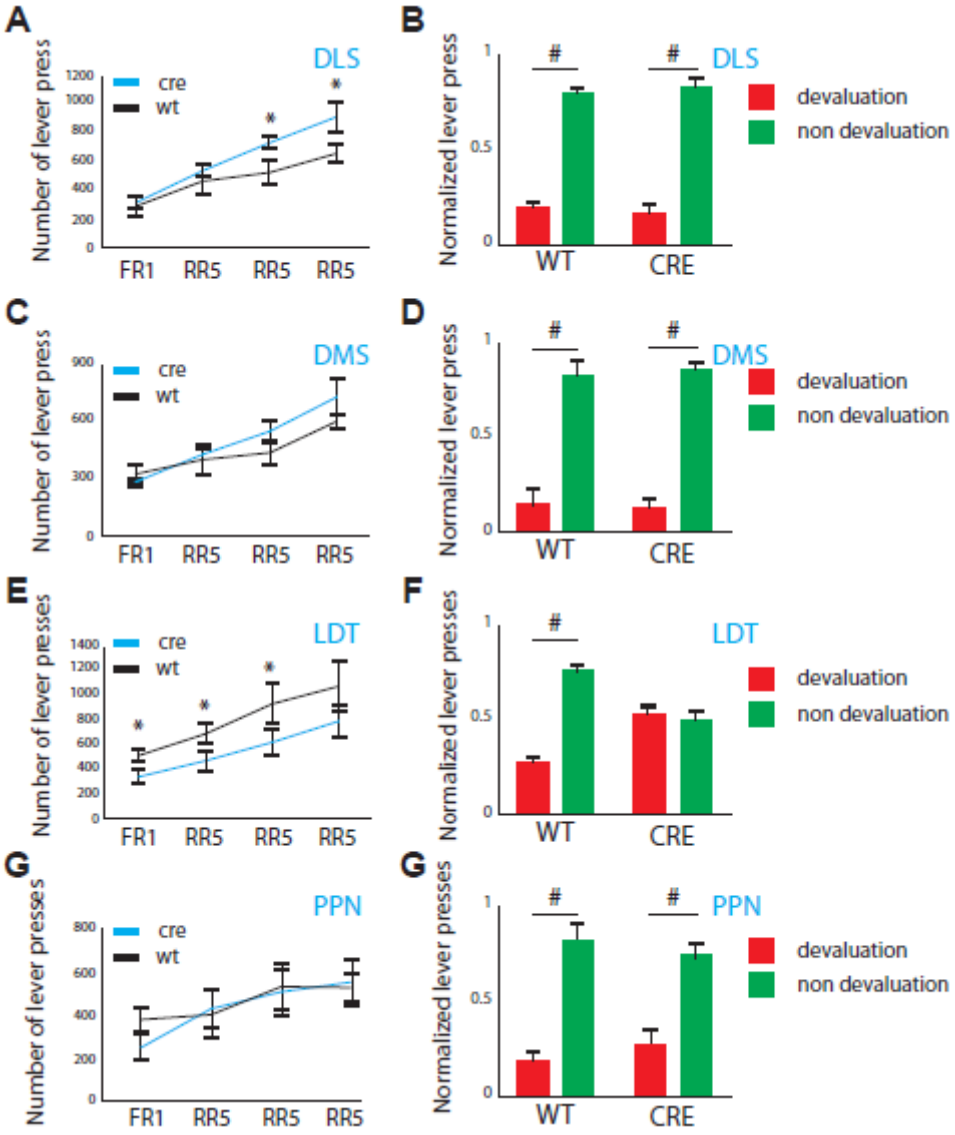

Figure 56: Cholinergic activity in DMS from LDT is needed for goal-directed acquisition. A significant difference was found in the RR acquisition between DLS, LDT and their relative WT control (**A** and **E**). Blocking of CRE-DLS activity showed significantly more lever presses than WT-DLS. CRE-LDT showed slowest acquisition than WT-LDT. No significant difference was found for DMS (**C**) and PPN (**G**) with their respective controls. Following outcome devaluation, a significant difference in the normalised lever presses value was found between valued and devalued conditions in CRE-DLS (**B**), CRE-DMS (**D**), CRE-PPN (**H**) and all control (**B**, **D**, **F** and **H**). No significant difference was found in the normalised lever presses value for CRE-LDT (**F**) suggesting an impairment of the goal-directed acquisition. *: significant difference between ChAT::cre and WT ($p<0.05$). #: significant difference between valued and devalued session ($p<0.05$). Values are shown as mean ± SEM.

Habit learning

We then retrained the animals in a progressive VI schedule (see methods, **Fig 54E**). As previously reported, no difference was found in VI acquisition in WT rats undergoing virus injections in PPN, LDT, DMS or DLS or cannulation in the DMS or DLS (**Fig 55D and E**), as shown by a significant day effect ($F(7,15)=18.279$, $p<0.001$), a non-significant mixed ANOVA group effect on the number of presses per minute ($F(3,15)=1.855$, $p=0.178$) and no interaction ($F(21,15)=0.919$, $p=0.567$). Following the last VI60 session, animals were tested on outcome-extinction "valued" or "devalued" schedule as before. No significant condition, group effect or interaction was observed (stats). This demonstrates that WT animals were insensitive to devaluation and were thus habit-driven (**Fig 55F**).

We did not observe any significant difference in the number of lever presses per minute in the CRE-PPN (**Fig 57G**), CRE-LDT (**Fig 57E**), CRE-DMS (**Fig 57C**) or CRE-DLS (**Fig 57A**) animals as confirmed by the mixed ANOVA effect (day: $F(7,41)=12.398$, $p=0.01$; group: $F(4,41)=441.487$, $p=0.267$; day × group: $F(28, 41)=0.969$, $p=0.435$). Analysis of the normalised lever presses during "valued" and "devalued" days in ChAT::cre animals showed a significant one way ANOVA condition effect in PPN and DLS, but not DMS and LDT (DLS: ($F(1,41)=3.543$, $p=0.03$**, Fig. 57B**, PPN: $F(1,41)=4.59$, $p<0.001$, **Fig. 57H**, DMS:$F(1,41)= 2.64$, $p=0,796$, **Fig. 57D**, LDT: $F(1,41)=9.57$, $p=0.357$, **Fig 57F**).

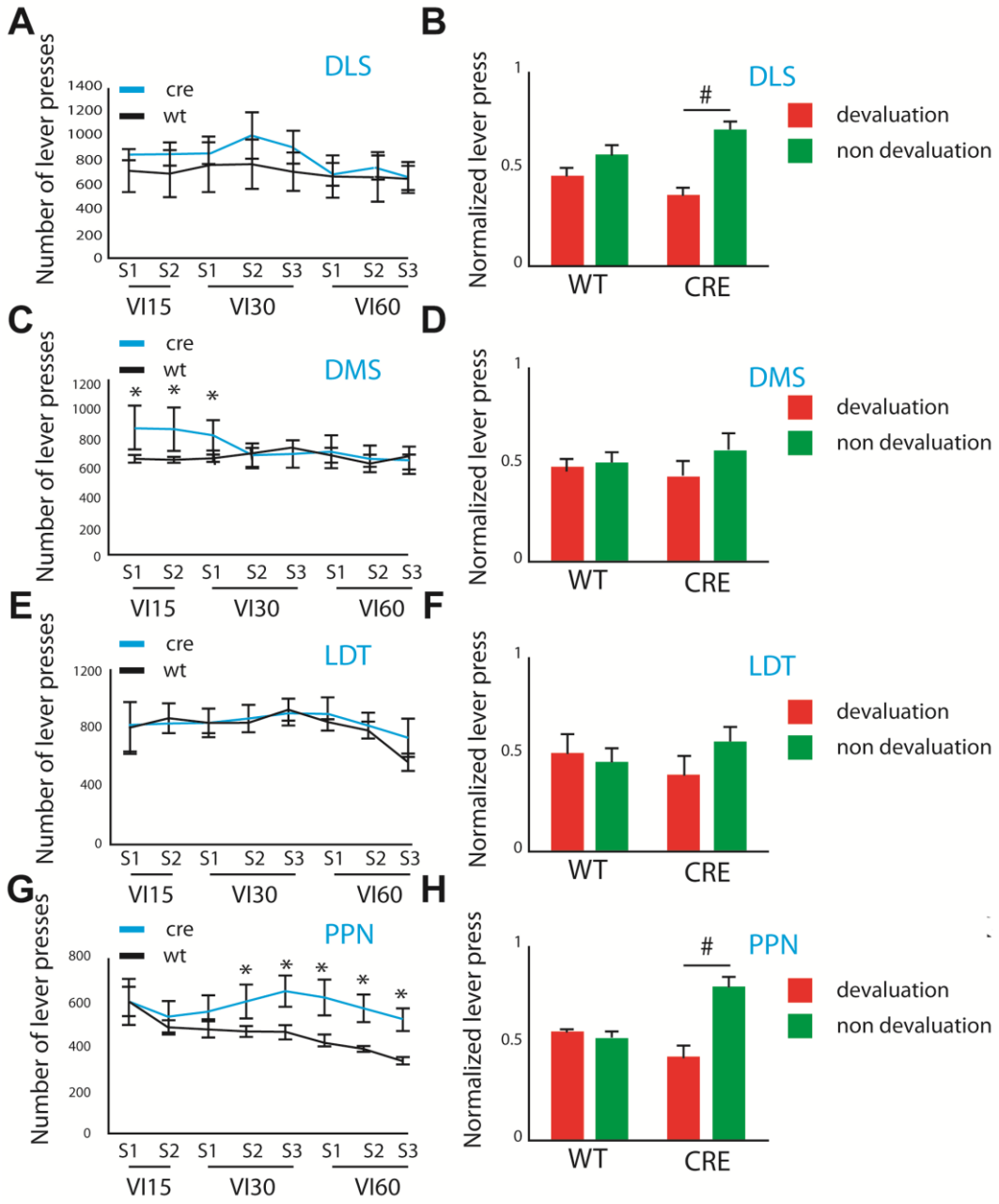

Figure 57: Cholinergic activity in DLS and PPN is needed for habit-driven learning. A significant difference was found in the RI acquisition between DMS, PPN and their relative WT control (**B** and **G**). Blocking of CRE-DMS activity showed significantly more lever presses than WT-DMS. CRE-PPN showed fastest acquisition than WT-PPN. No significant difference was found for DLS (**A**) and PPN (**E**) with their respective controls. Following outcome devaluation, a significant difference in the normalised lever presses value was found between valued and devalued conditions in CRE-DLS (**B**) and CRE-PPN suggesting an impairment of the habit learning. No significant difference was found in the normalised lever presses value for CRE-DMS (**D**), CRE-LDT (**F**) and all control (**B**, **D**, **F** and **H**). * Significant difference between ChAT::cre and WT ($p<0.05$). # Significant difference between valued and devalued session ($p<0.05$). Values are shown as mean ± SEM.

Analysis of the total number of presses showed no significant group effect ($F(3,45)=0.911$, $p=0.467$). These results suggest that cholinergic axons from the CINs in the DLS and from PPN are important for the switch between goal-directed and habit learning.

Reversal training

The outcome devaluation showed that the ACh activity in CINs located in the DMS was not needed for action-outcome association. However, the ACh from the LDT axons in the DMS appears to be necessary for goal-directed learning. To assess the role of DMS ACh in behavioural flexibility, we tested the same animals in a reversal task (Castane et al., 2010; Okada et al., 2011). For this, we retrained the animals on a FR1 schedule, where two levers were extended, but only one is rewarded (**Fig 60A, top**). On day 1, the lever used in previous training was rewarded whereas pressing on the other lever had no consequences. This was reversed on days 2 and 3 (**Fig 60A, bottom**). During reacquisition (day 1), no difference was observed in the total number of presses between all groups (WT, ChAT-PPN, ChAT-LDT, ChAT-DMS and ChAT-DLS) as shown by the one way ANOVA ($F(4,44)=1.504$, $p=0.219$). When levers were reversed (days 2 and 3), we observed a significant decrease of the number of rewards. As shown by the day1 × group mixed ANOVA (days effect: $F(2,80)=8.035$, $p=0.001$), group effect $F(4,80)=8.822$, $p<0.001$, day × group interaction ($F(8,80)=5.266$, $p<0.001$). Tukey post hoc tests showed significant differences between WT and DMS ($p<0.001$) and between WT and LDT ($p=0.041$). No significant difference was observed between WT and DLS ($p=0.486$) or between WT and PPN ($p= 0.998$). On the second reversal session the number of presses increased in all groups and showed a day effect (reversal1 vs reversal2:

F(1,40)=6.178, p=0.017), a group effect (F(4,40)=10.123, p<0.001) and a day × group interaction (F(4,40)=4.120, p=0.007). We followed up the group effect by post hoc Tukey tests, and saw a significant difference between WT and DMS (p<0.001) and between WT vs LDT (p=0.024). These results suggest that cholinergic activity in DMS arising from CINs or LDT slow down the place discrimination learning (**Fig 60B**).

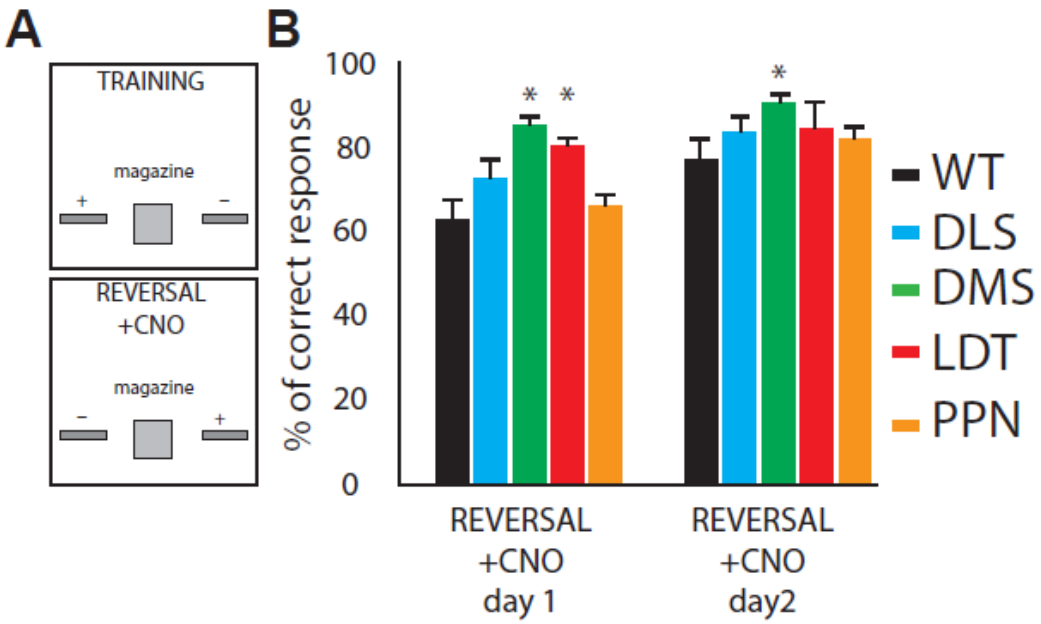

Figure 58: Blocking of acetylcholine release in the DMS by CINs or LDT enhance place discrimination. (A) Schematic representation of the reversal learning strategy. First animals injected in the DLS, DMS, PPN, LDT or controls were trained in a two-lever operant chamber to press one lever (correct; "+") for reward delivery (top panel). Following acquisition, both levers were inverted and animals received CNO injections. The positions of the initial correct levers were changed in a pseudorandom order. (B) Reversal flexibility was enhanced on the two consecutive reversal sessions in the rats injected in DMS and LDT and receiving CNO injection in the DMS. * is used for significant difference between mutants and WT (p<0.05). Values are shown as mean ± SEM.

Locomotion and sugar consumption

Both DMS and DLS are involved in locomotion and motivation (Wiltschko et al.,

2010; Schmidt et al., 1982; Clement et al., 2012). To rule out the contribution of overall motivation and locomotion on the effects observed above, we measured the total distance travelled in 15 min of open field sessions after CNO or saline injections in a new group of animals. No significant difference of the total distance travelled was observed between saline and CNO condition and between ChAT::cre-PPN and WT. These results were confirmed by the two-way ANOVA drugs × group showing a non-significant drug effect and a significant group effect ($F(2,12)=1.198$, $p=0.71$) (**Fig 59**). However, a significant difference between in locomotion PPN and WT groups was observed in saline conditions. No clear explanation could be found and data need to be replicated to confirm the effects. However, the experiment confirmed that CNO injected locally in the striatum does not have effect on the locomotion.

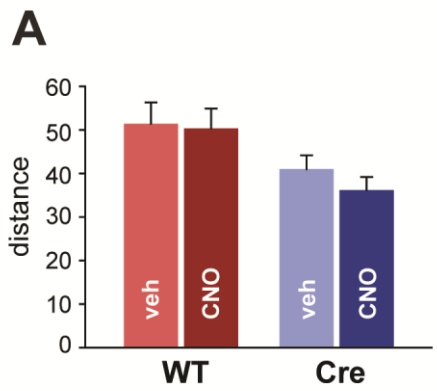

Figure 59: Blocking of cholinergic activity in DLS from PPN does not affect locomotion. Open field locomotion over 15 min session was recorded in ChAT::cre (blue) and WT (red) receiving CNO (deep red, deep blue) or saline (light red, light blue). No difference was found between WT and CRE and between saline and CNO condition in the total distance travelled (**A**). Values are shown as mean ± SEM.

Previously, non-specific lesions of PPN cholinergic neurons have been shown to reduce sugar consumption (Stefurak and Van der Kooy, 1994). Here, we recorded the

amount of sugar pellets (in g) consumed by ChAT::cre and WT animals injected in the PPN after CNO injection. We did not see any significant effects for group (F(1,11)=2.512, p=0.539), drugs (F(1,11)= 9.650, p=0.389) or interaction (F(1,11)=14.486, p=0.816) between PPN and WT (**Fig 63**).

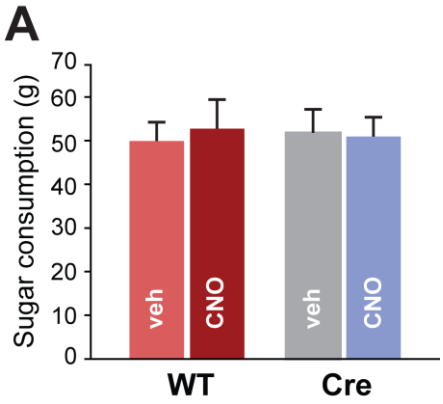

Figure 60: Blocking of acetylcholine release in the DLS from PPN does not affect sugar consumption. We recorded the amount of sugar consumed over 60 min by mutant (blue) and WT (red) receiving CNO (deep red, deep blue) or saline (light red, light blue). No difference was found between WT and CRE and between saline and CNO condition in the sugar consumption (g). Values are shown as mean ± SEM.

Discussion

By investigating the response of striatal neurons to optogenetic stimulation or pharmacogenetic inhibition of PPN, LDT or CINs axons *in vivo*, we made several observations. First, PPN and LDT cholinergic axons in the Str are functional, and optogenetic stimulation of their terminals inhibits MSNs activity and increases CINs activity. These findings support the observations made by electron microscopy and suggest that brainstem cholinergic axons in the Str make synaptic contact with MSNs and CINs (Dautan et al., 2014, see Chapter 2). In addition, we confirmed in an *in vivo* preparation the observation made from *in vitro* experiments (Witten et al., 2011) that activation of CINs produce an inhibition of MSNs and other CINs. The inhibition of CINs was fast and only apparent during optogenetic stimulation, while the response of MSNs was slow and extended after the stimulation. We also confirmed that CINs activation does not change significantly the activity of other GABAergic interneurons.

Using pharmacogenetic inhibition of cholinergic activity in the DLS and DMS, we observed parallel effects between PPN and CINs in the DLS, or between LDT and CINs in the DMS. Indeed, as previously confirmed, activation of CINs in DLS showed an important functional role in the habit learning (Tricomi et al., 2009), while the CINs in DMS showed no functional role in goal-directed learning (Bradfield et al., 2013; Shan et al., 2014). Furthermore, we showed that a decrease of the ACh activity arising from LDT in DMS impaired goal-directed acquisition, while the decrease of the ACh activity arising from PPN in the DLS impaired habit learning. This suggests that PPN cholinergic input in DLS is important for the switch from goal-directed to habit learning (Gremel and Costa, 2013). Next, we showed that inhibiting cholinergic activity in the DMS enhances behavioural flexibility in an

operant procedure, for both LDT and CIN inputs. Finally, by testing sugar consumption and locomotor activity, we confirmed that the impairment in goal-directed and habit learning is not due to a lack of motivation or to motor impairment.

MSNs activity is modulated by acetylcholine

MSNs are the only output of the Str, and have been shown to be differentially modulated by the inputs to the Str. Indeed, activation of DAergic axons in Str results in an increase of D1-MSNs activity, while D2-MSNs are inhibited (Surmeier et al.; 2007; Dreyer et al., 2010; see review: Keeler et al., 2014). Cortical and thalamic activation results in an increase of the activity of both D1- and D2-MSNs (Wilson, 1993; O'Donnell and Grace, 1995; Sharott et al., 2012, Ellender et al., 2013; Doig et al., 2010). Activation of cholinergic interneurons results in a strong inhibition of MSN activity (Witten et al., 2011; Tozzi et al., 2011). However, the synchronisation of the MSN activity appeared to be dependent on the activity of PV and CINs striatum interneurons (Damodaran et al., 2014; Mallet et al., 2006; Cui et al., 2013; Hjorth et al., 2009; Witten et al., 2011; Adler et al., 2013). Here, we showed that PPN and activation of LDT cholinergic projections will decrease the activity of D1- and D2-MSNs, suggesting the first inputs to the striatum that inhibit the direct and the indirect pathway in a similar way. In addition, we showed that the ACh arising from the brainstem plays a similar role on MSN activity than the ACh from CINs.

It has been shown *in vitro* that activation of CINs results in inhibition of the surrounding CINs probably by muscarinic M2 receptors and at a lower degree by M4 receptors (Ding et al., 2006). No functional role of excitatory cholinergic receptors in CINs has been observed (Bennett et al., 2000; Wilson et al., 2005; Sullivan et al., 2008). Here, the stimulation of brainstem cholinergic axons increases CINs activity

most likely by a nicotinic mechanism (M1 agonists do not produce significant effects on CINs activity). Brainstem cholinergic axons in the Str showed synaptic contact with spines, suggesting a direct contact with MSN. These synaptic contacts explain partially the response of MSNs to brainstem cholinergic axons stimulation. Cholinergic receptors have been found on GABAergic, glutamatergic and DAergic terminals (McGehee et al., 1995; MacDermott et al., 1999; Marchi et al., 2002; Threlfell et al., 2012). Indeed, brainstem cholinergic axons in the striatum can decrease MSNs activity through modulation of presynaptic AChRs. Indeed, activation of mAChRs reduce striatal glutamate release (Malenka and Kocsis, 1988) and decrease MSNs activity (Calabresi et al., 1998a). A more detailed analysis of the mechanism by which PPN/LDT cholinergic axons in Str modulate the striatal outputs seems to be necessary.

PPN and LDT are involved in behavioural functions implicating segregated striatal regions

Cholinergic axons from the brainstem to the Str appear to be highly organised (Dautan et al., 2014): PPNr cholinergic axons project to the DLS and LDT cholinergic axons project to DMS and NA. DLS has been described as having a sensorimotor function, while DMS shows an associative and limbic role (Sabol et al., 1985; Devan et al., 1999; Yin et al., 2004; Yin et al., 2005a). However, LDT cholinergic neurons projecting to DMS have collaterals to VTA and/or the parafascicular thalamic nucleus (Dautan et al., 2014). Similarly, single PPN cholinergic neurons can project to the SNc and/or the reticular thalamic nucleus. In parallel, PPN lesions impair sensorimotor-related behaviour (MacLaren et al., 2014), while LDT lesions impair limbic and associative functions (Winn, 2006; Nelson et al., 2007; Schmidt et al., 2009). Here, we showed that PPN and LDT cholinergic

projections to the Str are involved in the association between an action (lever press) and the outcome associated (reward). Similar observations have been found with lesioning or activating thalamic projection to DLS or DMS (Bradfield et al., 2013; Brown et al., 2010; Yin et al., 2005b) as well as as cortical-striatal projections (Gremel and Costa, 2013; Hollerman et al., 2000; Asplund et al., 2010). The role played by PPN/LDT cholinergic projections to the striatum might be complementary to the role played by PPN/LDT cholinergic projections to the VTA/SNc and the thalamus.

Finally, previous studies using electrophysiology and reversal training have showed goal-directed behaviour to be highly dependent on a balance between the synchronisation of MSNs in DMS and in DLS. In a goal-directed behaviour MSNs located in the DMS show higher synchronisation of their activity than the ones located in the DLS (Gremel and Costa, 2013). Here we confirmed that inhibition of LDT cholinergic projections in the DMS will retard the acquisition of a goal-directed behaviour, while inhibition of PPN cholinergic projections in the DLS, and inhibition of CINs in the DLS will decrease the acquisition of a habit-driven behaviour. These results suggest that LDT/PPN cholinergic projections to the striatum might modulate synchronicity of MSNs, respectively in the DMS and the DLS.

General discussion

Overall aims

The aim of this work was to examine the cholinergic modulation of the mesostriatal pathway, with emphasis on:

1) Electrophysiological properties and behavioural significance of the brainstem cholinergic projections to the dopaminergic midbrain.

2) Electrophysiological properties and behavioural significance of the brainstem cholinergic projections to the striatum.

3) Interactions of cholinergic inputs to the striatum with discrete cell populations.

Anatomical, electrophysiological and behavioural properties of the cholinergic brainstem projections onto VTA neurons

Firstly, I set out to describe the anatomy of the cholinergic projections from the brainstem to the midbrain and the striatum. The only source of ACh in the DAergic midbrain nuclei (**VTA/SNc**) has been described as arising from the brainstem, the **PPN** and the **LDT (**Holmstrand et al., 2011**)**. I observed no significant difference in the amount of VTA-cholinergic projections arising from the PPNc compared to the LDT. Optogenetic activation of PPNc cholinergic axons showed an excitatory response in DA neurons, a response that was maintained over the entire duration of the laser stimulation. The optogenetic activation showed both excitatory and inhibitory responses on non-DAergic neurons. We also observed that PPN cholinergic axon stimulation was sufficient to switch the firing pattern of DA neurons

in the VTA from non-bursting to bursting mode. Activation of LDT cholinergic axons in the VTA showed a similar excitatory response of DA neurons, but a larger proportion of neurons were responding to the laser stimulation compared to PPNc experiments. Some DA neurons showed a delayed response occurring after the stimulation of LDT axons, which was not observed in PPNc experiments. Significantly fewer non-DAergic neurons were activated during LDT cholinergic axon stimulation compared to PPNc experiments. In LDT experiments, a larger proportion of non-DAergic neurons showed a decrease of their spiking activity during laser stimulation compared to PPNc experiments.

During LDT axon stimulation, I observed that DA neurons which were bursting during baseline showed an increase in their bursting activity. However, during PPNc axons stimulation, I observed a switch from non-bursting to bursting mode of DAergic neurons. Increases in the number of bursts and the burst probability during LDT cholinergic axons stimulation seems to be due to an increase in the number of spikes within bursts and a decrease in the number of inter-burst spikes, while the changes in the burstiness of DAergic neurons during PPN cholinergic axons stimulation appears to be due to a disruption of the burst organisation. Further, I showed that the excitatory and inhibitory responses of DA and non-DA neurons during optogenetic activation of cholinergic axons were almost completely blocked by local iontophoretic injection of cholinergic receptor antagonists. Our electron microscopy, electrophysiological and pharmacological results suggest that the effects on VTA neurons are due to a release of ACh directly onto DAergic and non-DAergic neurons and not only due to a facilitation of glutamate or GABA release in the VTA, as previously suggested (Good and Lupica, 2009; Mansvelder and McGehee, 2000; Marchi et al., 2002). Using retrograde tracers, I showed that LDT cholinergic axons

innervate NA-projecting VTA neurons while the targets of PPN cholinergic axons in the VTA remain unknown. Some recent evidence suggests that PPN cholinergic axons target amygdala-projecting VTA neurons (Beier et al., 2015). Further, LDT input modulates DAergic neurons which are excited by aversive stimuli more strongly, while PPN axons show a stronger modulation of DA neurons that were inhibited by aversive stimuli (Brischoux et al., 2009; Ungless et al., 2004).

Lastly, I tested the effect of the activation of cholinergic axons in the VTA on behaviour. PPN cholinergic neurons have been shown to be involved in locomotor function, while LDT cholinergic neurons seem to be involved in reward-related behaviour. Interestingly, both motor and limbic functions are mediated by dopaminergic neurons in the VTA (Ungless et al., 2010). Some of these functions may depend on brainstem cholinergic input to the VTA. In combination with findings implicating LDT in psychostimulant related locomotion, the overall increase in locomotion found here appears to be related to an increase in dopaminergic activity. During stimulation, the increase of locomotion observed in PPN experiments and the decrease of locomotion observed in LDT experiments seems to be related to a short orienting response. Further, in a lever press task, stimulation of both LDT and PPN cholinergic inputs to the VTA retarded extinction.

Anatomical, electrophysiological and behavioural properties of the cholinergic brainstem projections onto striatum neurons

Following the description of cholinergic projections of the PPN and the LDT (Chapter 2), I found that the cholinergic neurons of the PPN form not only the cholinergic input to the VTA/SNc but also provide a major cholinergic innervation of the DS and the NA. I observed that those projections were not only avoiding the Str

striosomes, but were also topographically organised with the PPNr preferentially innervating the DLS and the LDT preferentially innervating DMS and NA. The PPNc constitutes a transition region between PPNr and LDT and innervates the DS and NA equally. Interestingly, I observed that cholinergic neurons from the LDT projecting to the NA also give rise to cholinergic collaterals projecting to VTA/SNc or to the thalamus.

Electron microscopy analysis revealed that cholinergic axons in the Str make synaptic contact with spines and dendritic shafts. Synaptic contacts with spines and dendritic shafts are more likely on MSNs and aspiny interneurons. In order to confirm that PPN and LDT were the only cholinergic inputs to the Str and NA, I targeted the 6 other cholinergic groups. Using scans of entire brains, I observed that, with the exception of PPN and LDT, no other cholinergic group gave rise to cholinergic axons in the Str and the Na. I showed that stimulation of PPN and LDT cholinergic inputs to the Str activates cholinergic interneurons, and that brainstem cholinergic neurons as well as cholinergic interneurons inhibit MSNs (Chapter 4).

The rat striatum underlies reward-driven operant behaviour (Grace et al., 2007; Dickinson and Balleine, 1993; Cui et al., 2013). Specifically, lesion experiments have demonstrated that the DLS mediates habitual responding in lever pressing tasks, which is insensitive to the reinforcer. On the other hand, the DMS mediates goal-directed responding which is driven by a psychological representation of the value of the reinforcer and is sensitive to a decrease in reinforcer value (Gremel and Costal, 2013; Shan et al., 2014; Bradfield et al., 2013; McDonald et al., 2001; White and Rebec, 1993; Yin et al., 2004). I tested the extent to which CINs or brainstem cholinergic projections mediate these discrete functions of DLS vs. DMS. I found

that both PPN inputs to DLS and CINs located in DLS are involved in the acquisition of habit. This effect demonstrates behavioural functions of the PPN-DLS cholinergic system. It remains to be answered whether specific interactions between PPN afferents and CINs mediate these effects. This can be answered in the future using disconnection studies. On the other hand, cholinergic inputs from LDT to DMS, but not CINs located in DMS, were implicated in goal-directed behaviour suggesting a role of direct LDT inputs on DMS principal neurons. This result suggests a competition between CINs in the striatum. The role of cholinergic transmission in the striatum is complex. The results of my behavioural experiments are coherent with observations made in DMS/DLS CINs lesion experiments (Okada et al., 2014). However, other studies report no effect or decrease of the behavioural flexibility following CINs lesions (Ragozzino et al., 2002). The different effects can be explained by differences in the task design. Indeed, most of the reversal set-shifting studies use radial mazes whereas for this work, an operant chamber was used. In contrast to reversal set-shifting in a radial maze, in the operant chamber the animal remains in a similar spatial orientation from trial to trial, providing it with fewer numbers of strategies (Floresco et al., 2008), reducing the bias related to locomotion or anxiety.

Patterns and significance of PPN and LDT innervation of BG

Originally, studies describing the basal ganglia focused almost exclusively on the dorsal basal ganglia (DS, globus pallidus, substantia nigra pars compacta, substantia nigra pars reticulata and subthalamic nucleus), mostly because of the role of SNc and DLS in neurodegenerative diseases (see review: Humphries and Prescott, 2010; Parent and Hazrati, 1995; Bolam et al., 2000; Gurney et al., 2001a). While, the ventral components of the basal ganglia (NA core, NA shell, VTA, medial SNc and

ventral pallidum) were implicated in reward-driven behaviour and locomotion (Goto and Grace, 2005; Niv et al., 2006; Mulder et al., 1998; Kelley and Mittleman, 1999; Bauter et al., 2003; see review: Pennartz et al., 1994) (**Fig 64**).

Recent findings, including the results presented here, suggest that because of its anatomical and functional interconnections with the basal ganglia, PPN should be considered as a component of the basal ganglia system (Mena-Segovia et al., 2004). Indeed, PPN cholinergic projections were found in DS, SNc, STN and GP, additional projections were also found in the somatosensory cortex and lateral thalamus (Mena-Segovia et al., 2008; Dautan et al., 2014; Dautan et al., 2015). I also observed a gradient of projections between the PPNr and PPNc. Indeed, cholinergic neurons from PPNr appear to project mostly to the DLS, lateral SNc and controlateral thalamic nucleus. Cholinergic neurons from the PPNc also send inputs to the VTA, DMS, NA and mediodorsal thalamic nucleus. Thus PPNr cholinergic neurons innervate only the dorsal parts of the BG, whereas PPNc projects to the entire BG.

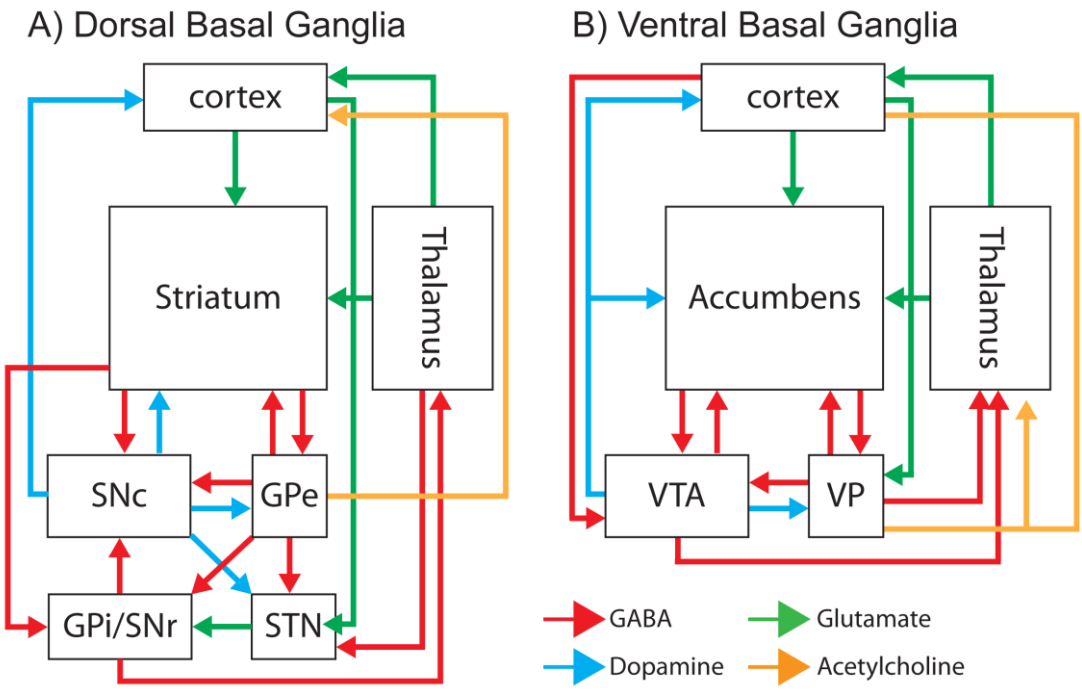

Figure 61: Parallel circuitry of the ventral and basal ganglia. Simplified model of the dorsal (**A**) and ventral (**B**) basal ganglia circuitry. Major DAergic (blue), GABAergic (red), glutamatergic (green) and cholinergic (orange) innervations were represented. Cholinergic and non-cholinergic projections from the brainstem are illustrated on the next figure. Abreviation: VTA ventral tegmental area, VP: ventral pallidum, GPe: globus pallidum external segment, GPi: globus pallidus internal segment, SNr: substantia nigra pars reticulata, SNc: substantia nigra pars compacta and STN: subthalamic nucleus.

The mapping of cholinergic efferents from LDT shows projections to the NA, the VP, the VTA, the mediodorsal thalamus, gigantocellular tegmental field, GP, visual and cingulate cortex (Holmstrand and Sesack, 2011; Mitani et al., 1988; Higo et al., 1996; Motts and Schofield, 2009; Shiromani et al., 1992). This pattern of projection suggests an interconnection of the LDT with the ventral aspect of the BG.

The projection of PPN and LDT within the BG suggests a dorsal-to-ventral organisation of brainstem cholinergic inputs (**Fig 65**). Typically, PPNr cholinergic neurons send sensorimotor-related information to the dorsal BG; PPNc cholinergic neurons carry sensorimotor and limbic information to the dorsal and ventral BG while the LDT cholinergic neurons carry limbic and motivational inputs to the ventral BG. These observations were confirmed by the functional role of cholinergic projections to the DLS (PPNr), DMS (PPNc and LDT) and VTA (PPNc and LDT) showed in this thesis.

In vivo, the responses of CINs to salient stimuli are dependent upon dopamine release (Aosaki et al., 1994) which can also be mediated by glutamatergic inputs (Wang et al., 1991; Marti et al., 2002). MSNs exhibit short response latencies to salient stimuli in the range 100-150 ms (Hikosaka et al., 1989). An important question to resolve is whether the thalamo-striatal or mesostriatal projections could mediate these short-latency responses of striatal neurons directly. Since the latencies

of thalamic neuron responses to salient stimuli are in the range 150-200 ms and the latencies of DAergic neuron responses are normally in the range 70-100 ms (Schultz et al., 1998; Doing et al., 2014), striatal neuron responses must be initiated by a short-latency signal input that drives striatal neurons and pathways to the striatum. Brainstem cholinergic neurons response latencies are always shorter than the ones observed in DAergic, thalamic and striatal neurons, generally 70 ms (Kobayashi and Okada, 2007). In a series of observations, I proposed the cholinergic brainstem as a source of short-latency salient stimuli input that drives striatal neuron responses. First, as described in Chapter 1, a direct brainstem-striatum projection has been described. Second, local activation of brainstem cholinergic axons in the VTA increases DAergic neurons burstiness. Third, local activation of brainstem cholinergic axons in the striatum produces short-latency responses in CINs and slow responses of MSNs. Consequently, I proposed the brainstem cholinergic neurons as a source of short-latency responses to salient stimuli to DAergic midbrain. In addition, cholinergic neurons seem to prepare the striatum for dopaminergic and glutamatergic release following salient stimuli. However, further experiments are needed to evaluate the axonal conduction time, the receptors involved in each structure and their dynamic. In addition, a salient stimulus predicting reward does not evoke dopamine release in all regions of the striatum (increased DA release in NA core and DMS) (Brown et al., 2011), consistent with the suggestion that dopamine function relates to the multifaceted nature of salience. On the other hand, non-rewarding salient stimuli activate dopamine neurons (Horvitz, 2000; Ungless, 2004). First, more detailed studies are needed regarding the cholinergic modulation of the dopamine neuron responses to salient stimuli. For example, it will be important to determine whether dopamine neurons latency to salient stimuli is affected by PPN/LDT

cholinergic-specific lesions. Second, a more detailed understanding is needed regarding a putative modulation of dopamine release by brainstem-striatum pathway. Finally, a clearer understanding is needed of the role of Ach on thalamus activity.

Functional significance of brainstem input to VTA

Brainstem cholinergic neuron activity is related to locomotion and to salient stimuli. For example, PPN cholinergic neurons degenerate in PD (Hirsch et al., 1987; Zweig et al., 1987; Jellinger, 1988), and PPN cholinergic and non-cholinergic neurons project to motor regions of the spinal cords, such as the gigantocellular nucleus (Skinner et al., 1990; Martinez-Gonzalez et al., 2014). PPN electrical stimulation affects posture, muscle tone and locomotion (Reese et al., 1995; Steriade and McCarley, 1990; Lai and Siegel, 1990).

PPN descending projections to the reticular nucleus are implicated in locomotion whereas ascending projections (to the VTA/SNc or STN) are inplicated in the control of gait and muscle tone (Kringelbach et al., 2007; Thevathasan et al., 2012; Moro et al., 2010; Garcia-Rill et al., 1990). Bilateral lesions of PPN cholinergic neurons are sufficient to induce gait and balance disorders (Karachi et al., 2010). In PD patients, symptoms associated with loss of PPN cholinergic lesions were resistant to DA treatment (Karachi et al., 2010; Ryczko et al., 2013), suggesting that gait and balance disorders observed in faller PD patients were dependant of PPN cholinergic activity, while symptoms in non-faller PD were DA related (Karachi et al., 2010; Grabli et al., 2013).

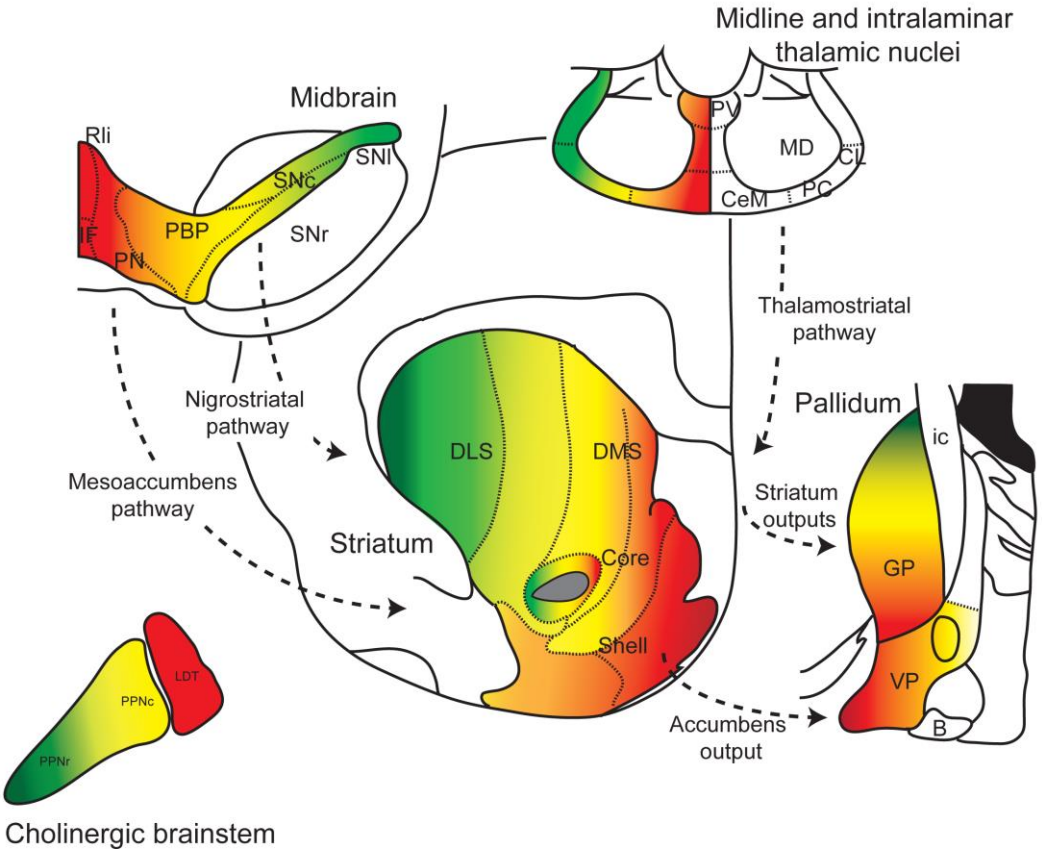

Figure 62: Innervation of the basal ganglia by the cholinergic brainstem. Cholinergic inputs to the midbrain (top left), the thalamus (top right), the Str (centre) and the pallidum (bottom right) are distributed along a medio-lateral extends. DLS, substantia nigra, dorsal pallidum and lateral thalamus nuclei receive cholinergic inputs mostly from the PPNr (green). DMS, lateral accumbens, medial substantia nigra, ventral tegmental area, medial thalamic nucleus and medial ventral pallidum receive cholinergic inputs mostly from the PPNc (yellow). Finally, DMS, NA, olfactory tubercle, VTA, centro-medial thalamic nucleus and lateral ventral pallidum receive cholinergic inputs mostly from the LDT (red). Dashed lines indicate main projections within BG. Based on Voorn et al., 2004; Dautan et al., 2014; Dautan et al., 2015; Holmstrand and Sesack, 2011.

LDT cholinergic neurons have also been implicated in locomotion. Amphetamine, morphine or nicotine–induced locomotion is mediated by DAergic neurons in VTA (Clarke and Kumar, 1983a; Clarke and Kumar 1983b; Nakahara et al., 2001; Panagis et al., 1996). However nicotine administration also induces activation of LDT neurons (Lanca et al., 2000). LDT lesions reduce psychostimulatant-produced locomotion (Forster et al., 2002). These effects have been shown to be almost

exclusively associated with cholinergic neurons (Alderson et al., 2005). Thus psychostimulatant effects on locomotion may involve the mesoaccumbal pathway through the activation of LDT cholinergic neurons. Indeed, dorsal striatal CINs lesions do not show effects on locomotion whereas NA CINs lesions reduce psychostimulant-produced locomotion by reducing DA release (Okada et al., 2011; Laplante et al., 2013).

All together, these results suggest independent mechanisms of PPN and LDT produce locomotion: PPN appears to be involved in gait and posture and these effects may be independent of the mesolimbic pathway. On the other hand, LDT appears to be involved in psychostimulant-produced locomotion and this effect is most likely to be dependent on the mesolimbic pathway.

Functional significance of striatal cholinergic activity

Experimental evidence suggests dissociation between cognitive function mediated by DMS and DLS, and also by MSNs and CINs (Groenewegen et al., 1990; Devan et al., 2011; White and Rebec, 1993; Kim et al., 2009; Mizumori et al., 2009). Anatomically, a clear-cut separation between DLS and DMS is not straight forward. No clear distinction, based on inputs to the Str, has been found between DMS and DLS (Haber et al., 2000; Lynd-Balta and Haber, 1994; Zahm et al., 1996) and no difference has been observed in the number of FSI and MSNs (D1 or D2) between DLS and DMS (Gertler et al., 2008; Gerfen et al., 1990; Wu and Parent, 2000). Interestingly, however CINs are more densely distributed in the DLS than DMS (Bernacer et al., 2007; Altar et al., 1991; Phelps et al., 1989).

Modulation of excitatory inputs to DLS or DMS showed the role of ACh in habit and goal-directed behaviour (Gremel and Costa, 2013; Yin et al., 2004;

Bradfield et al., 2013). Goal-directed behaviour, while dependent on DMS, does not seems to be affected by DMS CINs lesions but appears to be dependent on DMS MSNs (Fanelli et al., 2013; Macpherson et al., 2014; Kim et al., 2013). In addition, habit learning behaviour appears to be affected by loss of CINs in the DLS (or thalamic input to CINs), and also by a loss of MSNs (or MSNs activity) in the lateral Str (Bradfield et al., 2013; Kimchi et al., 2009; Thorn et al., 2010). The findings presented in Chapter 4 confirm that PPN and LDT cholinergic projections to the striatum are involved in opposite, but complementary, behaviour.

Future directions and conclusion

The conclusions reached here present some testable hypotheses that need more experiments. The first is that brainstem projections to the VTA and the Str act together, with a strict temporality, to modulate the release of dopamine in the striatum. Suggesting that direct brainstem-striatum pathway will prepare the release of dopamine by pre-activating CINs. This could be further investigated and tested by: 1) Fast scan cyclic voltammetry, to measure the dopamine release following stimulation of cholinergic axons in the midbrain and in the striatum. A high level of dopamine release in the striatum following stimulation of both outputs would establish the temporal interaction of brainstem-striatum and brainstem-midbrain-striatum pathway, 2) Using behavioural disconnection studies to test if interhemispheric blocking of midbrain and Str Ach release arising from the brainstem will show a stronger behavioural effect than intrahemispheric blocking.

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Appendix 1: Summary of all injections coordinates (distance from midline and surface of the brain).

	AP	ML	DV	Angle
Ch1 (medial septum)	**0.4**	**1.6**	**-5.7**	**15**
Ch2 (vertical band)	**0.8**	**2**	**7.3**	**15**
Ch3 (Horizontal band)	**1**	**1.9**	**8.5**	**0**
Ch4 (nucleus basalis)	**1.2**	**3**	**7.3**	**0**
Ch5 (PPN)	**-7.3**	**1.8**	**7.2**	**0**
PPNc	**-7.8**	**1.8**	**-6.5**	**0**
PPNr	**-6.8**	**1.8**	**-7.5**	**0**
Ch6 (LDT)	**-8.5**	**0.9**	**6**	**0**
Ch7 (medial habenula)	**-3.5**	**0.3**	**-4**	**0**
Ch8 (PBN)	**-4.5**	**4.3**	**-5.5**	**0**
DLS	**0.5**	**3**	**4.5**	**0**
DMS	**0.5**	**1.8**	**4.5**	**0**
NA	**1.5**	**1.8**	**6.7**	**0**
lateral shell of NA	**1.2**	**2.7**	**6.8**	**0**
medial shell of NA	**1.5**	**0.9**	**7.2**	**0**
medial core of NA	**1.5**	**0.9**	**6.5**	**0**

Appendix 2: Statistics summary behaviour Chapter 3

overall locomotion	distance (pre vs stim)	mixed ANO-VA	treatment	F(1,29)=9,005	p=0,005
			group	F(2,29)=6,027	p=0,006
			interaction	F(2,29)=6,594	p=0,004
		Tukey	WT vs PPN		p=0,843
			WR vs LDT		p=0,009
			PPN vs LDT		p=0,024
	mean speed (pre vs stim)	mixed ANO-VA	treatment	F(1,29)=5,702	p=0,038
			group	F(2,29)=6,108	p=0,038
		Tukey	WT vs PPN		p=0,839
			WR vs LDT		p=0,007
			PPN vs LDT		p=0,018
drug effect	distance (stim vs stim+drugs)	mixed ANO-VA	treatment	F(1,19)=0,769	p=0,391
			group	F(2,19)=0,703	p=0,508
			interaction	F(2,19)=1,405	p=0,270
	mean speed	mixed ANO-VA	treatment	F(1,19)=1,043	p=0,320
			group	F(2,19)=0,746	p=0,486
			interaction	F(2,19)=1,746	p=0,201
inst, locomotion	distance (pre vs stim)	mixed ANO-VA	treatment	F(1,29)=1,362	p=0,108
			group	F(2,29)=364,306	p=0,006
		Tukey	WT vs PPN		p=0,044
			WR vs LDT		p=0,029
			PPN vs LDT		p<0,001
drug effect	distance (stim vs stim+drugs)	mixed ANO-VA	treatment	F(1,19)=3,617	p=0,09
			group	F(2,19)=1,412	p=0,268
		Tukey	WT vs PPN		p=0,465
			WR vs LDT		p=0,271
			PPN vs LDT		p=0,881

Appendix 3: Statistics summary electrophysiology Chapter 4

MSN	firing (pre vs stim)	one way ANOVA	PPN	F(1,38)=2,097	p=0,043
			LDT	F(1,37)=2,21	p=0,033
	stim x group	mixed ANOVA	stim	F(2,37)=11,372	p<0,001
			group	F(1,37)=6,032	p=0,713
			interaction	F(1,37)=4,98	p>0,05
	basal firing rate	t-test	PPN vs LDT	t(37)=0,230	p=0,820
	stim firing rate	t-test	PPN vs LDT	t(37)=0,696	p=0,491
	response	mixed ANOVA	stim	F(1,37)=3,697	p>0,05
			group	(F1,37)=0,023	p=0,982
CINs	firing (pre vs stim)	one way ANOVA	PPN	F(1,12)=23,97	p<0,001
			LDT	F(1,7)=9,779	p=0,017
CINs-eYFP	response x cell type	mixed ANOVA	response	F(2,18)=1,17	p=0,132
			cell type	F(2,18)=9,65	p=0,018
			interaction	F(2,18)=5,503	p=0,003
		Tukey	YFP+ vs YFP-		p=0,002
	basal firing rate	t-test	PPN vs LDT	t(19)=0,397	p=0,696
	stim firing rate	t-test	PPN vs LDT	t(19)=0,07	p=0,945
	response vs post	one way ANOVA	PPN	F(1,12)=0,426	p=0,526
			LDT	(F1,7)=0,006	p=0,940
PV	baseline	t-test	PPN vs LDT	t(15)=0,743	p=0,469
	firing (pre vs stim)	one way ANOVA	PPN	F(1,7)=0,363	p=0,566
			LDT	F(1,8)=5,32	p=0,05
	stim firing rate	t-test	PPN vs LDT	t(15)=1,083	p=0,296
	response vs post	one way ANOVA	PPN	F(1,7)=0,315	p=0,592
			LDT	F(1,8)=3,72	p=0,09
CINs to PV	stim x group	mixed ANOVA	stim		p>0,05
			group		p>0,05
			interaction		p>0,05
Latency (z-score)	MSN response	mixed ANOVA	group	F(2,76)=0,344	p=0,710
			stim	F(1,76)=35,746	p<0,001
			interaction	F(2,76)=1,051	p=0,355

Appendix 4: Statistics summary behaviour Chapter 4

Control	RR training	mixed ANOVA	day x group	day	F(3,16)=23,370	p<0,001
				group	F(3,16)=3,340	p=0,797
				interaction	F(9,16)=2,090	p=0,049
	devaluation	mixed ANOVA	condition x group	condition	F(1,41)=21,124	p<0,001
				group	F(4,41)=352,03	p>0,05
				interaction	F(4,41)=42,69	p>0,05
	RI training	mixed ANOVA	day x group	day	F(7,15)=18,279	p<0,001
				group	F(3,15)=1,855	p=0,178
				interaction	F(21,15)=0,919	p=0,567
	devaluation	mixed ANOVA	condition x group	condition	F(1,41)=0,235	p=0,815
				group	F(4,15)=9,36	p>0,05
				interaction	F(4,15)=1,09	p>0,05
Mutant	RR training	mixed ANOVA	day x group	day	F(3,41)=60,637	p<0,001
				group	F(4,41)=278,017	p=0,568
				interaction	F(12,41)=36,51	p=0,218
	devaluation	mixed ANOVA	condition x group	group	F(4,45)=2,110	p=0,097
		tukey	mutant vs ctrl	DLS		p<0,001
				DMS		p<0,001
				PPN		p<0,001
				LDT		p=0,416
	RI training	mixed ANOVA	day x group	day	F(7,41)=12,398	p=0,01
				group	F(4,41)=441,487	p=0,267
				interaction	F(28,41)=0,969	p=0,435
	devaluation	mixed ANOVA	condition x group	group	F(3,45)=0,911	p=0,467
		tukey	mutant vs ctrl	DLS		p=0,03
				DMS		p<0,001
				PPN		p=0,796
				LDT		p=0,357
Reversal	reacquisition	one way	WT vs mutant		F(1,44)=1,504	p=0,219
	reac vs day 1	mixed NOVA	day x group	day	F(2,80)=8,035	p<0,001
				group	F(4,80)=8,822	p<0,001
				interaction	F(8,80)=5,266	p<0,001
		tukey		WT vs DMS		p<0,001
				WT vs LDT		p=0,041
				WT vs DLS		p=0,486
				WT vs PPN		p=998
	day 1 vs day 2	mixed ANOVA	day x group	day	F(1,40)=6,178	p=0,017
				group	F(4,40)=10,123	p<0,001
				interaction	F(4,40)=4,120	p=0,007
		Tukey		WT vs DMS		p<0,001
				WT vs DLS		p>0,05
				WT vs LDT		p=0,024
				WT vs PPN		p>0,05