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Dichotomy between motor and cognitive functions of midbrain cholinergic neurons

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Abstract

Cholinergic neurons of the pedunculopontine nucleus (PPN) are interconnected with all the basal ganglia structures, as well as with motor centers in the brainstem and medulla. Recent theories put into question whether PPN cholinergic neurons form part of a locomotor region that directly regulates the motor output, and rather suggest a modulatory role in adaptive behavior involving both motor and cognitive functions. In support of this, experimental studies in animals suggest that cholinergic neurons reinforce actions by signaling reward prediction and shape adaptations in behavior during changes in environmental contingencies. This is further supported by clinical studies proposing that decreased cholinergic transmission originated in the PPN is associated with impaired sensorimotor integration and perseverant behavior, giving rise to some of the symptoms observed in Parkinson's disease and progressive supranuclear palsy. Altogether, the evidence suggests that cholinergic neurons of the PPN, mainly through their interactions with the basal ganglia, have a leading role in action control.

Keywords

pedunculopontine; cholinergic; movement; behavioral arrest; gait; balance

Introduction

There has been an ongoing debate on whether cholinergic neurons of the pedunculopontine nucleus (PPN) have motor or cognitive roles. From the early experiments exploring the effects of electrical stimulation of the brainstem in decerebrate cats in the 1960s, to the varied outcomes of deep brain stimulation (DBS) in the PPN of Parkinson's disease (PD) patients in recent years, the question about the nature of cholinergic neurons in motor functions remains unsolved. On this matter, divergent conclusions from a wide variety of experimental approaches, ranging from lesion studies to pharmacological approaches, together with the neurochemical heterogeneity that characterizes the PPN, suggest that some of the motor functions assumed to be associated with the cholinergic neurons may be carried out by other cell populations. Even more elusive has been the role of cholinergic neurons in

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cognitive functions given the difficulties in dissecting the cholinergic nature of the contributions of the PPN to behavior. New technologies and experimental advances have produced exciting data in the past years. On this basis, we aimed to reassess the question of whether cholinergic neurons of the PPN contribute to cognitive or motor functions by revisiting classic PPN studies in light of recent publications and evaluate the evidence that support each case. We then use evidence gathered from clinical studies to interpret the contribution of cholinergic neurons to normal brain operations and the consequences of their dysfunction in disease, setting all this in the context of the experimental data generated in animals. Finally, in the conclusions, we debate on whether a dichotomic approach is the best way to understand the PPN cholinergic neurons.

Motor functions of cholinergic neurons

The PPN, together with the cuneiform nucleus (CnF), traditionally constitute the mesencephalic locomotor region [MLR, (Whelan, 1996)]. This region, located in the midbrain, is defined functionally rather than anatomically: electrical stimulation of the MLR and surrounding areas of decerebrate animals induces stepping on a treadmill. Graded electrical stimulation increased the extensor tone and with continuous stimulation this evolved into coordinated stepping. Increasing stimulation elicited faster gait patterns (Shik et al., 1966). However, in the past, several experimental papers challenged the question of whether the PPN is indeed directly involved in motor control and if it should be considered part of the MLR, and very thorough reviews have been published on that topic (Winn, 2006, 2008). Neither partial, nor complete, nor selective cholinergic lesions of the PPN caused changes in spontaneous locomotion, in locomotion elicited by d-amphetamine or apomorphine, or deficits in individual gait parameters (Gut and Winn, 2015; Inglis et al., 1994a, 1994b; MacLaren et al., 2014; Steiniger and Kretschmer, 2004; Swerdlow and Koob, 1987). In recent years, however, new evidence shed light onto the question of the role of the PPN in motor functions.

PPN cholinergic neurons mediate motor inhibition

Recent optogenetic experiments have shown that activation of glutamatergic neurons of the MLR, but not cholinergic, produces a locomotor response. Transduction of the excitatory opsin channelrhodopsin-2 (ChR2) in the glutamatergic neurons of the PPN and CnF elicited an increase in locomotion that resembles the dynamics of the activation induced by electrical stimulation (Roseberry et al., 2016). The effect on motor activity was observed both, in animals that were not moving, and in animals that were already moving, thus suggesting a role in initiation and maintenance of locomotion. Subsequently, a different study aimed to separate the differences between the two structures that compose the MLR and reported functional differences between PPN and CnF. Using targeted injections for each structure and optogenetic stimulation during a locomotor task, the authors showed that CnF induces higher running speeds than PPN and drives a switch in the locomotion pattern, from walking to gallop. In contrast, PPN was shown to increase locomotion gradually and it was proposed to mediate exploratory locomotion. After chemogenetic inhibition of glutamatergic neurons of either the CnF or PPN, only glutamatergic PPN inhibition decreased overall locomotion and exploratory head-dips in a hole-board test, designed to encourage exploratory behavior

(Caggiano et al., 2018). More recently, another study aimed to compare the effects of PPN and CnF activation on muscle activity. Similarly, using an optogenetic strategy, the authors show that only CnF is able to initiate locomotion whereas activation of glutamatergic neurons of the PPN inhibits locomotion by disrupting the locomotor cycle [i.e., disruption of the extensor-flexor cycle; (Josset et al., 2018)]. Altogether, the studies above confirm the existence of the MLR as a functionally-defined area involved in the control of locomotion through its descending projections and suggest that the effects of their activation are correlated with their pattern of connectivity with descending motor systems. In summary, these data put the glutamatergic neuron population at the center of the functional role of the MLR. It then remains to be answered what the role of the cholinergic neurons in the function of the MLR is, and whether they can be considered part of this functionally specialized area.

Studies by Grillner and colleagues in the lamprey demonstrated that, in fact, PPN cholinergic neurons do not belong to the MLR. In an elegant study they show that the connectivity of the MLR largely avoids the cholinergic neurons of the midbrain, otherwise identified as PPN. After injections in the middle rhombencephalic reticular nucleus, no cells were labeled within the area that is defined as the PPN. Only labeled fibers are observed passing through the PPN, thus supporting the claim that PPN and MLR are two separate structures, at least in terms of connectivity. However, cholinergic neurons of the isthmic cell group have descending projections and might intermingle with PPN cholinergic neurons in other species (StephensonJones et al., 2012). Interestingly, there has been some debate about the existence of an area so-called midbrain extrapyramidal area (MEA), which situates medially to the PPN in the rodent, receives dense innervation from the output of the basal ganglia (Rye et al., 1987; Sherman et al., 2015; Steiniger and Kretschmer, 2004; Steininger et al., 1992) and is involved in locomotion (Churchill and Kalivas, 1999). The function of the MEA, however, is not well understood. What is clear from these early studies is that PPN and MEA are different areas: MEA does not contain cholinergic neurons and, despite MEA receiving denser innervation from the substantia nigra pars reticulata and the subthalamic nucleus, PPN maintains close reciprocal connections with the basal ganglia, which rely predominantly on the cholinergic neurons (see the following section). *Is there a motor role of PPN cholinergic neurons?* PPN cholinergic neurons have descending projections that target lower brainstem regions, including the nucleus pontis oralis (PnO) and the nucleus pontis caudalis (PnC) (Garcia-Rill et al., 2001), the gigantocellular nucleus (Grofova and Keane, 1991; Martinez-Gonzalez et al., 2014) and medial reticular formation (Nakamura et al., 1989). Furthermore, axon collaterals from PPN cholinergic neurons reach the medulla and spinal cord (Rye et al., 1988; Skinner et al., 1990; Spann and Grofova, 1989). A series of experiments in the decerebrate cat by Takakusaki and colleagues have shown that electrical stimulation of the PPN elicit a reduction of the muscle tone of the soleus muscle that is sensitive to the cholinergic muscarinic antagonist atropine sulfate (Takakusaki et al., 2003, 2016). Recent optogenetic experiments in awake mice have shown mixed results following the activation of PPN cholinergic neurons on locomotor activity, ranging from a mild increase in locomotion in head-fixed animals on a trackball only when mice were already moving (Roseberry et al., 2016), to a reduction in locomotion in freely-moving mice (Josset et al., 2018). The latter experiment went further to examine the muscle groups that were susceptible of cholinergic manipulation and identified an increase in the activity of

extensor muscles without significantly affecting the flexors. This translated in a reduction in motor activity when PPN cholinergic neurons were activated. It is important to note that the interaction between cell types in the PPN needs to be further characterized in order to rule out the possibility that the effects observed are the consequence of such interaction.

In agreement with the above findings, a series of experiments have explored the role of the ascending collaterals of PPN cholinergic neurons on basal ganglia activity. The collaterals of cholinergic neurons that innervate basal ganglia targets are believed to originate from the same neurons that also innervate descending targets, according to single-cell reconstructions (MenaSegovia et al., 2008). Thus, PPN cholinergic neurons provide afferents to all the components of the basal ganglia: striatum (Dautan et al., 2014, 2016a), external globus pallidus (Charara and Parent, 1994; Eid et al., 2016; Woolf and Butcher, 1986), internal globus pallidus (Shink et al., 1997), subthalamic nucleus (Bevan and Bolam, 1995; Kita and Kita, 2011), substantia nigra pars reticulata [SNr; (Gould et al., 1989; Lavoie and Parent, 1994)] and substantia nigra pars compacta [SNc; (Bolam et al., 1991; Clarke et al., 1987)]. However, only a few of these projections have been functionally characterized. In the striatum, for instance, our lab has identified a direct connection between PPN cholinergic neurons and different types of striatal neurons, including spiny projection neurons (SPNs, the main striatal output neuron) and cholinergic interneurons (CINs). Activation of PPN cholinergic axons *in vivo* produces an increase in the firing rate of CINs and a decrease in the discharge of SPNs (Dautan et al., 2018). While the full impact of the functional consequences of such activation still needs to be determined, these results suggest that PPN cholinergic neurons are able to interfere with the striatal output by directly inhibiting its projection neurons. This may represent a mechanism by which PPN is able to interrupt motor programs in striatal circuits. In line with this, a recent study elegantly demonstrated a cholinergic-mediated mechanism by which PPN afferents to the SNr are able to act upon the axon terminals from direct pathway SPNs. Thus, modulation of M4 muscarinic receptors decreases the inhibitory drive from SPNs on the SNr, therefore disinhibiting the GABAergic output from the SNr and in turn decreasing the motor output from the basal ganglia (Moehle et al., 2017). Altogether, the combined evidence suggests that axon collaterals from PPN cholinergic neurons may be acting postsynaptically on SPNs to reduce their firing rate, and presynaptically on the SPN's axon terminals in the SNr to reduce the release of GABA. Other connections originated from cholinergic axons collaterals remain to be explored in detail. For example, while it is known that PPN excites STN neurons (Capozzo et al., 2009; Hammond et al., 1983), the effect of such activation on the basal ganglia network activity is not yet known. Based on the predictions of the basal ganglia connectivity model, excitation of STN neurons would increase activity in SNr (Alexander and Crutcher, 1990; Obeso and Lanciego, 2011), therefore synergizing with the reduced inhibition arising in direct pathway SPNs. In summary, the net effect of PPN cholinergic activation on basal ganglia circuits seems to reduce motor behavior.

Nevertheless, an important component of the basal ganglia circuitry has not yet been considered in the premises above. Cholinergic neurons project to the dopamine neurons of the midbrain (SNc and ventral tegmental area, VTA), which in turn provide dense innervation to the dorsal and ventral striatum (see details below). In terms of motor behavior, activation of cholinergic axons arising in the PPN and the laterodorsal tegmental nucleus

(LDT) elicit locomotion in an open field (Xiao et al., 2016), and this is likely mediated by dopamine release in the striatum. If this were a pure motor response, it would result counterintuitive in light of the evidence of motor inhibition described above that seems to be mediated and complemented by both ascending (through striatal targets and basal ganglia output nuclei) and descending targets (mediating increased extensor activity and disruption of the muscle cycle). Based on all the above, we propose that the role of cholinergic neurons is not to mediate motor activity but rather to select and reinforce selective behavioral outputs, thus playing a central role in adaptive behavior.

Cognitive functions of cholinergic neurons

There is abundant evidence that supports a role for cholinergic neurons in cognitive functions beyond their traditionally accepted involvement in saliency and arousal. An extensive number of papers have aimed to dissect the functions of different subtypes of PPN neurons and, more recently, the contribution of cholinergic neurons to adaptive behavior. For example, non-selective lesions of PPN neurons impair the learning of operant responding for rewards. When the task was simple (e.g. every lever press delivers a pellet), or when the animals were pretrained prior to the lesions, they could execute the task in order to receive rewards (Alderson et al., 2002, 2004). However, when the contingencies changed (e.g., when the reinforcement schedule became more complex and the animals received the reward after a random number of presses but on average every 8 presses) or during extinction, the lesioned rats were not able to successfully update their behavior and/or made many errors of perseveration and anticipation (Alderson et al., 2002; Wilson et al., 2009). Future experiments should define the specific role of cholinergic neurons in this function and whether local inflammation may have spread into neighboring regions. In the following sections, we will expand on recent evidence that suggest that PPN, and in particular cholinergic neurons, play a role in signaling reward prediction and shape behavioral flexibility.

Contribution of cholinergic neurons to reward prediction error signaling

Dopaminergic reward prediction error (RPE) signaling is fundamental to understand the role of the PPN in associative learning. The most prominent feature of dopamine neurons is their change in discharge mode from tonic to burst firing in response to unexpected rewards or a reward predicting stimulus (conditioned stimulus, CS) once this association has been established (Hyland et al., 2002; Schultz, 1998). When an expected reward is omitted, neuronal firing of dopamine neurons is suppressed. This RPE, which signals the mismatch between expectation and outcome, is crucial for reward-related associative and reinforcement learning. It informs the organism which stimulus is associated to a reward – in the case of a *positive* RPE – and which stimulus is no longer followed by a reward – constituting the *negative* RPE. The capability of dopamine neurons to provide such teaching signal therefore depends on their encoding of the probability (or rather uncertainty) of a reward and the value of the signal.

Neurons that encode a reward prediction need to be able to distinguish between the probability of reward delivery and, therefore, uncertainty. Indeed, Schultz and colleagues

have shown that dopamine neurons can differentiate between different reward delivery probabilities. Fully predicted rewards were followed by little to no response in the firing of dopamine neurons, whereas rewards that followed stimuli that did not fully predict a reward, elicited phasic activation of dopamine neurons. The more unlikely (lower probability) the delivery of a reward after a stimulus, the higher the response to the reward (Fiorillo et al., 2003). However, some dopamine neurons do not show phasic, but rather sustained activation after stimuli with variable probability for a reward. The higher the uncertainty (e.g. 50% probability of reward delivery), the larger the change in neuronal activity.

To signal the value of an upcoming outcome, dopamine neurons also need to be able to distinguish between positive and negative outcomes. As mentioned above, dopamine neurons increase their firing in response to a positive outcome (i.e., a reward). In order to signal the value of an outcome, dopamine neurons should in addition be inhibited by negative outcomes. Whilst some studies could support this theory by showing that dopamine neurons are inhibited by aversive stimuli (Tan et al., 2012; Ungless et al., 2004), others have shown that some neurons were also excited by aversive stimuli [such as airpuff-predicting stimuli, (Brischoux et al., 2009; Matsumoto and Hikosaka, 2009)]. Matsumoto and colleagues classified dopamine neurons in two groups: in the first group, neurons represent motivational value, thus increasing their activity during reward expectation and decreasing activity during punishment or stimuli predicting aversive events; in the second group, neurons increase their firing rate also during both punishments (e.g. an airpuff) or their predictive stimuli after conditioning. The authors speculate that this group of dopamine neurons indicate the motivational salience of the CS. This way, dopamine neurons could also be indicating the need for a behavioral (orienting) response in response to salient stimuli, without encoding for the value of the information carried by the stimulus (Matsumoto and Hikosaka, 2009). Different dopamine neuron populations (based on projection target or input sources) might therefore integrate information differently and have different functions in the mechanism with which the organism orients its behavior towards salient stimuli.

Cholinergic neurons innervate dopamine midbrain neurons, as mentioned before. Microinjections of muscarinic and nicotinic agonists into the SNc resulted in extended dopamine efflux in the striatum. Blaha and colleagues showed that this cholinergic effect was associated with PPN activity: electrical stimulation of the PPN elicited a tri-phasic response of dopamine efflux that was mediated by nicotinic, muscarinic and glutamatergic receptors in the midbrain (Blaha and Winn, 1993; Forster and Blaha, 2003). Local administration of scopolamine into the PPN produced a similar effect on dopamine release in the striatum in a dose-dependent manner (Chapman et al., 1997). Accordingly, stimulation of the PPN by local injection of bicuculline caused an increase in dopamine neurons burst firing, whilst inactivation of the PPN by means of muscimol decreased bursting activity in the VTA (Floresco et al., 2003). More recent studies expanded on these results and offered a more detailed insight into the mechanisms of PPN influence over dopamine neurons activity. The PPN provides fast excitatory input to midbrain dopamine neurons mediated by glutamatergic transmission (Dautan et al., 2016b; Galtieri et al., 2017), and a slower and sustained excitatory input mediated by acetylcholine (Dautan et al., 2016b), thus influencing their firing mode by eliciting burst firing and inducing dopamine release in the striatum. Optogenetic stimulation of PPN cholinergic terminals in the SNc and VTA increases

dopamine neuron firing (Dautan et al., 2016b; Xiao et al., 2016) and switch their firing pattern from tonic to phasic, or vice versa, depending on the previous state of the neuron (Dautan et al., 2016b). Notably, stimulation of cholinergic PPN neurons triggers locomotion only in the SNc but not in the VTA [(Xiao et al., 2016); only LDT axons had an effect on locomotion in the VTA; (Dautan et al., 2016b)]. In contrast to these locomotor effects triggered by the PPN in the SNc, stimulation of PPN cholinergic axons in the VTA caused conditioned place preference (CPP) in a biased version of the CPP task (Xiao et al., 2016) and attenuated extinction when the natural reward was replaced by optogenetic stimulation (Dautan et al., 2016b). Furthermore, photoinhibition of cholinergic PPN neurons that are projecting to the VTA caused severe learning deficits in an appetitive Pavlovian conditioning task (Yau et al., 2016).

The dynamics of PPN neurons is remarkably similar to the response to rewards and conditioned stimuli observed in dopamine neurons. Okada and Kobayashi have shown in a series of studies that PPN neurons in monkeys performing reward-biased saccade tasks respond robustly to the coding of reward-related cues and the value of the reward that follows the cue. PPN neurons showed tonic increases or decreases during task execution. Further, some of the tonic excitatory neurons differentially encoded for predicted reward magnitude, showing a stronger response to large reward predicting cues than to small reward predicting cues, which indicates the ability to predicting the value of a future reward (Okada et al., 2009). In a recent study, PPN neurons that were monosynaptically connected to SNc dopamine neurons, as determined by modified rabies virus-based retrograde trans-synaptic tracing in genetically-tagged dopamine neurons, PPN neurons were observed to be functionally heterogeneous (Tian et al., 2016). Their activity was recorded during a stimulus-outcome association task. PPN neurons were very heterogeneous in their activity during the task. In response to reward-predicting cues some neurons showed phasic, others sustained excitation; some showed inhibition or even bi-phasic responses. It needs to be pointed out that this study did not differentiate between the different cell-types of the recorded neurons. Further, the activity of SNc-projecting PPN neurons did not only change in response to conditioned stimuli (i.e. encoding expectation) but also to reward or both. This means that the PPN as a structure is able to provide the two types of information to dopamine neurons that they need to compute RPEs: the value of the predicted reward and the actual value of the delivered reward. A proportion of PPN neurons were observed to code positively for reward-predicting cues, meaning increasing their firing rate response with increasing reward probabilities.

Because of the dynamic of activation of cholinergic neurons in response to rewarding events (Okada et al., 2009; Pan and Hyland, 2005; Thompson and Felsen, 2013) and because of the excitatory effects on dopamine phasic transmission (Dautan et al., 2016b; Xiao et al., 2016), it is possible that the signaling of positive RPE may originate in cholinergic neurons, but further experiments are necessary to generate conclusive evidence. The evidence presented here then supports a role for cholinergic neurons in signaling a better-than-expected outcome and therefore promoting actions that lead to better outcomes; in order words, PPN cholinergic neurons may be reinforcing actions.

Role of the PPN cholinergic neurons in behavioral flexibility

Cholinergic signaling in the brain has been associated with behavioral flexibility. Across different brain areas, release of acetylcholine or pharmacological activation of cholinergic receptors have been shown to facilitate the integration of new learning into neuronal ensembles encoding old learning. Conversely, disruption of cholinergic transmission impairs the ability to switch between behavioral sequences. Neurological conditions where the cholinergic neuronal systems are impaired illustrate this further (e.g., Alzheimer disease, cholinergic-based dementia, progressive supranuclear palsy; see next section). Among these cholinergic systems, the role of the midbrain is not well understood yet. This is due in part to the complex connectivity of its neurons, which provide a sparse, long-range axonal innervation of a wide variety of heterogeneous targets [for a review see (Martinez-Gonzalez et al., 2011)]. Recent studies, however, support their involvement in behavior, and in particular, in cognitive processes associated to the adaptation of behavior in response to changing contingencies, or in other words, cognitive flexibility.

Acetylcholine in the striatum has been shown to mediate place and response reversal learning, attentional and behavioral set-shifting, strategy shifting and reversal learning of contingencies (Aoki et al., 2015; Apicella, 2002; Bradfield et al., 2013; Brown et al., 2010a; Ragozzino and Choi, 2004; Ragozzino et al., 2002, 2009; Tzavos et al., 2004; Zucca et al., 2018). As described in the previous section, striatal acetylcholine was believed to originate exclusively from CINs, but an unaccounted source was found to arise in the cholinergic midbrain (Dautan et al., 2014) and target CINs, thus providing an additional mechanism to regulate cholinergic transmission in striatal circuits. Experiments in our lab were recently aimed to determine whether the actions from these two cholinergic systems (intrinsic vs extrinsic) were complementary or served similar functions. In an instrumental task to evaluate action shifting dependent on cholinergic transmission, the release of acetylcholine was selectively blocked during the training of the task in either striatal neurons or PPN/LDT neurons using a chemogenetic strategy. Subsequently, the animals were pre-exposed to the reinforcer and tested in the same tasks they were trained in. The results revealed that acetylcholine in the dorsolateral striatum is critical to encode habitual learning, whereas in the dorsomedial striatum is necessary to encode goal-directed learning. Furthermore, the results showed that the same effects were obtained by either blocking the activity of CINs or blocking the activity of PPN/LDT (Dautan et al., 2018). Because the latter is capable of modulating the activity of CINs, this suggests a hierarchical control of midbrain projections over CINs and, consequently, over the striatal output, since CINs are able to modulate the activity of SPNs. In summary, these results suggest that PPN/LDT exert control over action selection at the level of the striatum. Notably, the same experimental manipulation (i.e. inhibition of acetylcholine release in the striatum from midbrain axon terminals) did not produce any effect on locomotor activity, thus supporting the contribution of midbrain cholinergic neurons to the *selection* of behavior.

In addition to their direct influence over striatal circuits, midbrain cholinergic neurons can also potentially influence behavior through thalamic circuits. The thalamus has traditionally been considered a structure that conveys subcortical information to the cerebral cortex and relays efferent copies of motor programs originated in the cortex (Sherman and Guillery,

2002, 2011). Neurons of the parafascicular thalamic nucleus (PF) selectively activate CINs by means of glutamate release (Ding et al., 2010). The activation of CINs mediated by PF neurons has been associated with cognitive flexibility (Brown et al., 2010b), suggesting that the role of CINs in cognitive flexibility is regulated by thalamic inputs. Indeed, pharmacological inactivation by means of the GABA agonists baclofen and muscimol of PF neurons impaired specifically reversal learning in a T-maze task: the same manipulation had no effect on the mere acquisition of place discrimination in the maze. However, baclofen/muscimol-injected rats required more trials to learn the reversed contingencies and made significantly more regressive errors than controls. In the same lines, inactivation of the PF inhibited the increase in dorsomedial striatal cholinergic efflux during the reversal learning phase observed in controls, but not during the first acquisition phase (Brown et al., 2010a). Activation of thalamostriatal projecting neurons caused a burst/pause firing pattern in CINs, which led to a transient interruption of cortical signaling to SPNs through presynaptic M2 receptors followed by a period of enhanced responsiveness in D2 (but not D1) SPNs through postsynaptic facilitation. Given that this facilitation was occurring during the firing pause of the CINs, this could constitute a mechanism of redirection of attention and disrupting of ongoing motor behavior (acting on the indirect pathway) with the presentation of a salient stimulus: The fast activation of presynaptic corticostriatal M2 receptors reduces glutamatergic release at corticostriatal terminals on SPNs, whilst the responsiveness of D2 SPNs enhances the ‘no go’ signal (Ding et al., 2010), causing the required cessation of ongoing behavior necessary for updating and changing motor behavior in response to changing contingencies. In a series of carefully designed experiments, Balleine and colleagues showed that manipulating the thalamostriatal pathway with consequential disturbance of cholinergic function in the striatum impaired rodents’ ability to update initially learned action-outcome sequences (Bradfield et al., 2013). Rats with PF lesions showed no difficulties learning a task but were unable to adapt to changes in the contingencies, whether these were changes due to contingency degradation, extinction or reversal of contingencies.

Thus, cholinergic axons from the PPN and LDT innervate profusely several thalamic structures and are able to modulate the thalamic communication with the cortex and the striatum. In particular, cholinergic axons densely innervate the PF. This cholinergic input is believed to produce an excitatory effect that triggers fast-frequency oscillations typically associated with arousal signals. Whether the PPN cholinergic input determines the activity of thalamostriatal neurons that in turn regulate the activity of CINs is not known yet, but the dense innervation of the PF (Parent and Descarries, 2008) suggests that this may be the case. Data from behavioral experiments supports this assumption: PPN non-selective lesions or pharmacological inactivation lead to increased perseverance in an operant task and impaired action-outcome associations (Maclaren et al., 2013; Wilson et al., 2009), suggesting that PPN has a strong modulatory influence over behavioral set shifting, possibly mediated via its connectivity with PF thalamostriatal neurons and therefore suggesting a role in cognitive flexibility.

In summary, the available evidence suggests the existence of two mechanisms by which midbrain cholinergic neurons arising in the PPN and LDT influence the activity of CINs in the striatum and shape behavioral flexibility. The first one is *direct* through the modulation

of the activity of CINs by monosynaptic connections, and the second one is *indirect* through the putative activation of thalamic circuits that in turn project to the striatum and activate striatal interneurons, most notably CINs. Together with the evidence from experimental manipulations in animals described above, suggesting a thalamic role in behavioral flexibility [i.e. (Bradfield et al., 2013; Brown et al., 2010a)], further evidence arises from human studies in patients who have suffered neurological damage in thalamic structures. For example, lesions in the anterior thalamic groups have been associated with perseverative behavior (Carrera and Bogousslavsky, 2006). Moreover, additional evidence is supported by the diminished cholinergic transmission observed in some neurodegenerative disorders, as will be further developed in the following section. Therefore, both experimental and clinical evidence support a role of cholinergic transmission in the thalamus in cognitive flexibility.

Dysfunction of midbrain cholinergic neurons

Similar to the debate regarding motor functions of the PPN, there has been a prominent interest in whether the PPN plays a significant role in the development of certain movement deficits in PD and progressive supranuclear palsy (PSP). In the PPN, there is cholinergic and noncholinergic (glutamatergic and GABAergic) cell loss in idiopathic PD, as well as in PSP and multiple system atrophy (MSA), which are neurological disorders that can develop into atypical parkinsonian forms where severe impairment of gait and balance are prominent features (Hirsch et al., 1987; Jellinger, 1988; Rinne et al., 2008; Schmeichel et al., 2008). Human *post mortem* brainstem samples revealed a lack of markers of glutamate transporters in the PPN in PSP (Eser et al., 2018; no data is available in PD), suggesting a reduced glutamatergic function, which was combined with a high proportion of tau-bearing neurons. In PD, the activity of the remaining neurons in the PPN has been shown to be altered, the techniques used in these studies did not allow to differentiate between different neuronal subtypes (Aravamuthan et al., 2008; Mitchell et al., 1989; Orieux et al., 2000). In old, but not young, MPTP-treated monkeys, dopaminergic cell loss was accompanied by loss of PPN cholinergic neurons (Karachi et al., 2010), although this has not been a consistent finding. Nevertheless, old MPTP monkeys show more marked postural deficits than the younger MPTP-treated monkeys, where PPN degeneration has not been observed. After lesioning the PPN (without causing DA cell loss), gait and postural deficits developed (Karachi et al., 2010). Because PD is associated with impaired activity in the basal ganglia circuit (Avila et al., 2010; Brown, 2006; Weinberger et al., 2009), this evidence suggests a role of the PPN in the pathophysiology of the movement disorders that characterize parkinsonism. Nonetheless, non-selective lesions of the PPN in rodents did not lead to any measurable gait or postural deficits (Gut and Winn, 2015). Neither combined dopamine denervation with partial PPN lesions, nor PPN lesions alone, showed any noticeable changes in gait deficits in comparison with the traditional model of nigrostriatal degeneration (6-hydroxydopamine lesions of the SNc). Notably, however, the additional damage produced by the implantation of an electrode for DBS in the PPN induced freezing of gait (even off-stimulation), a cardinal sign of PD. The presence of freezing of gait was only during contextual movement, or in other words, when animals were trained to run along a corridor. While the lesions were not cell-type specific, these results suggest that the contribution of the PPN to motor behavior is

contingent on the environmental demands and the required adaptations to elicit a behavioral output.

As part of the symptomatology that appears in the late stages of PD, falls can develop from different complications and are often the result of postural instability or the occurrence of freezing of gait. Freezing of gait is a complex symptom that occurs in the absence of L-dopa medication (off-medication) and occasionally in some patients also during L-dopa treatment. It is described as "*an episodic absence or marked reduction of forward progression of the feet, despite the intention to walk*" (Nutt et al., 2011). This motor feature is often triggered during particular circumstances, such as when patients are challenged by an obstacle in their path, when crossing a door frame, when turning direction or during dual-task situations. During these challenging moments, the additional sensory inputs that need to be integrated with the motor program do not seem to be processed efficiently. Interestingly, patients with significant thalamic cholinergic denervation showed more pronounced deficits of signal detection in a signal detection task that measures perceptual sustained attention under additional attentional challenges (using perceptual distractors), compared to PD patients with normal thalamic cholinergic signaling or healthy controls (Kim et al., 2017). This suggests that difficulties to integrate sensory information into motor control due to decreased thalamic cholinergic innervation might be a contributing factor for gait and postural symptoms. In support of this, Mueller and colleagues found that only thalamic cholinergic signaling correlated significantly with the ability to use sensory feedback to maintain balance (Muller et al., 2013).

Despite the extensive literature on the impairment of PPN function in PD, clinical and *post mortem* studies are limited in determining how much of the PPN-driven dysfunction is due to cholinergic cell loss. As reviewed in the previous sections, PPN glutamatergic neurons play a fundamental role in the control of movement as part of the MLR. In contrast, the experimental evidence discussed earlier suggests that the role of cholinergic neurons may be more closely related with cognitive functions. In line with this, cognitive symptoms seem to correlate with a loss of cholinergic tone in the basal ganglia and the thalamus. The variable pattern of basal forebrain and brainstem cholinergic denervation, measured as a lower acetylcholinesterase (AChE) activity in cortex and thalamus, respectively, seems to correlate with clinical features of PD patients. Low cortical AChE activity occurs more prominently in patients with lower cognitive performance whilst patients with low thalamic AChE activity show a higher propensity for falls (Bohnen et al., 2012). In an earlier study, Bohnen and colleagues investigated the relationship between cholinergic activity in cortex and thalamus and the propensity of falls in PD patients. They report that patients with a history of falls had significantly lower cortical and thalamic AChE activity than non-fallers or control subjects (Bohnen et al., 2009). In line with these findings, *post mortem* studies reported more severe PPN cholinergic cell loss in PD fallers compared to non-fallers (Karachi et al., 2010). Additional clues of the role of the midbrain cholinergic function may arise from the evidence in PSP.

PSP is an atypical parkinsonian syndrome with severe cognitive and motor impairments, the latter characterized by alterations of gait and balance that are typically non-responsive to dopaminergic medication. The neuropathology of PSP is characterized by a marked neural

degeneration and widespread atrophy in the brainstem, basal ganglia and intralaminar thalamus (of which the PF forms part; (Henderson et al., 2000; Steele et al., 1964)). What distinguishes this disorder and makes it relevant for the current review is that the degeneration occurs first and predominantly in cholinergic neurons of the midbrain over the course of the disease (Hirsch et al., 1987). Immunohistochemical detection of the synthetic enzyme of acetylcholine (choline acetyltransferase, ChAT) has been reported to be markedly reduced in the PPN and LDT (Kasashima and Oda, 2003), but also in the striatum, nucleus accumbens and substantia innominata (Ruberg et al., 1985). The reduction in the number of PPN cholinergic neurons was found to be as high as 60%, according to *post mortem* studies (Warren et al., 2005), which was accompanied by a significant number of tau-positive neurons (Eser et al., 2018). Furthermore, thalamic AChE activity is lower in PSP than in PD patients, as measured by AChE positron emission tomography (Gilman et al., 2010), indicating a greater loss of cholinergic PPN neurons in PSP. In addition to the parkinsonian motor symptoms, PSP patients show prominent cognitive impairments, including dementia, and perseverant responses (Brown et al., 2010b). Approximately 70% of PSP patients will show a form of dementia and executive function deficits are the most common cognitive deficit. Apathy and impulsivity are features often observed in PSP and support its diagnosis (Burrell et al., 2014). Thus, these cognitive symptoms in PSP patients are in agreement with the effects of reduced cholinergic transmission in basal ganglia and thalamic circuits and further support the role of PPN cholinergic neurons in action control.

In summary, both the clinical and the experimental data point towards a complex influence of the PPN on motor output, suggesting that the major impairment lies in the integration of new sensory input and adaptation to changes in the environment, which require an adjustment of the motor output and the selection of a suitable motor program.

Conclusions

Here we have reviewed evidence that supports roles of cholinergic neurons in both motor and cognitive functions. Those motor functions, however, do not fit with the classic notion of the MLR and they rather seem to contribute to motor inhibition. In agreement with this, the ascending connectivity of cholinergic neurons with basal ganglia structures support a role in stopping actions (at the level of the SNr and the striatum). Then, on the other hand, cholinergic neurons contribute to goal-directed locomotion and the reinforcement of actions through their connections with dopamine neurons. How can all this evidence be put together in a single theory of cholinergic neurons?

When environmental contingencies change and a certain action no longer leads to a positive outcome, i.e. is no longer adaptive, the organism needs to pay attention to the new stimuli and/or the changed outcomes that are related to a specific action, in order to change the response selection. Stopping an old action and reinforcing a new action are key mechanisms that underlie flexible behavior. PPN cholinergic neurons may contribute to both mechanisms by constituting an interface between motor and cognitive functions, thus accessing the motor systems that are recruited during changing contingencies. In this context, cholinergic neurons may be required to provide a constant update of action-outcome associations, which requires them to signal motivational salience and valence of a stimulus to the dopaminergic

system. They further need to signal a mismatch between expected and real outcome, stop specific actions, for the organism to pay attention to new stimuli and changed contingencies, and to reinforce new actions. When the mechanisms that are dependent on cholinergic neurons fail, the updating process is incomplete, leading to impaired sensorimotor integration (e.g. freezing of gait and falls) and perseverant behavior, as observed in both PD with cholinergic degeneration and PSP, which are characterized with disrupted activity in basal ganglia circuits. New directions in PPN research should thus aim to test the role of cholinergic neurons of the midbrain as critical integrators in the basal ganglia that are necessary for efficient updating of action-outcome associations for adaptive response selection.

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