The cellular diversity of the pedunculopontine nucleus: relevance to behavior in health and aspects of Parkinson's disease

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The Cellular Diversity of the Pedunculopontine Nucleus: Relevance to Behavior in Health and aspects of Parkinson's disease

Running title: Behavioral relevance of PPN cellular heterogeneity

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Abstract

The pedunculopontine nucleus (PPN) is a rostral brainstem structure that has extensive connections with basal ganglia nuclei and the thalamus. Through these the PPN contributes to neural circuits that effect cortical and hippocampal activity. The PPN also has descending connections to nuclei of the pontine and medullary reticular formations, deep cerebellar nuclei and the spinal cord. Interest in the PPN has increased dramatically since it was first suggested to be a novel target for treating patients with Parkinson's disease (PD) who are refractory to medication. However, application of frequency-specific electrical stimulation of the PPN has produced inconsistent results. A central reason for this is that the PPN is not a heterogeneous structure. Here we review current knowledge of the neurochemical identity and topographical distribution of neurons within the PPN of both humans and experimental animals, focusing on studies that used neuronally-selective targeting strategies to ascertain how the neurochemical heterogeneity of the PPN relates to its diverse functions in relation to movement and cognitive processes. If the therapeutic potential of the PPN is to be realized, it is critical to understand the complex structure-function relationships that exist here.

Introduction

The pedunculopontine nucleus (PPN), synonymous with the terms pedunculopontine tegmental nucleus (PPTg or PPT), tegmental pedunculopontine nucleus (TPP) or nucleus tegmenti pedunculopontinus (NTPP), is a brain structure that has been highly conserved in the animal kingdom throughout evolution. In humans, it is located in the caudal pontomesencephalic tegmentum, between the substantia nigra (SN) and the parabrachial nuclei, lying lateral to the ascending limb of the superior cerebellar peduncle. Its rostral end commences just below the red nucleus, dorsal to the SN, continuing caudally to the level of the locus coeruleus. Medially, the PPN is bounded by fibers of the brachium conjunctivum. The medial lemniscus forms the lateral and ventral borders, with the cuneiform and subcuneiform nuclei that form the dorsal aspect.

The PPN occupies a strategic position for modulating a range of physiological and behavioral functions (reviewed by Gut and Winn 2016) (Figure 1). It has a strategic position in basal ganglia circuitry, having reciprocal connections with key output nuclei, including direct output to sites of motor and autonomic control in the brainstem, cerebellum and spinal cord. This realization has encouraged the belief that the PPN is essentially a basal ganglia output station, but its multiple other connections suggest wider functionality. For instance, it has limbic inputs from the basal forebrain/ventral pallidal region as well as the lateral hypothalamus, and multimodal sensory input from the brainstem and midbrain. Indeed, PPN neurons respond to auditory stimuli, in particular those with very short latencies (~8 ms); indicating that very fast sensory processing happens here (Dormont and others 1998). Perhaps the single largest PPN connection is with the thalamus, via which the PPN exerts significant influence over cortical activity (Heckers and others 1992).

Extensive neuronal death occurs within the PPN during the early stages of neurodegenerative diseases such as Parkinson's disease (PD), progressive supranuclear palsy (PSP), dementia with Lewy bodies (DLB) and multiple system atrophy (MSA) (Hirsch and others 1987; Pienaar and

others 2013). The activity of the remaining neurons is also changed due to disturbed basal ganglia outflow (Mitchell and others 1989). These pathological data provided the rationale for targeting the PPN for alleviating specific symptoms of advanced PD by using deep brain stimulation (DBS), where electrodes are implanted unilaterally or bilaterally (Plaha and Gill 2005; Mazzone and others 2005; Stefani and others 2007; for recent reviews see Collomb-Clerc and Welter 2015; Mazzone and others 2016). PPN-DBS has been reported as alleviating dopamine-resistant axial motor abnormalities, including freezing, gait abnormalities and postural abnormalities (Collomb-Clerc and Welter 2015) (Figure 2; Table 1), although some studies showed that gait amelioration was only transient and there were no motor benefits (Ferraye and others 2010). PPN-DBS induced amelioration of non-motor symptoms, particularly improvements in executive functions, verbal fluency (Zanini and others 2009), working memory and sleep architecture (Alessandro and others 2010). The variety of outcomes from PPN-DBS is striking. One possibility for this is that the relatively small numbers of PPN-DBS studies performed so far, with varied patient inclusion criteria, stage of illness and electrode placement (to entail different likelihoods of stimulating local fibre systems) mean that varied outcomes are inevitable. Moreover, the fact that there is neuronal loss from the PPN will add a further complicating factor to the likelihood of achieving positive clinical outcomes from DBS in PD cases. On the other hand, the variability might reflect observations from experimental animal studies showing diverse functions for PPN neurons that cover motor and also cognitive domains (reviewed by Gut and Winn 2016).

A major obstacle preventing PPN-DBS from achieving its full therapeutic potential is determining the optimal placement for stimulating electrodes and selection of appropriate stimulation parameters. Computational modeling suggests that errors as small as 1 mm for surgically placing electrodes could produce substantial decrements in PPN activation (Zitella and others 2013). Furthermore, side-effects such as paraesthesia and oscillopsia may result from unintended stimulation of surrounding structures (Ferraye and others 2009). To guide correct

placement, studies of the topographical organization of the human PPN are needed. For instance, electrode placement into the caudal versus rostral PPN may explain variance in clinical effects. Also, studies using experimental animals (discussed below) showed that PPN sub-regions have different functions. Hence, given the subtlety of internal organization, it is highly likely that stimulating PPN subfields could result in different but potentially predictable outcomes. Here we summarize the current state of knowledge, first regarding the neurochemical composition and topological distribution of neurons in the PPN and secondly the behavioral functions of the PPN, relating PPN structure and function to potential involvement in PD.

The neurochemical composition of the PPN

Classical neurotransmitters

The PPN of humans, non-human primates and rodents contain neurons expressing the neurotransmitters acetylcholine (ACh) (Hirsch and others 1987; Pienaar and others 2013), gamma-aminobutyric acid (GABA), glutamate (Wang and Morales 2009; Mena-Segovia and others 2009; Barroso-Chinea and others 2011; Martinez-Gonzalez and others 2012), and glycine (Pienaar and others 2013). ACh-producing cholinergic neurons are the ones that have been most closely investigated, with recent studies that identified previously unknown parcelation of cholinergic inputs to the midbrain dopamine-containing neurons (Dautan and others 2016a) as well as direct cholinergic projections to the striatum (Dautan and others 2014; Dautan and others 2016b). Such studies are making it increasingly clear that both cholinergic and non-cholinergic neurons contribute significantly to the PPN's control over behavior and cortical state (Ros and others 2010; Petzold and others 2015).

PPN neurons co-expressing classical neurotransmitters have also been described, although this does not necessarily imply co-release (Stamatakis and others 2013). In the cat PPN, co-reactivity for choline acetyltransferase (ChAT) (the transferase enzyme responsible for ACh synthesis and therefore an immunohistochemical marker for cholinergic neurons) and GABA

was seen in neuronal somata, dendrites and axon terminals (Jia and others 2003). In addition, Lavoie and Parent (1994) found ChAT and glutamate co-expression within the PPNs of squirrel monkeys, as well as intermingling of cholinergic and noradrenergic dendrites. Similar connectivity was described in rats between serotonergic neurons projecting from the dorsal raphe and cholinergic and non-cholinergic PPN neurons (Steininger and others 1997), while analysis of guinea pig PPNs found histaminergic afferents from the posterior hypothalamus (Khateb and others 1990). Demonstrations of cholinergic–noradrenergic–serotonergic–histaminergic interactions reinforce the long-standing idea that the PPN plays a role in the ascending reticular activating system (ARAS).

Neuropeptides

Neurons projecting to or from the PPN express various neuropeptides, including orexin/hypocretin (HCRT), galanin, substance P, enkephalin and dynorphin. Darwinkel and colleagues (2014) showed double-labelling of nitric oxide synthase (NOS) and orexin-1 receptors in neuronal cell bodies and fibers of the mouse PPN, while Peyron and others (1998) showed that the PPN receives HCRT input from hypothalamic neurons. Double-labelling immunohistochemistry in rat PPN detected intermingling of fibers containing HCRT and dopamine-beta-hydroxylase, a marker of noradrenergic neurons. HCRT neurons are implicated in regulating arousal, reward and motivation, the sleep-wake cycle and appetite (Chen and others 2015); functions the PPN is implicated in, whilst such neurons are also affected in progressive PD (Drouot and others 2003). Galanin localises in human PPN neurons containing the neurokinin, substance P (Gai and others 1993). A substantial loss of substance P-containing PPN neurons was seen in the post-mortem PPNs of PD patients (Halliday and others 1990), although the impact this has on motor-, cognitive- and autonomic nervous system functions in PD patients remain to be determined. Also of significance, cholinergic neurons in the PPN and the adjacently lying laterodorsal tegmental nucleus (LDT) are unique in the pontomesencephalic tegmentum for

expressing urotensin II (UII) receptors. Not only does urotensin in the PPN control behavioral state (Huitron-Resendiz and others 2005), the uniqueness of its actions on PPN cholinergic neurons has been exploited in creating the fusion toxin, diphtheria toxin (Dtx) /UII (Clark and others 2007) (Table 2).

Opioid peptides are expressed in rat PPN neurons (Fallon and Leslie 1986), including enkephalin and dynorphin, which may inhibit neuronal excitation, thereby modulating the release of neurotransmitters involved in pain relief and euphoria. Opioid peptides have also been implicated in the pathophysiology of PD, with Rinne and others (1983) reporting supersensitivity of enkephalin receptors in the striatum and limbic system of post-mortem PD brains.

Calcium binding proteins

Disturbed calcium homeostasis can render certain neurons vulnerable to neurodegeneration. As such, studies correlating expression profiles of calcium-binding proteins with different neuronal subtypes in the PPN at progressive stages of PD may be important in understanding local cell death. In squirrel monkeys, PPN cholinergic neurons express calcium-binding proteins such as calbindin (Côté and Parent 1992), while in cynomolgus monkeys a proportion of PPN cholinergic neurons co-express calretinin, another calcium-binding protein (Fortin and Parent 1999). The calcium-binding albumin protein, parvalbumin, is expressed in rat PPN (Dun and others 1995), albeit at lower levels than calbindin and calretinin. Parvalbumin plays a key role in GABA neurotransmission, the main inhibitory neurotransmitter in the CNS. GABA release was increased in mice with reduced parvalbumin expression, prompting the suggestion that reduced parvalbumin expression may be a compensatory mechanism to enhance GABA release (Collin and others 2005). More detailed physiological and anatomical studies in experimental models of PD are awaited to elucidate how neurotransmitter release in the PPN, a calcium-dependent process, regulates specific behavior.

Neurochemical subdivisions of the PPN

The PPN is classically defined as containing a *pars compactus* (PPNc), comprising the caudal half of the nucleus, and a *pars dissipatus* (PPNd), comprising the rostrocaudal axis of the PPN (Olszewski and Baxter 1982). The PPNs of rodents show a peculiar organization pattern: in rats the PPNd consists of an abundance of GABAergic neurons, but sparse populations of cholinergic or glutamatergics. In contrast, the PPNc contains a dense population of cholinergic and glutamatergic neurons, with a lower density of GABAergic neurons (Wang and Morales 2009; Mena-Segovia and others 2009; Martinez-Gonzalez and others 2012). It remains to be established whether the rostrocaudal distribution pattern for neurons with various neurotransmitter identities is rodent-specific or is repeated in human and non-human primates.

Cholinergic neurons appear to be the most vulnerable (Pienaar and others 2013), although exactly why remains unexplained. One possibility is that only PPN cholinergic neurons contain NOS, which synthesizes the gaseous neuromodulator nitric oxide (NO). Neurons expressing NOS actively resist the toxic effects of excitatory amino acids (Contestabile and Ciani 2004) but can mediate excitotoxic damage at high concentrations (Dawson and others 1991). In this regard it is worth noting that a post-mortem study found that key proteins of the mitochondrial electron transport chain were significantly downregulated in PPN cholinergic neurons of PD but not control patients (Pienaar and others 2013). Since NO can trigger mitochondrial signal transduction events relating to cellular defense and –adaptation (Finocchietto and others 2009), it could be inferred that defective NO signaling, resulting in mitochondria-mediated cell arrest and programmed cell death could underlie PPN cholinergic neuronal loss in PD patients.

Functional heterogeneity of the PPN

The PPN is anatomically divisible into the PPNc and PPNd. Given that the PPN contains a neurochemically diverse neuronal population and has an extensive array of afferent and efferent connections, both ascending and descending, it is likely that further subdivision could eventually

become apparent. In this section we will examine PPN behavioral functions across species and the extent to which these can associate with particular PPN neurons.

Behavioral state control

The PPN seems to play a pivotal role in regulating sleep states. REM sleep resembles wakefulness, with similar cortical electroencephalogram (EEG) recording spectra. However, during REM sleep, but not the waking state, there is suppression of brainstem and spinal motor-reflexes to stop movement being initiated. A high proportion of PPN cholinergic neurons send dual projections to the thalamus and pontine reticular formation (PRF), suggesting that the PPN has the potential to simultaneously modulate cortical activity via the thalamus, and motor activity via the PRF (Semba and others 1990; Lydic and Baghdoyan, 2003; Fuller and others 2007).

PD patients and animal models of PD present with a range of sleep-related symptoms, including excessive daytime sleepiness, a marked increase in the number of nocturnal awakenings and rapid eye movement (REM) sleep disorder (Gagnon and others 2002). In a striking demonstration of sleep dysfunction in an animal model of PD, Belaid and others (2014) performed long-term continuous EEG monitoring of vigilance states in macaques at baseline, after 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) intoxication, after administering Levodopa (at a clinically effective dose) as well as following cholinergic PPN lesions with Dtx/UII, selective for PPN cholinergic neurons (Table 2). MPTP intoxication resulted in excessive daytime sleep episodes, sleep fragmentation, a reduction of sleep efficiency, a reduction in time spent in REM- and slow-wave sleep, an increase in muscle tone during REM and non-REM sleep episodes and in the number of awakenings and movements during sleep. Levodopa treatment resulted in a partial but significant improvement in almost all sleep parameters, whilst the cholinergic PPN lesion produced a transient decrease in REM- and slow-wave sleep, as well as slightly improved sleep quality.

Although involved, the PPN is not a master switch for sleep–wake transitions (Saper and others 2010). Excitotoxic lesions of the PPN have little effect on spontaneous sleep activity but do affect rats' ability to recover properly from sleep deprivation (Deurveilher and Hennevin 2001). Traditionally the PPN is viewed as part of the ARAS, which is involved in sleep regulation, operating in parallel with brain systems in the hypothalamus and basal forebrain. Cholinergic neurons are the most numerous in the ARAS, found in both the PPN and the LDT, and referred to as the Ch5 and Ch6 groups respectively (Mesulam and others 1989). Using selective optogenetic activation in mice to clarify the role of cholinergic neurons in the PPN during REM sleep, Van Dort and others (2015) showed that selectively increasing cholinergic tone in the PPN or LDT during non-REM sleep increased the number of REM sleep bouts but did not affect REM episode duration. Notably though, the authors acknowledged that the transgenic mice used in the experiments, expressing an excitatory opsin in all cholinergic neurons, were found to express extra copies of the vesicular ACh transporter gene as well as showing increased cholinergic tone (Kolisnyk and others 2013).

Sleep involves interactions between monoamine neurotransmitters of the ARAS and hypothalamic hypocretin neurons projecting to the PPN (Peyron and others 1998). Pal and Mallick (2006) showed in freely behaving rats that noradrenergic neurons projecting from the locus coeruleus to the PPN can tonically inhibit REM sleep. Likewise, Strecker and colleagues (1999) measured extracellular serotonin 5-hydroxytryptamine (5-HT)) levels in the PPN of naturally sleeping cats and found that levels were highest during wakefulness, lower during slow-wave sleep and lowest during periods of REM sleep. These results suggest that decreased 5-HT levels in the PPN during sleep may be a critical determinant in timing REM sleep cycles. Studies have shown 5-HT’s potent hyperpolarization of cholinergic PPN neurons, a mechanism that appears central to presenting working models of REM sleep control (Leonard and Llinas 1994). Bilateral PPN microperfusion of the serotonin type 1A (5-HT_{1A}) receptor agonist, 8-OH-DPAT, into rats revealed that 5-HT_{1A} receptor-responsive PPN neurons become maximally

active immediately before and during REM (Grace and others 2012). Note though that this result contradicts what was the prevailing model of REM sleep generation, which predicted that serotonergic inhibition of PPN neurons should suppress REM sleep (Thakkar and others 1998).

Beyond the monoamine neurotransmitters of the ARAS, it is well established that $GABA_A$ ionotropic and ligand-gated ion channel receptors induce sleep, with several hypnotics targeting $GABA_A$ receptor-mediated inhibitory processes to decrease waking, increase slow-wave sleep and enhance the intermediate stage between slow-wave sleep and paradoxical sleep (reviewed by Gottesmann 2002). Some authors have suggested that REM sleep-active PPN neurons are predominately GABAergic (44-76%) (Maloney and others, 1999), and it has also been suggested that the physiological activity of GABA in the PPN is important for REM sleep. REM sleep was shown to be increased following intra-PPN microinjection of $GABA_A$ receptor agonists in freely moving rats (Pal and Mallick 2009). The same authors suggested that GABA could inhibit the release of noradrenaline for regulating REM sleep (Pal and Mallick 2006). The role of non-cholinergic PPN neurons in behavioral state regulation was also highlighted by Verret and others (2005), with analysis of immediate early-gene expression to highlight neuronal activation which showed that in rats deprived of REM sleep, the vast majority of activated PPN cells were non-cholinergic. Further studies are required to determine the role of different PPN neurons in the onset and maintenance of sleep architecture and the pathological mechanisms that underlie sleep disruption in PD.

This brief summary presents a high-level view of how different neuronal-types in the PPN and their various inputs relate to the control of behavioral state. For more detailed information, readers should consult the recent comprehensive overview of the role of the entire ARAS in health and disease presented by Garcia-Rill (2015).

Locomotion

The PPN has long been associated with movement. However, whether it is essentially a regulator of coordinated locomotion, part of what was termed the mesencephalic locomotor region, or whether it has a role in more complex processing, involving not just the initiation of locomotion but also action selection or decision making, remains to be firmly established. There are clearly connections between the PPN and motor control systems. Skinner and others (1990) established that the PPN can control locomotion via descending projections. PPN neurons that innervate motor structures are predominantly situated in the caudal part of the rodent PPN (Scarnati and others 2011) from where descending projections innervate the oral pontine reticular nucleus, the gigantocellular nucleus and the medioventral medulla. Projections to the spinal cord have also been described (Scarnati and others 2011). Other connections include the dentate nucleus of the cerebellum (Vitale and others 2016) and the vestibular system (Aravamuthan and Angelaki 2012). All these connections to brain sites that are established regulators of motor functions are consistent with the idea that the PPN has a role to play in coordinated movement. Equally important, it has been established that muscle tone can be regulated by neurons in the PPN (reviewed by Takakusaki and others 2016). In human patients, neuronal activity has been recorded that shows PPN to be associated with gait (Tattersall and others 2014) and walking (Yeo and others 2011) and it is clear that PPN neurons are relevant to controlling locomotor activity. However, studies on experimental animals repeatedly illustrate that the relationship between the PPN and control of movement is not simple. The literature concerning non-primate experimental animals is clear and has been reviewed previously (Gut and Winn 2016). Briefly, excitotoxic lesions of the PPN using either ibotenate, N-methyl-d-aspartate (NMDA) or quinolinate did not impair either spontaneous or drug-induced whole body locomotion. Excitotoxic lesions also did not impair reaching or grasping (Dunbar and others 1992), gait or posture (Gut and Winn 2015) (Figure 4 provides examples of tests for measuring a broad range of motor and cognitive behavioral domains in rodent models of PD). Examination of the effects

of excitotoxic lesions on non-human primate PPNs have produced clear data, but conflict with the outcomes of studies that made use of other species. Studies have demonstrated motor impairments following kainate-induced excitotoxic lesions (Kojima and others 1997; Munro-Davies and others 1999). However, due to its extreme potency, no study yet has utilized kainate for lesioning the rodent PPN to study the impact on locomotion. As such, whether the data gathered from non-human primate studies reflects a genuine species difference (compared to rodents) or is simply due to different experimental methodologies, remains uncertain. For further discussion of this matter, see Gut and Winn 2016.

Interestingly, a recent study suggests that the PPN has a role in relation to movement and navigation. Navigation is thought to rely on interplay between the medial entorhinal cortex (MEC) and hippocampus. The MEC contains grid cells which systematically represent an animal's displacement in the environment. Alongside these (to enable proper use of positional representation by grid cells) are neurons whose activity relates to running speed (Kropff and others 2015). A likely source of information regarding locomotor speed is the PPN. Electrophysiological single-unit activity recordings made in rats performing a spatial navigation task (Norton and others 2011) showed that a substantial proportion of PPN neurons held a correlation between firing rate and velocity, with this correlate that was unaffected by the spatial context, including reward. Similarly, Lee and others (2014) showed a relationship between running stimulated from the region of the PPN and cortical activity in mice. Rats bearing PPN excitotoxic lesions were substantially impaired in making appropriate choices in an 8-arm radial maze but were quicker than controls in moving from arm to arm (Keating and Winn 2002), possibly suggesting that PPN neurons normally brake run speed. In a simple runway task, similarly lesioned rats were able to increase running speed when reward value increased (Ainge and others 2006), suggesting that while decision making was impaired, motivation to run was unaffected.

Axial motor functions

Although PD-related motor symptoms respond well to dopamine replacement therapy, axial abnormalities including gait dysfunction, gait freezing and postural instability, respond poorly. The PPN has been adopted as a therapeutic target but its role in gait pathology is disputed. A recent study in PD patients showed PPN-DBS having no effect on gait parameters, although the intervention improved anticipatory postural adjustments and gait postural control (Collomb-Clerc and Welter 2015). This contrasts with DBS of the subthalamic nucleus (STN) and globus pallidus interna (GPi), which did improve gait in PD patients (Weiss and others 2015) (Figure 2; Table 1). Interestingly, Weiss and colleagues (2015) noted a correlation between improvement in imagined gait during STN-DBS and an increase in regional cerebral blood flow in a brain region which includes the PPN. Whether this implicates the PPN as a gait modulator of STN-DBS or whether it reflects a more general activated state remains to be determined.

Given the inconsistent patient data, a number of labs have used animal models to better understand the role the PPN plays in the behavioral features of PD. We focus on three recent approaches: (1) DBS in rats bearing combined dopamine depletion and PPN lesions; (2) the lactacystin rat model of PD (Table 2), and (3) use of the fusion toxin Dtx/UII.

PPN-DBS was studied by Gut and Winn (2015) who analysed gait in rats harboring either partial or full bilateral PPN ibotenate lesions. Lesioned rats were then bilaterally implanted with wireless DBS electrodes in either anterior or posterior PPN (corresponding approximately to the PPNd and PPNc, respectively) for chronic stimulation using clinically-relevant frequencies. The PPN was found to not be critical for normal gait, with addition of a partial PPN lesion to a dopamine-depleting nigral one that did not worsen gait parameters further than those produced by dopaminergic depletion alone. The combined lesion model showed a correlation between cholinergic survival and a deficit in forelimb gait stability, but not other parameters. However, the group that received the combined partial PPN lesion and striatal dopamine depletion showed

pronounced freezing of gait (FOG). In addition, in dopamine-depleted rats with an intact PPN, posterior PPN-DBS ameliorated some gait deficits, whilst anterior PPN-DBS worsened FOG. Gait disturbance is not considered to be purely a motor phenomenon but a complex interplay between motor, cognitive and affective factors (Heremans and others 2013), which may explain why FOG was not visible when rats were in their home cages, only becoming apparent when they were in the narrow test runway.

Pienaar and others (2015a) showed that rats intranigrally (unilaterally) infused with the proteasome inhibitor lactacystin manifest severe motor disabilities in tasks involving the contralesional fore- and hindlimbs. The lesion resulted in destruction of SN dopaminergic neurons (Vernon and others 2011; Pienaar and others 2015a), as well as neurons in the adjacently-lying PPN (Pienaar and others 2015a). Further work revealed that lactacystin toxicity was not specific to cholinergic neurons when administered *in vivo* to rats (Elson and others 2015) but overexpression of the presynaptic protein α-synuclein (resembling Lewy body pathology seen in parkinsonism) predominantly accumulated within PPN cholinergic neurons rather than non-cholinergic ones. Direct stimulation of remaining PPN cholinergic neurons in rats that had been intra-nigrally lesioned with lactacystin was achieved using hM3Dq. Essentially a floxed muscarinic G protein-coupled receptor, with the coding gene modified via insertion of 2 point-mutations (a so-called Designer Receptor Exclusively Activated by Designer Drug (DREADD)), to allow for neuronal depolarization upon binding to the ligand clozapine-N-oxide (CNO) (Figure 3). Rats were ChAT::Cre+, so that all cholinergic neurons expressed Cre-recombinase, restricting hM3Dq expression to cholinergic neurons. This cholinergic-specific excitation of remaining PPN cholinergic neurons dramatically reversed parkinsonian-like motor deficits, particularly gait and postural instability (Pienaar and others 2015b).

The fusion toxin Dtx/UII has been used to examine normal functions of PPN cholinergic neurons. A study by Karachi and others (2010) revealed gait and postural deficits by macaques

after injection of Dtx/UII, accompanied by cholinergic and non-cholinergic neuronal damage in the PPN. The authors suggested that the loss of non-cholinergic PPN neurons may have been secondary to the primary loss of PPN cholinergic neurons, but caution in interpretation is required since the concentrations of Dtx/UII used (and at which behavioral deficits appeared) are known to generate non-selective lesions in rodent. Studies reporting cholinergic-selective lesions in rats showed no impairments in spontaneous or nicotine stimulated locomotion, or learning impairments (MacLaren and others 2016). With regards to drug rewards, Steidl and colleagues (2014) showed that there was no impairment in performance despite almost complete cholinergic neuronal loss being accompanied by significant loss of PPN glutamate and GABA containing neurons. This low degree of selectivity was possibly a product of employing multiple rather than single toxin injections, with larger doses causing non-selective damage (Clark and others 2007). Other studies reported that motor deficits appear after Dtx/UII lesions, including impairments in the accelerating rotarod test at higher speeds and in fine motor control (MacLaren and others 2014a). These might reflect specific motor problems, but could alternatively be accounted for by disruption of the attentional processes required for these tasks. In this regard, deficits in sustained attention (Cyr and others 2015) and acoustic startle responding have both been reported after Dtx/UII lesions (MacLaren and others 2014b).

The question raised at the start of this section was whether the PPN is essentially a regulator of coordinated locomotion or whether it has a more complex role in generating actions. The underlying neural substrate for the effects described here likely involves extensive reciprocal connections with corticostriatal systems, with implications for cognition and emotional reactivity. Given the damage to dopamine-containing neurons in both the lactacystin and PPN-DBS studies, one possible explanation for the effects of PPN-DBS and for the restorative power of DREADD-mediated cholinergic activation after lactacystin lesioning is stimulation of ascending cholinergic projections to the thalamus. Such stimulation might correct dysfunctional oscillatory activity in corticostriatal circuits, known to be essential for producing coordinated

movement (Brown 2007). This would be consistent with the deficits in attention and acoustic startle reactivity that follow Dtx/UII lesions in PPN. It would be intriguing to know whether DREADD-mediated restoration of movement is accompanied by restoration of both corticostriatal oscillatory activity and the cognitive deficits that appear after dopamine-depleting lesions. Figure 4 gives examples of tests used for measuring specific motor behavioral domains in rodent models of PD, including for assessing the axial parkinsonian symptoms referred to in this section.

Cognitive functions

For many neuroscientists the idea that the PPN, a brainstem structure, is involved in cognitive functions will be surprising, but data from PPN-DBS studies in parkinsonian patients and a wealth of literature using experimental animals evidence this convincingly. Electrophysiological data from primates and rodents shows complex processing. An early primate study demonstrated that arm movements relate to firing of PPN neurons (Matsumura and others 1997) but later work by Okada and others (2009), also recording from the non-human primate PPN revealed more complex responses, with PPN neurons responding to either the predicted or actual scale of rewards. These are not intrinsically motor signals and hint strongly at more subtle processes occurring in the non-human primate PPN.

Similar predictive responding by PPN neurons has been shown in rodents. Norton and others (2011) recorded from rat PPN neurons and correlated reward acquisition (which was sensitive to changes in context), speed and direction. The authors concluded that PPN neurons played a role in sensory-motor gating and were involved in reinforcement learning. Later work from the same group (Redila and others 2015) showed that neurons in the LDT were also responsive to movement and reward but were less influenced by current sensory input. Similarly, Thompson and others (2016) showed that PPN neuronal activity related to decision making. In particular, at

the point of action selection, mouse PPN neuronal activity related to the previous choice and its outcome, with the strength of this response that predicted the next decision.

Although none of these studies were able to identify the neurochemical identity of the neurons being recorded, they highlight the fact that computations occurring in the PPN and LDT do not only relate to sleep or movement *per se*, but rather indicate complex integrative properties. The electrophysiological data are supported by numerous lesion studies. Over the last 20 years a variety of studies from multiple labs using excitotoxic lesions (which, as noted above, do not effect basic locomotor functions) show that the PPN has a role in reward and reinforcement (Olmstead and Franklin 1994), learning and memory (Keating and Winn 2002; Wilson and others 2009; MacLaren and others 2013) and attention (Inglis and others 2001; Kozak and others 2005). Such discoveries in experimental animals are matched by clinical studies on PD patients who underwent PPN-DBS. As noted above, as well as effecting sleep structure (Alessandro and others 2010) this treatment has led to improved verbal fluency and use of language (Zanini and others 2009) and improved performance in working memory tasks (Alessandro and others 2010).

Many PD patients suffer from dysexecutive syndrome, with some studies suggesting that PPN-DBS in advanced PD patients ameliorates executive function deficits, along with improving delayed recall and verbal fluency (Alessandro and others 2010). In another study, PD patients that had received bilateral stereotactic PPN implantations were subjected to 18-fludeoxyglucose (18F)-Positron Emission Tomography (PET) for identifying sites of abnormal glucose metabolism. Using a battery of cognitive testing, Ceravolo and others (2011) showed that delayed recall and executive functions improved as a result of bilateral low frequency stimulation (LFS)-PPN in PD patients. In the same study, these authors also showed increased glucose utilization in bilateral prefrontal areas as well as in the left ventral striatum, left

subgyral, right insula and right superior temporal gyrus, strongly supporting the contention that PPN stimulation improves cortical function.

Of particular interest to this review are studies that looked at the effects of excitotoxic lesions in restricted areas of the PPN, and studies that attempted selective destruction of cholinergic neurons. Ibotenate lesions of the posterior but not anterior PPN disrupt nicotine self-administration, perhaps unsurprisingly given the density of PPN cholinergic neurons (Alderson and others 2006). Rats with similar lesions of the posterior PPN were slower at learning to lever press with food reinforcement and had lower rates of lever pressing. Those with anterior PPN lesions worked at normal rates for food reinforcement but tended to perseverate and make anticipatory errors, and also persisted with responding in extinction (when no reinforcement was present and terminating lever pressing was appropriate). These deficits were characterized by Wilson and others (2009) as indicative of learning impairments mediated by the posterior PPN with the anterior PPN involved in selecting appropriate and avoiding inappropriate actions. This is supported by a study showing that inactivation of the posterior PPN by local injection of the selective $GABA_A$ receptor agonist, muscimol, impairs performance in a contingency degradation programme, which is taken as a key indicator of action-outcome association learning (MacLaren and others 2013). However, studies with the selective fusion toxin Dtx/UII cloud this picture, with such lesions having no effect on learning to lever press for food rewards (MacLaren and others 2016) but having effects consistent with impaired attention, as noted above (Cyr and others 2015). Overall, the data shows there are regional differences in PPN function: non-selective lesions of the posterior but not anterior PPN create clear learning deficits, but lesions that destroy only the cholinergic population of the posterior PPN produce attentional deficits, with no impact on learning ability per se. One might speculate that the posterior PPN (PPNc) cholinergic neurons therefore regulate attentional functions, posterior PPN non-cholinergic neurons are involved in learning and that anterior PPN (PPNd) non-cholinergic neurons are

involved with response selection. Examples of tests designed to assess cognitive behavioral domains relevant to PD in rodent models of the disease are illustrated in Figure 4.

Conclusions

The heterogeneous cellular architecture of the rodent PPN is topographically organized, reflecting its functional diversity. The neurochemical types are widely distributed and intermingled, but can also be seen to define subterritories, particularly along a rostrocaudal axis. The data from animal models have started to unpack the relationships between PPN regions and different neurochemically identifiable neurons within them. Critical here though has been the discovery – reinforced by PPN-DBS studies in patients – that the PPN has functions not only related to behavioral state control and movement but also to cognitive processes. For the immediate future, the PPN will remain the subject of intense study, as understanding of the role of its diverse neural elements in controlling behavior continues to expand. Central to this understanding is the accurate description of its complex organization. Optogenetic and chemogenetic techniques will almost certainly play an important role in coupling structure to function and may go on to fulfill a translational role, at the very least as an alternative to electrode-based DBS.

Declaration of Conflicting Interests

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Abbreviations

Acetylcholine, ACh; Ascending reticular activating system, ARAS; Choline acetyltransferase, ChAT; Clozapine-N-oxide, CNO; Deep brain stimulation, DBS; Dementia with Lewy bodies, DLB; Designer Receptor Exclusively Activated by Designer Drugs, DREADD; Diphtheria toxin, Dtx; 18-fludeoxyglucose, 18F; Electroencephalogram, EEG; Freezing of gait, FOG; Gamma-aminobutyric acid, GABA; Globus pallidus interna, GPi; 5-hydroxytryptamine (serotonin), 5-HT; Orexin/hypocretin, HCRT; Laterodorsal tegmental, LDT; Low frequency stimulation, LFS; 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, MPTP; Nitric oxide, NO; Nitric oxide synthase, NOS; Nucleus tegmenti pedunculopontinus, NTPP; Parkinson's disease, PD; Pedunculopontine nucleus, PPN; PPN pars compactus, PPNc; PPN pars dissipatus, PPNd; Pedunculopontinetegmental nucleus, PPTg/PPT; Pontine reticular formation, PRF; Positron Emission Tomography, PET; Progressive supranuclear palsy, PSP; Rapid eye movement, REM; 6-hydroxydopamine, 6-OHDA; Substantia nigra, SN; Subthalamic nucleus, STN; Tegmental pedunculopontine nucleus, TPP; Urotensin-II, UII

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Figure legends

Figure 1: An overview of the major efferent projection pathways originating in the PPN, with information on their neurochemical identity. In rats, the PPN sends ascending efferent fibers to the thalamus and the basal ganglia complex, which travel via the *ansa lenticularis* and the *lenticular fasciculus*. Further ascending efferents from the PPN include the entopeduncular nucleus (EP, homologous to the GPi in primates) and SNpc. Descending efferents project to the cerebellum, reticular formation (RF), locus ceruleus (LC), raphe nuclei (RN) and spinal cord (SC). Arrow colors represent the main neurochemical phenotype of the neuronal projections, but the neurochemical nature of the majority of PPN projection neurons remain to be fully identified (black arrows indicate that these have only so far been identified as being 'non-cholinergic'). The figure also shows the differential distribution of neurons within the PPN according to neurotransmitter expression, along the rostrocaudal axes of the PPN, which divides the PPN into the PPNc and PPNd. The distribution gradients of prominent neuronal populations (cholinergic (green), glutamatergic (blue), GABAergic (red) neurons and unspecified neurochemical cell type (black)) indicate functional territories within the PPN, with each neuronal subgroup demonstrating unique connectivity to neuronal structures involved in regulating specific behavioral effects. In rats, using the accepted means of defining the PPN borders according to the cholinergic (Ch5) neurons, total unilateral PPN neuronal counts have been estimated at ~19,028 (Pienaar and van de Berg 2013). Of these, the GABAergic population is highest at the rostral pole, contrasting to the glutamatergic and cholinergic neuronal groups, which are more densely concentrated at the caudal pole of the nucleus (Wang and Morales 2009; Mena-Segovia and others 2009). Discrepancy exists relating to the estimated number of cholinergic and non-cholinergic neurons in the human PPN, due in large-part to the ill-defined boundaries of the PPN in humans (Jenkinson and others 2009). See text for further discussion. Abbreviations used: Cerebral cortex, Cerebral cx; Entopeduncular nucleus, EP; Inferior colliculus, IC; Lateral hypothalamus, LH; Locus ceruleus, LC; PPN pars compactus, PPNc; PPN pars dissipates, PPNd;

Raphe nuclei, RN; Reticular formation, RF; Spinal cord, Sc; Substantia nigra pars compacta, SNpc; Subthalamic nucleus, STN; Superior colliculus, SC; Ventral tegmental area, VTA.

Figure 2: DBS is a well-established therapeutic option for PD patients. A more tailored approach to DBS therapy, encompassing all potential combinations of stimulation parameters (contact, voltage, pulse width, pulse frequency) improves overall patient outcomes. When formulating a DBS treatment plan, patient symptoms guide target choice. The figure portrays the four possible brain target sites for placement of stimulating electrodes (orange), namely **(A)** the ventrolateral subdivision of the thalamus, also referred to as the ventral intermediate nucleus (Vim), **(B)** the STN, **(C)** the GPi and **(D)** the PPN. Abbreviations used: Central tegmental tract, CTT; Cranial nerve, CN; Decussation of the superior cerebellar peduncle, SCP, Dec; Globus pallidus externa, GPe; Globus pallidus interna, GPi; Lateral lemniscus, LL; Medial lemniscus, ML; Spinothalamic tract, STT; Substantia nigra pars reticulata, SNr.

Figure 3: (A) Mechanism of action for DREADDs in controlling activity of specific cell types in the brain. DREADDs belong to the metabotropic class of receptors that use G proteins as their signaling mechanism and are dubbed hM3Dq (human M3 muscarinic DREADD receptor coupled to Gq signaling) and hM4Di (human M4 muscarinic DREADD receptor coupled to Gq signaling). Upon being activated by an agonist, G protein-coupled receptors (GPCRs) interact with the trimeric G-protein alpha/beta/gamma complex. The figure illustrates the GPCR's seven transmembrane domain structure, into which two point-mutations (red arrows) have been inserted, causing a loss of responsiveness to the cognate agonist ACh. However, when such mutant GPCRs are expressed in a particular tissue, the ligand CNO can activate the mutant receptor, with *in vivo* activation of neurons overexpressing hM3Dq that induces neuronal excitation through whilst CNO activation of neurons overexpressing hM4Di that induces neuronal silencing by hyperpolarizing and attenuating neuronal firing. In experimental animals, use of *Cre*-dependent adeno-associated viruses by which to restrict

DREADD expression to cells that selectively express *Cre* selectively is an increasingly popular method expressing DREADDs in specific cell populations. Spatial specificity of DREADD expression can be further refined through stereotaxic microinjection of the virus into a target region. Ultimately, behavior change is monitored as this represents the output of specific cells, which are manipulated using a DREADD-CNO approach. CNO possesses excellent oral bioavailability and CNS penetration; hence DREADDs offer a means for remotely-induced stimulation/inhibition in different neuronal types that are interdigitated in the same brain areas e.g. the PPN. **(B)** The left figure illustrates the highly non-specific stimulation that is applied to all cell types within the stimulation target. On the other hand, as shown on the right, DREADDs offer more specific effects than electrical stimulation, by altering the firing of specific neuronal types to evoke a change in the behavior of experimental animals and ultimately perhaps in humans also; hence offering an alternative to DBS. However, a significant barrier before DREADD-based therapeutic approaches can reach their full potential is presented by the challenge of virally expressing designer receptors in adult human neurons.

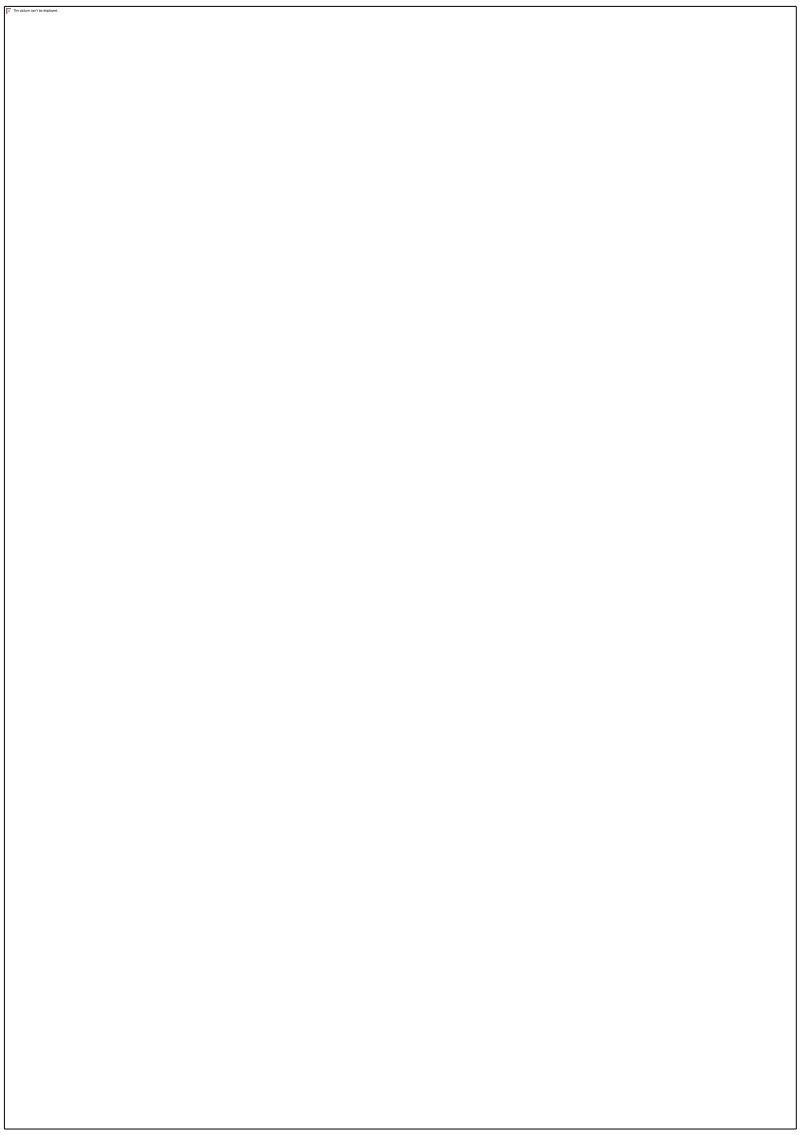
Figure 4: The images portray standardized behavioral tests to measure, in animals that model PD, the functional deficits resulting from damage to the CNS. Development of such tests represents efforts to obtain objective and quantitative readouts. **(A - E)** Motor tests provide a good read-out of neurological function. Several well-validated tests have been developed that assess motor phenotype in rodents, with impaired locomotor function that is evaluated by using scoring systems or biomechanical measures. **(A)** This test assesses balance impairment as an axial symptom of PD resulting from the loss of postural reflexes, seen during the later stages of PD. **(B)** A sensitive and easy to execute test for detecting sensorimotor function, by assessing the ability of rodents to reliably place a forelimb on a tabletop, when the contralateral vibrissae are contacted. **(C)** Gait impairment, including shuffling, short steps and slow walking speed features frequently in PD patients and is mimicked in rats rendered hemiparkinsonian via stereotaxic intracranial delivery of toxins. To record gait pattern changes in rats, several investigative

systems have been developed, the most conventional being use of footprint analysis, where an animal's paws are covered by ink, to create footprints on a paper strip, as the animal traverses a narrow walking track. **(D)** This test exploits the natural exploratory tendency of rodents by evaluating its spontaneous forelimb use and how this is impaired in motor system injury models that depict stroke and PD. For this, an animal is placed in a transparent Plexiglas cylinder for observations. **(E)** Motor coordination can be assessed in rodents by means of the rotarod test, where the animal is placed on a horizontal rod that rotates about its long axis. In order not to fall off, the animal must walk forward. Two versions of the rotarod, set- and accelerating speed, is available. **(F - I)** Many behavioral paradigms have been developed to examine the contribution of various neural systems implicated in learning and memory function in rodents. **(F)** Rodents have a natural exploratory drive to explore new environments (for food and shelter) and to avoid open and brightly lit spaces (exposure to predators). By placing rodents into a relatively large, brightly lit novel context of a circular or rectangular shape, locomotor activity, exploratory drive, neophobia and certain aspects of anxiety are measured. **(G)** Rodents are deemed to display anxiety-like behavior when it spends an increased amount of time and makes multiple entries into the closed arms (that represent rodents' preference for protected areas) of the elevated plus maze. In contrast, its innate motivation to explore novel environments is measured as activity in the open arms of the maze. **(H)** A test of spatial learning, where rodents are tasked at locating a submerged escape platform in an opaque swimming pool, by relying on distal navigation cues placed around the perimeter of the swimming arena. As performance in this task requires strength and coordination, evaluators need to dissect out the motor from the cognitive impairments when assessing rodent models of neurological disease. Although a detailed explanation of the procedures used in executing the tests exemplified here lies outside the scope of the current article, we refer the interested reader to articles that review behavioral outcome measures used in animal models of PD (e.g. Bury and Pienaar 2013; Pienaar and others 2012). **(I)** A cognitive function (especially executive function, learning and memory) assessment tool

based on rodents that interact with an automated touchscreen platform, which is gaining popularity, as various assays can be run similarly to human subjects, thereby allowing translation of laboratory animal results to humans. A positive (appetitive) reinforcement, rather than an aversive one is used for stimulating performance, which minimizes stress in the animals. Further information and precise protocols for performing touchscreen assays using experimental animals can be found in Horner and others (2013).

Table 1: A summary of the different sites for implantation of a DBS electrode for alleviating PD symptoms. Target choice and optimal stimulation parameters should be tailored to a patient's individual needs. Although rare, practitioners should be aware that the procedure is not risk free, but candidacy for DBS surgery should be deemed based on whether a patient’s needs and expected benefits outweigh the risks of surgery. Information presented in the table are discussed in detail in several recent review articles. The interested reader is referred to Papageorgiou and others (2016) and Verhagen Metman and others (2016).

Table 2: Toxins that recognize a broad range of cell types including neuronal cells, but show preferential toxicity for specific neurons, with this feature that can be exploited for studies investigating the relative contribution made by these neurons towards behavior mediated by the PPN.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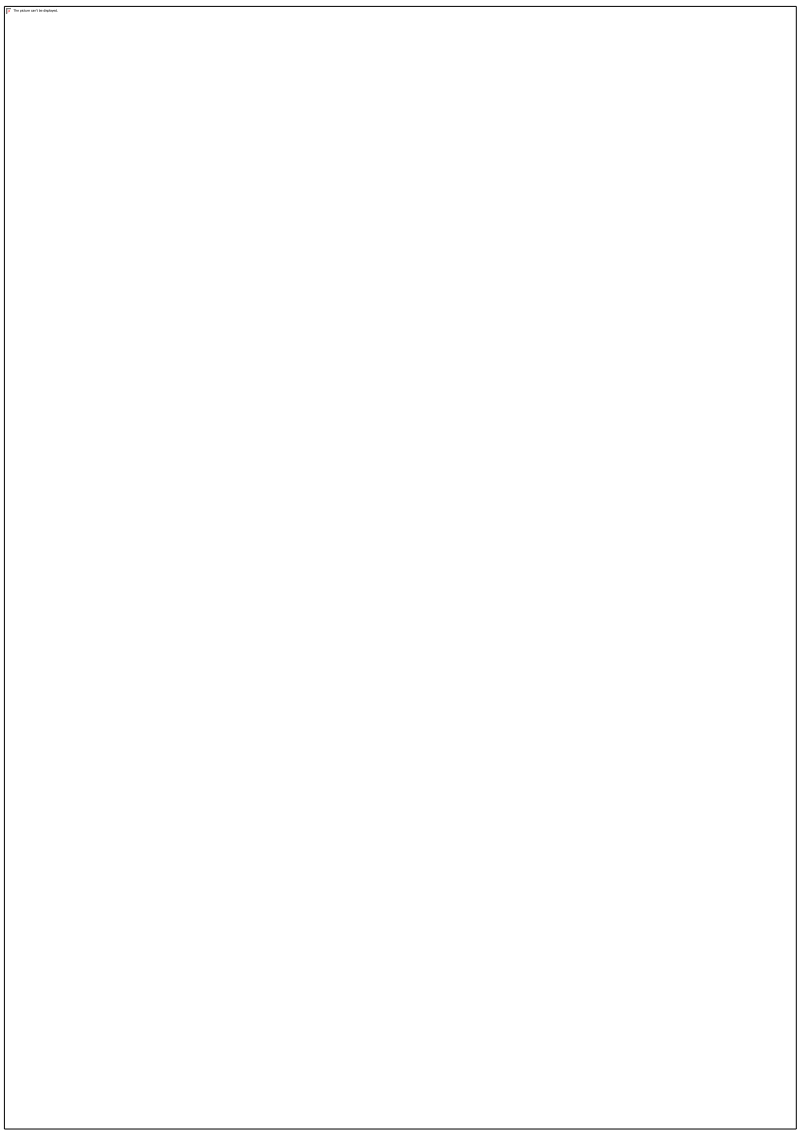
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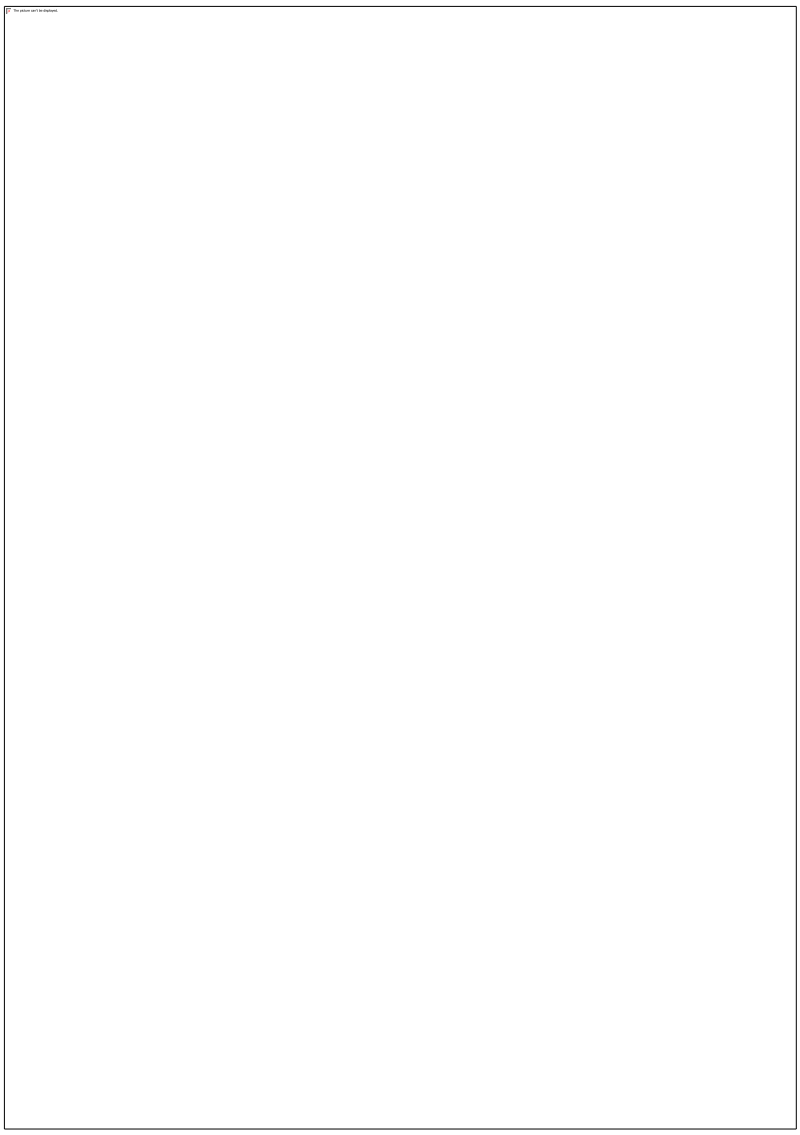
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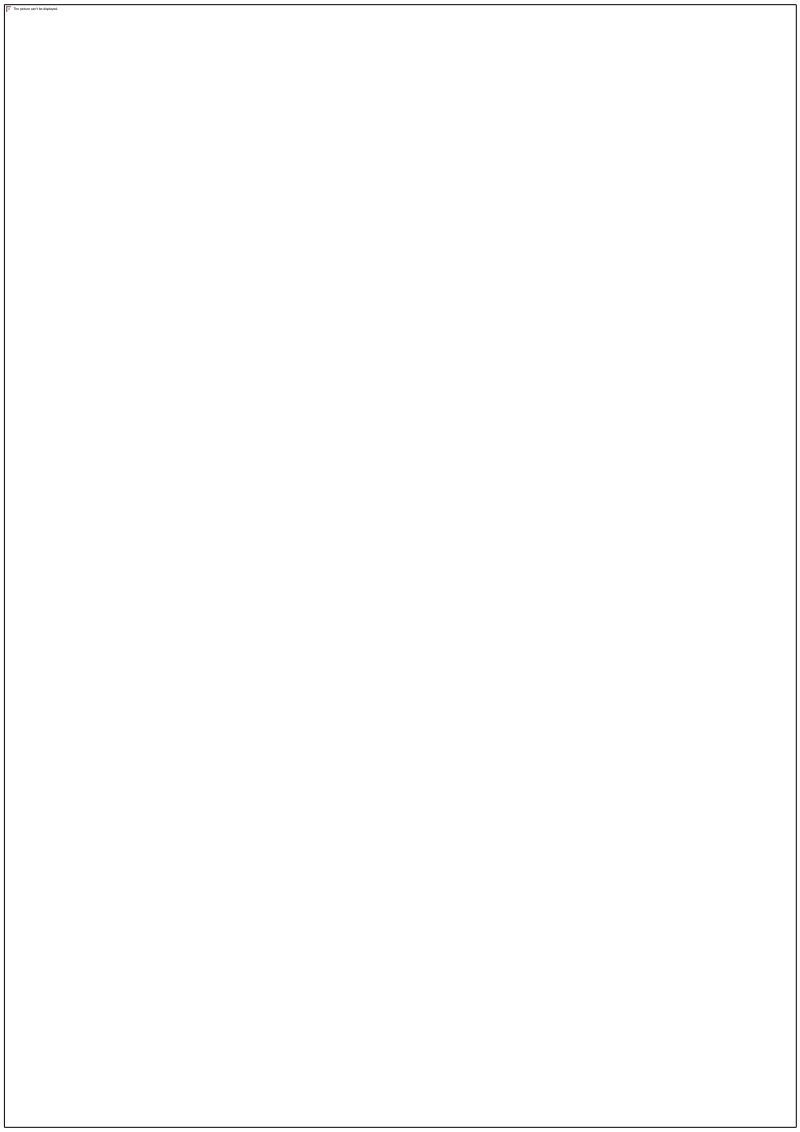

Table 1:

Target (and key reference)	**Major indications**	**Electrical stimulation parameters**	**Potential side-effects resulting from the DBS procedure**	**Contraindications**

Vim nucleus of the Thalamus	• PD with essential tremor
	• PD with orthostatic tremor
		High frequency stimulation	• Surgery-related complications
			  ○ Minimal tissue damage
			  ○ Intracerebral hemorrhage
			  ○ Chronic subdural hematoma
			• Device-related complications
			  ○ Device infection
			  ○ Pulse generator malfunction
			• Stimulation-related complications
			  ○ Intractable dyskinesia
			  ○ Apraxia of eyelid opening
			  ○ Back pain
			  ○ Restless leg syndrome
			• Psychiatric symptoms
			  ○ Impulse control disorder
			  ○ Depression (most common)
			  ○ Delusion
			  ○ Euphoria
			  ○ Disinhibition
			  ○ Suicide attempts
			• Other
			  ○ Tolerance may develop in patients that require adjustment of stimulation parameters
				• Non-drug induced preoperative psychiatric and/ cognitive dysfunctions
				• Caution should be applied in PD patients co-diagnosed with diabetes (increased predisposition for device-induced infection) and/hypertension (increased predisposition for intraoperative hemorrhage from implanting a DBS electrode)

STN	• PD with rigidity • PD with tremor • PD with slowness of movement (bradykinesia)	High frequency stimulation		
GPi	• PD with dystonia • PD with tremor • PD with rigidity • PD with bradykinesia • PD with dyskinesia • PD with gait difficulties	High frequency stimulation		
PPN	PD with FOG	Low frequency stimulation		

Table 2:

Toxin	Source	Toxic mode of action	Cellular specificity
Diphtheria toxin – Urotensin II (Dtx-UII)	Diphtheria toxin fused to urotensin-II (Clark and others 2007)	Dtx-UII comprise of the endogenous ligand for urotensin-II receptors that are expressed by PPN cholinergic neurons but not by glutamatergic or GABAergic ones.	Cholinergic neurons. As with other fusion toxins, high doses can cause death of other neuronal types.
Lactacystin	Bacteria belonging to the genus *Streptomyces*	Inhibits the proteasome, with clasto-lactacystin β-lactone that comprise the cell-permeable, active form of lactacystin.	Several studies have shown that lactacystin's effect extend beyond the injection site and exerts a weaker toxic effect on non-dopaminergic than dopaminergic neurons. In rats, stereotaxic lesioning of the nigrostriatal system results in destruction of dopaminergic neurons and formation of ubiquitin/α-synuclein immunopositive inclusions in surviving ones (McNaught and others 2002). Lactacystin was not fully selective to dopaminergic neurons, but also moderately reduced the number of non-dopaminergic ones in the SN (Konieczny and others 2015). This study also showed slightly reduced GABA levels in the SN following the lesion, while others showed that striatal GABA medium spiny neurons appear resistant to this toxin, when delivered *in vivo* (Lorenc-Koci and others 2011). As an example of the toxin's effects beyond the injection site, Harrison and others (2016) observed neurodegeneration within the SN, and degeneration of dopaminergic neurons of the nearby ventral tegmental area (VTA). However, loss of VTA

			dopaminergic neurons was less severe than in the SN pars compacta. When injected directly into the rat SN, preferential retrograde toxicity is shown towards cholinergic neurons in the PPN with less destruction of PPN non-cholinergic neurons (Elson and others 2015). It is likely that lactacystin's selectivity with regards to neuronal types and brain region depends on the dose and infusion rate; aspects which require further investigation in future studies.
MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine)	A street-drug contaminant deriving from underground laboratory preparations of a potent analog of meperidine (Demerol).	MPTP itself does not appear to be toxic, but its oxidized product (1-methyl-4-phenylpyridinium (MPP+)), is toxic. Energy-driven mitochondrial uptake of MPP^+ results in sufficiently high concentrations of the toxin to interfere with mitochondrial respiration in the cell.	Appears to specifically target neurons involved in PD pathology, including nigrostriatal dopaminergic and brainstem cholinergic neurons
Excitotoxins The most commonly used are **ibotenic acid** (ibotenate), **kainic acid** (kainate), **N-methyl-D-aspartate** (NMDA) and **quinolinic acid** (quinolinate)	Rigid structural analogs of glutamate, derived from various sources. Ibotenate is a secondary metabolite of the mushroom *Amanita muscaria*; kainate is found in seaweeds; NMDA is a synthetic product, not naturally	Binding to glutamate receptors typically result in increased influx of chloride and calcium ions, excess water entry, to finally result in osmotic lysis of cells. Different excitotoxins have different binding profiles to the various types of glutamate receptor and are effective at	Excitotoxins cause neuronal death but spare fibers of passage (e.g. Brace and others 1997).

	found in biological tissues; quinolinate is naturally synthesized in the kynurenine metabolic pathway.	different concentration ranges.	
Transient lesions using muscimol	Muscimol is a natural psychoactive constituent of the mushroom species *Amanita muscaria* (as is ibotenic acid) and *Amanita pantherina*.	A potent $GABA_A$ receptor agonist, with $GABA_A$ comprising the receptor for GABA, the CNS's principal inhibitory neurotransmitter.	It is used as a *de facto* transient lesion. By acting as a GABA agonist, muscimol inhibits all neurons in the region of injection. Unlike a local anesthetic, it does not affect local fiber systems.