Cholinergic system changes in Parkinson's disease: Emerging therapeutic targets

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Regionally heterogeneous cholinergic system changes are seen in Parkinson's disease and correlate with a range of motor and non-motor dopaminergic therapy refractory clinical features. These can be conceptualized within a system-level framework in which nodal deficits produce circuit dysfunctions. The topographies of cholinergic changes overlap with neural circuitries involved in cognitive, motor, perceptual, sleep, and autonomic functions. Cholinergic deficits within cognitive control network hubs predict cognitive deficits better than global PET brain volume-of-interest neocortical cholinergic deficits. Postural instability and gait difficulties are associated with cholinergic system changes in thalamic, caudate, limbic, neocortical and cerebellar nodes. Cholinergic system losses also involve peripheral organs in Parkinson's disease. Possible hypercholinergic activity of the mesopontine cholinergic neurons in isolated REM sleep behavior disorder, as well as in the hippocampus of cognitively normal Parkinson's disease, suggests early compensation during the prodromal and early stages of Parkinson's disease. Novel pharmacologic, deep brain stimulation, non-invasive vagus nerve, caloric vestibular, and transcranial direct current neurostimulation approaches may allow targeting of hypocholinergic brain regions to treat motor and non-motor features of Parkinson's disease.

Panel 1: **Research in context**

Selectivity of cholinergic therapeutic approaches and neural circuitry in Parkinson's disease

Effects of cholinergic degeneration cannot be viewed in isolation but should be placed in the context of severe dopaminergic losses and other pathologies in Parkinson's disease. Topographically distinct cortical and subcortical cholinergic system changes in Parkinson's disease reflect differential vulnerability of cholinergic systems that associate with specific clinical features of these disorders. Neural circuitries involve a variety of neurotransmitters. For example, cholinergic neurons in the pedunculopontine nucleus project to GABAergic and glutamatergic neurons in the mesencephalic locomotor region, the medulla (reticulospinal tract), medial vestibular nuclei, dopaminergic midbrain neurons (substantia nigra pars compacta and ventral tegmental area) and the thalamus (1, 2). Similarly, neurostimulation targeting of brain regions with impaired cholinergic systems will induce cholinergic and non-cholinergic effects that may conjointly improve neural circuitry functions relevant to specific clinical features of dementia, and postural instability and gait difficulties, the hypocholinergic subtype in Parkinson's disease (3).

Scientific evolution from a diffuse neuromodulator to regionally specific deterministic functions of the basal forebrain cholinergic systems.

Brain cholinergic systems are traditionally viewed as diffuse neuromodulator system, but recent evidence points to basal forebrain cholinergic projections exhibiting specific connectivity (4). These neurons give rise to extensive, multi-branch projections with subpopulations of neurons innervating limited numbers of cortical fields (5). The governing rule may be that basal forebrain cholinergic neurons are organized as clusters with members of each cluster innervating strongly interconnected cortical regions. It is neither necessary nor probably correct to assume that acetylcholine acts at a relatively low temporal resolution (tonic), but that fast (phasic) cholinergic signaling is a sufficient framework to conceptualize our current understanding of the basal forebrain cholinergic system (6).

Panel 2: **Cholinergic neuron groups**

- Ch1 (medial septal nuclei) projecting to hippocampus (7)
- Ch2 (vertical limb nucleus of the diagonal band of Broca projecting to the hippocampus and hypothalamus (7)
- Ch3 (horizontal limb nucleus of the diagonal band of Broca) projecting to the olfactory bulb and piriform cortex (7)
- Ch4 (nucleus basalis of Meynert) projecting to the cortical mantle, amygdala and midline thalamic complex (7)

- Ch5-6 (pedunculopontine nucleus-laterodorsal tegmental complex) projecting to the thalamus, striatum, subthalamic nucleus, both pallidal segments, both substantia nigra components, basal forebrain, brainstem, and spinal cord) (7, 8)
- Ch7 (medial habenula) projecting to the interpeduncular nucleus (9)
- Ch8 (parabigeminal nucleus) projecting to the superior colliculus (10).
- Striatal cholinergic interneurons (11)
- Medial vestibular nucleus (pons) projecting to the vestibulocerebellum (12). Additional sources of cerebellar cholinergic afferents include the prepositus hypoglossi nucleus (identified only in rats (13)) and the basal interstitial nucleus of the cerebellum (14)
- Brainstem and spinal motor neurons
- Dorsal motor nucleus of the vagus with efferents providing parasympathetic innervation to the heart and GI tract from the lower esophagus to the proximal colon.
- Cholinergic enteric neurons and glial cells (15)
- Sacral cholinergic preganglionic neurons providing parasympathetic innervation to the bladder and distal colon (16)
- Interomediolateral preganglionic sympathetic neurons
- Spinal cord cholinergic interneurons (17)

Introduction

Parkinson's disease is a progressive neurodegenerative disorder with motor and non-motor morbidities. Distal limb bradykinesia is the disease-defining motor feature with variably associated rigidity, resting tremor, and balance and gait disturbances. Dopaminergic pharmacotherapy is the mainstay of management in Parkinson's disease, with considerable efficacy for bradykinesia but it is ineffective for several disabling features, including dementia, and postural instability and gait difficulties (18). Dementia and these motor features, leading to falls and freezing of gait, are present in the majority of patients 10 to 15 years after onset (18), although it can occur at earlier stages in many patients. Emergent postural instability and gait difficulties usually coincide with dementia emergence, suggesting shared pathophysiology (19). The dopaminergic treatment refractory nature of these motor and non-motor impairments implies extra-striatal and/or non-dopaminergic pathophysiology. Post-mortem neuropathology and *in vivo* imaging studies identified cholinergic system changes associated with dementia, falls, and freezing of gait in Parkinson's disease ((20-22), see also (3) for review). These correlates of cholinergic deficits, along with severe olfactory dysfunction, REM sleep behavior disorder, and some neuropsychiatric features comprise a malignant hypocholinergic subtype of Parkinson's disease (3). Cholinergic system changes cannot be understood in isolation and need to be viewed in a wider pathophysiological context, in particular interactions with dopaminergic deficits in Parkinson's disease (see panel #1 for research context). Our goal is to provide an update of cholinergic systems and disease-specific changes in cholinergic systems in Parkinson's disease and isolated REM sleep behavior disorder (a Parkinson's disease prodrome). We discuss regional changes in overlapping hubs of functionally distinct neural networks and the potential application of novel pharmacologic, and therapeutic deep brain and non-invasive neurostimulation approaches that may selectively target hypocholinergic circuits of both the central and peripheral nervous systems. Figure 1 provides an overview of relevant cholinergic systems anatomy.

Overview of brain and enteric nervous cholinergic systems

Brain cholinergic neurons are mainly projection neurons bridging different CNS regions, with motor and some autonomic neurons interfacing between the CNS and peripheral nervous system. The important exception is striatal cholinergic interneurons. The striatum exhibits the highest density of cholinergic markers in the brain, underscoring the critical but incompletely understood role of cholinergic interneurons in striatal function (11). The most extensive brain cholinergic projection system is the basal forebrain complex, also containing important populations of GABAergic and glutamatergic neurons. Associated with attention, memory, and learning, cholinergic basal forebrain neurons project to the whole cortical mantle. These neurons exhibit topographic organization with recent studies revealing clusters with specific connections to cortical targets (see panel #2 for overview of different cholinergic groups; see also panel #1 for context).

As a critical link to the peripheral autonomic system, dorsal motor nucleus of the vagus neurons are cholinergic in nature and provide parasympathetic innervation of several organs, including the gastro-intestinal tract from the lower esophagus to the proximal colon. The distal colon and other organs receive parasympathetic innervation from sacral cholinergic preganglionic neurons (16).

Acetylcholine receptors

Cholinergic signaling is mediated by both metabotropic (G-protein coupled; muscarinic [mAChR]) and ionotropic (nicotinic [nAChR]) receptors. There are 5 (M_1 – M_5) mAChRs (23, 24). M_1, M_3, and M_5 are coupled preferentially to $G_{q/11}$ G-proteins that activate phospholipase C, while M_2 and M_4 are coupled preferentially to $G_{i/o}$ G-proteins that inhibit adenylyl cyclase and modulate ion channel functions. nAChRs are excitatory, cation permeated ligand gated ionophores composed of 5 subunits (25). There are 10 α subunit genes, 4 β subunit genes, and genes for ε, δ, and γ subunits. Different subunit combinations lead to varying biophysical properties and with pentameric structure, the potential range of receptor permutations-subtypes is huge. Within the CNS, heteromeric $\alpha4\beta2^*$ (*potential inclusion of other subunits) and homomeric α7 receptors appear to be the predominant nAChRs with evidence of α6 containing receptors at some CNS synapses (26). Varying synaptic localization of both nAChRs and mAChRs further complicates cholinergic signaling. nAChR and mAChRs are expressed as postsynaptic receptors in cholinergic synapses. Both AChR classes are also expressed as presynaptic receptors with localization on both cholinergic (presynaptic homoreceptors) and non-cholinergic (presynaptic heteroreceptors) neuron terminals. An example of presynaptic AChRs potentially relevant to Parkinson's disease are heteroreceptor nAChRs expressed on glutamatergic thalamocortical terminals innervated by basal forebrain cholinergic cortical afferents. These $\alpha4\beta2^*$ nAChRs are thought to be important mediators of attentional signaling (27). Another potentially relevant population of presynaptic nAChR heteroreceptors are expressed on nigrostriatal dopaminergic terminals and activated by striatal cholinergic interneurons. Activation of these nAChRs, possibly containing α6 subunits, evokes dopamine release (28). Similarly, presynaptic M_4 heteroreceptors are found on glutamatergic corticostriate terminals and GABAergic terminals of striatal neurons projecting to the substantia nigra pars reticulata, presumably activated by acetylcholine from striatal cholinergic interneurons and pedunculopontine nucleus-laterodorsal tegmentum complex terminals, respectively. In both cases, M_4 stimulation results in diminished neurotransmitter release (24). M_4 heteroreceptors are not expressed on indirect pathway spiny projection neurons. There are likely additional complexities of information transmission by cholinergic neurons. nAChRs and mAChRs are widely distributed in the CNS with variable receptor subtype expression between and within regions. One notable exception is M_5 whose expression is limited to dopamine neurons. Some cholinergic neurons also use other small molecule neurotransmitters and many co-express peptide neuromodulators.

Assessment of cholinergic system changes in Parkinson's disease

Cholinergic systems degeneration was identified as a major pathophysiologic component of cognitive impairments in Alzheimer's disease and Lewy body disorders, including Parkinson's disease (see (29) for review). Cholinergic system deficits were originally identified with post-mortem studies. Largely restricted to end-stage disease, post-mortem studies generally permit only qualitative correlations with disease features. *In vivo* radioligand imaging studies offer opportunities to investigate and define regional cholinergic system changes in patients at earlier disease phases, facilitating correlation with key clinical features. Traditionally, acetylcholinesterase radiotracers were used. However, new radioligands binding to the vesicular acetylcholine transporter, such as the [^{18}F]-

fluoroethoxybenzovesamicol (FEOBV) (30), represent a significant advance because of specific expression of vesicular acetylcholine transporters in cholinergic neurons, lower likelihood of medication-related regulation, and more reliable quantification of brain tracer binding, especially in high binding areas such as the striatum and cerebellum. Acetylcholinesterase is also expressed in multiple non-cholinergic neurons and even non-neuronal cells, including immune cells (31). [^{18}F]FEOBV PET imaging can also be performed using simplified imaging acquisition protocols. The following sections focus on cholinergic system changes associated with dopamine medication refractory symptoms of cognitive, and postural instability and gait difficulties, and autonomic functions in Parkinson's disease.

Cholinergic system changes and cognition in Parkinson's disease

Molecular imaging studies demonstrated more severe cholinergic synapse deficits, especially of posterior cortices, in Parkinson's disease with dementia compared to Parkinson's disease with normal cognition (32-34). These results are consistent with post-mortem data indicating preferential loss of nucleus basalis of Meynert and medial septal-vertical limb of the diagonal band cholinergic cells in Parkinson's disease with dementia compared to controls, whereas Parkinson's disease patients without dementia had more limited nucleus basalis of Meynert losses only (35). MRI studies have confirmed robust correlations between the volume or integrity of the basal forebrain with cognitive impairments in Parkinson's disease (36). Consistent with imaging evidence of diminished cholinergic synapses, studies of cholinergic receptors in basal forebrain target regions demonstrated reduced receptor density in Parkinson's disease with cognitive impairments (37, 38).

There is evidence for the existence of robust and specific associations between the magnitude of cholinergic deficits and severity of cognitive impairments across the Parkinson's disease spectrum from intact cognition to dementia, independent of nigrostriatal terminal losses (33, 39). Cholinergic deficits preferentially affect the domains of memory, attention, and executive function (40, 41) as well as visuospatial function (40). Regional correlations indicate spatial overlap of cholinergic systems node deficits associated with memory, attention, executive function, and language dysfunctions, suggesting shared neural circuitry vulnerability driving deficits in different cognitive domains. Overlapping regions include thalamic nuclei, especially the lateral geniculate nucleus, hippocampus, caudate nucleus, cingulum, and lateral cortical territories encompassing the prefrontal, insular and opercular regions (41). The topography of shared cholinergic deficits coincides with nodes of the salience, cingulo-opercular, and default mode neural networks (42). Cholinergic deficits within these cognitive control network hubs predict cognitive deficits better than global PET brain volume-of-interest neocortical cholinergic deficits (41). Cholinergic deficits in control networks may also be linked to visual dysfunction in Parkinson's disease (43-45).

Early vulnerability of visual cortical cholinergic synapses (34) and correlation of visual thalamus deficits with cognitive deficits (41) indicates an important role of visual system cholinergic deficits in Parkinson' disease. Cholinergic neurotransmission plays pivotal and complex roles in visual processing, likely at several levels of visual processing (43, 44). PET and SPECT imaging studies demonstrated cortical cholinergic deficits in Parkinson's disease patients with hallucinations compared to non-hallucinating patients (46). Similarly, greatest deficits of cholinergic receptors were found in dementia with

Lewy bodies patients with recent history of visual hallucinations (47). Functional connectivity studies indicate abnormal coupling of basal forebrain nuclei to visual cortices in Parkinson's disease and Dementia with Lewy bodies (48).

Cholinergic system changes associating with postural instability and gait difficulties in Parkinson's disease

Parkinson's disease progression is characterized by debilitating postural instability and gait difficulties, notably falls and freezing of gait (18). These posture-gait dysfunctions are strongly linked to cholinergic abnormalities and independent of dopaminergic deficits (49, 50). Recent studies with [^{18}F]FEOBV PET suggest that Parkinson's disease with isolated falls and those with freezing of gait share common cholinergic deficits within components of an attentional-motor interface network, including thalamic nuclei (esp. the lateral geniculate nucleus) and caudate nucleus with more extensive striatal, limbic, and cortical cholinergic deficits in those with freezing of gait (51). These data suggest striatal cholinergic interneuron dysfunction as a common denominator in the pathophysiology of falls and freezing of gait. A confluence of clinical and experimental data indicates that deficient striatal cholinergic interneuron integration of attention and motor functions underlies these important clinical features (52, 53). However, vesicular acetylcholine transporter binding in the striatum also includes signal components from projections from the pedunculopontine nucleus/laterodorsal tegmental complex. For example, an anatomic tracing study showed that the rostral pedunculopontine nucleus preferentially innervates the dorsolateral striatum, and the laterodorsal tegmental complex preferentially innervates the medial striatum and nucleus accumbens core (54). Therefore, striatal binding reductions in part may also reflect degenerating pedunculopontine nucleus/laterodorsal tegmental complex projections.

These results emphasize the necessity of understanding postural instability and gait features as products of cholinergic deficits within a framework of failing cognitive, particularly attentional, and motor integration. Isolated falls typically precede freezing of gait. With disease progression, these episodic disturbances become more frequent. Cholinergic deficits within the medial geniculate nucleus and the entorhinal cortex are robustly associated with non-episodic axial motor impairments (*i.e.*, all axial motor impairments except for falls and freezing of gait) (55). The medial geniculate nucleus is involved in multi-sensory (auditory, vestibular, and proprioceptive) processing. The entorhinal cortex plays a role in multisensory information processing relevant for spatial navigation, including vestibular and visuospatial signals (56). These results imply a significant role of impaired sensorimotor integration underlying non-episodic axial motor features in Parkinson's disease. A plausible model is that non-episodic postural instability and gait difficulties are underpinned by cholinergic deficits in the medial geniculate nucleus and the entorhinal cortex: emergence of falls by progression to involvement of the lateral geniculate nucleus and caudate nucleus, and progression to more diffuse striatal, limbic and cortical deficits underlying freezing of gait.

Cholinergic system changes and peripheral autonomic dysfunctions

Molecular imaging studies also allow assessment of parasympathetic innervation of internal organs. Reproducible estimates of cholinergic innervation were acquired with the PET radiotracer 5-[^{11}C]-methoxy-donepezil ([^{11}C]donepezil), a reversible

acetylcholinesterase inhibitor (57). [^{11}C]donepezil is a ligand-type tracer, i.e., it binds directly to a binding site on acetylcholinesterase. Thus, it is parallel to FEOBV in that it is a marker of the concentration (density) of acetylcholinesterase - in contrast to the classical substrate-like tracers such [^{11}C]PMP, which is a measure of enzyme hydrolysis rates. [^{11}C]donepezil PET studies showed peripheral autonomic changes in Parkinson's disease. Early-stage Parkinson's disease patients showed 22% loss of signal in the colon and 14% in the small intestine and renal cortex (58). In later-stage Parkinson's disease, there was 35% reduction in the small intestine and 22% loss in the pancreas (59). Subjects with isolated REM sleep behavior disorder showed marked reduction of [^{11}C]donepezil uptake in the small and large intestine, similar to that seen in moderate-stage Parkinson's disease, and exceeding reductions seen in *de novo* Parkinson's disease subjects without this parasomnia (60, 61). These observations support the hypothesis that Parkinson's disease includes a *body-first* subtype, characterized by prodromal autonomic denervation and isolated REM sleep behavior disorder, and a *brain-first* subtype characterized by nigrostriatal degeneration prior to the appearance of REM sleep behavior disorder and autonomic denervation (62).

Emerging evidence of bidirectional cholinergic system changes in prodromal and early-stage Parkinson's disease

Clinical manifestations in Parkinson's disease result from neurodegeneration, but some features may be palliated by neuronal compensatory processes. Older postmortem studies described larger quantities and sizes of cholinergic terminals in the substantia nigra pars compacta, speculated to partly compensate for nigral neuronal death (63). Recent PET studies suggested similar evidence of brain cholinergic upregulation in Parkinson's disease and prodromal Parkinson's disease. A dual ligand [^{18}F]-FEOBV and monoaminergic PET study showed higher striatal vesicular acetylcholine transporter binding in Parkinson's disease patients than in normal control persons, (and also as an inverse function of striatal dopaminergic binding) (64). These findings may provide some (limited) support for the historic striatal acetylcholine-dopamine balance (seesaw) model of striatal dysfunction. Moreover, a recent study of Parkinson's disease subjects with a muscarinic receptor ligand suggests bidirectional changes in cholinergic neurotransmission in several brain regions (38). These bidirectional changes may reflect a combination of denervation, regulation, or compensatory processes and possibly even with changes in muscarinic cholinergic receptors themselves. In a small number of patients with isolated REM sleep behavior disorder, PET imaging with [^{18}F]-FEOBV revealed increased uptake in several brain regions, which may indicate cholinergic terminal upregulation (65). These increases were notable in brainstem areas associated with the promotion of REM sleep and muscle atonia (see figure 2), and were significantly correlated with the abnormal muscle activity during REM sleep. Possible explanations include increased activity or compensatory sprouting of cholinergic terminals during early phases of neurodegeneration, a phenomenon suggested to occur also in prodromal stages of Alzheimer disease (66)(67). More recently, [^{18}F]-FEOBV uptake was found to be increased bilaterally in the hippocampus of Parkinson's disease patients without cognitive deficits but absent in cognitively impaired patients (68). These hippocampal features occurred in these patients despite cholinergic denervation in other parts of the brain that were of similar topography and severity with those observed in Parkinson's disease patients presenting with mild cognitive impairments. Compensatory

cholinergic mechanism might therefore underly normal cognition in Parkinson's disease. Increased acetylcholinesterase enzyme activity has been reported in Leucine Rich Repeat Kinase 2 (LRRK2) mutation carriers with prodromal Parkinson's disease, also suggesting a possible compensatory response (69).

Cholinergic system changes may provide novel therapeutic targets in Parkinson's disease

Knowledge of heterogeneous cholinergic system changes in Parkinson's disease is relevant for clinical practice. Although currently approved cholinesterase inhibitor drugs have limited effectiveness for dopaminergic medication refractory features (70, 71), use of these drugs is restricted by peripheral organ side-effects and poor brain penetrance. Cholinesterase inhibitor-induced increases in brain acetylcholine levels likely have unintended consequences because non-specific tonic stimulation of all cholinergic receptor subtypes, and may induce undesired effects (72). Given the lack of clinically available subtype specific cholinergic drugs, the most effective cholinergic drug intervention in Parkinson's disease may be de-prescribing non-selective anti-cholinergic drugs. This is particularly important for non-selective antimuscarinic drugs that may worsen cognition and increase risk of developing freezing of gait (73).

Novel pharmacological approaches

Non-selective mAChR antagonists have a long history of use in Parkinson's disease, and acetylcholinesterase inhibitors, though not highly efficacious, are extensively used for treatment of cognitive impairment in Parkinson's disease. As mAChRs and nAChRs are widely distributed in the CNS and other organs, these non-selective agents exhibit poor therapeutic indices due to undesirable side effects. Recently developed pre-clinical tool compounds suggest that subtype specific agents may have reduced adverse effects and increase patient tolerability. Development of subtype specific mAChR allosteric modulators has been a particular focus because the conservation of the acetylcholine binding site across mAChR subtypes is a significant obstacle to the development of highly subtype specific orthosteric agents. Due to breakthroughs in medicinal chemistry and pharmacology, the development of several mAChR subtype selective compounds for M_1, M_4, and M_5 have been reported. Despite some preclinical evidence suggests that M_1 antagonists have anti-parkinsonian efficacy. M_1 inhibition, however, may impair cognition. In contrast, M_1 positive allosteric modulators enhance cognition in a variety of preclinical models (23, 74). M_1 potentiation effects may correlate with enhanced function of cortical, hippocampal, and striatal circuits associated with executive function and cognition (23). An M_1 PAM ameliorated falls in a rat model of Parkinson's disease gait-balance disorders (75). Similarly, combination therapy with a cholinesterase inhibitor and a subtype selective serotonin receptor antagonist reduced falls in the same model (76). Basal ganglia M_4 mAChRs emerged as potentially important targets for treatment of Parkinson's disease. Activation of striatal M_4 mAChRs inhibits dopamine release and activation of substantia nigra pars reticulata M_4 mAChR presynaptic heteroreceptors inhibits motor function. Both M_4 negative allosteric modulators and newly developed M_4 antagonists have beneficial effects in Parkinson's disease clinical models (24, 77, 78). While initial studies involve domains modulated by dopaminergic therapy, M_4 selective inhibition may modulate

symptoms refractory to dopaminergic replacement (24). Cholinergic pharmacology research in Parkinson's disease largely focused on muscarinic agents, but there is emerging evidence for targeting nAChR subtypes. α4β2* nicotinic receptor agonists may improve gait and attention (71) and α7 activation may be pro-cognitive and reduce dyskinesias (79). α4β2* and α7 activation can release dopamine in the prefrontal cortex (80). Clever methods to manipulates nAChR subtype expression and trafficking may produce targeted nAChR pharmacology (81). Figure 3 provides a pictorial overview about the main cholinergic receptor interaction sites in the basal ganglia.

Non-invasive neurostimulation approaches

The presence of heterogeneous cholinergic system changes in Parkinson's disease may be an obstacle to effective systemic cholinergic pharmacotherapy. Negative effects might occur in brain regions with preserved or compensatory cholinergic function - an overdose effect - while potentially benefiting areas with cholinergic losses. A similar overdose effect exists for levodopa where cognitive side-effects may occur due to relatively spared ventral tegmental area dopaminergic projections (82). Selective targeting of hypocholinergic brain regions in Parkinson's disease might be feasible through repurposing of neuromodulatory approaches. The vagus nerve is a key conduit of the parasympathetic nervous system and acetylcholine is the primary neurotransmitter of vagal afferents. Vagal afferents project to the nucleus tractus solitarius within the brainstem, which in turn projects to a number of cortical and subcortical structures both directly and indirectly via the locus coeruleus (83). The locus ceruleus-norepinephrine axis has widespread projections, including to the cholinergic nucleus basalis of Meynert. Recent work supports vagus nerve stimulation in Parkinson's disease (84-86). Vagus nerve stimulation can be invasive or non-invasive, using transcutaneous stimulation of the auricular or cervical branches of the vagus nerve. Animal studies provide mechanistic insights into vagus nerve stimulation as it relates to Parkinson's disease. Repetitive auricular vagus nerve stimulation significantly improved motor function, increased α7 nicotinic receptor expression, and modulated inflammation in 6-hydroxydopamine-lesioned rats (87). Chronic implanted vagus nerve stimulation improved locomotion, neuroinflammation, and decreased α-synuclein expression in the substantia nigra in rats (88). Treatment with the muscarinic antagonist scopolamine and basal forebrain lesions attenuate vagus nerve stimulation effects in rats (89, 90), thought to be mediated indirectly by cholinergic anti-inflammatory pathways (91).

In humans, cholinergic central pathways may be activated during vagal nerve stimulation as demonstrated by functional MRI following transcutaneous cervical stimulation in healthy humans (92). Single or multiple doses of transcutaneous vagus nerve stimulation have effects on dopa-resistant gait characteristics in patients with Parkinson's disease (85). For example, a randomized double-blind, sham-controlled pilot study in Parkinson's disease demonstrated reduced step time and step length variability in the active group compared to the sham group (84). Another randomized controlled trial showed that transcutaneous vagus nerve stimulation delivered multiple times per day over one month improved overall motor function while serum tumor necrosis factor (TNF)-α and glutathione levels decreased and brain-derived neurotrophic factor levels increased significantly in patients with Parkinson's disease (86).

Electrostimulation of intrinsic auricular muscle zones is thought to activate the C2 spinal nerve, facial, trigeminal and vagus nerves (the latter via parasympathetic contribution),

together with activation of locomotor areas and the pedunculopontine nucleus (93). A placebo- and sham-controlled, double-blinded study demonstrated statistically significant and clinically significant improvement in motor scores following active treatment in patients with Parkinson's disease, which the authors postulate may be related to activation of the locus coeruleus-pedunculopontine nucleus axis (93). Another neuromodulatory approach that may enhance gait and balance in Parkinson's disease through enhancement of pedunculopontine nucleus connectivity is caloric vestibular stimulation (94, 95). In a double-blind, placebo controlled, randomized study, 33 Parkinson's disease participants completed eight weeks of treatment at home, with significant improvements in both non-motor (esp. cognitive) symptom burden and motor scores with active compared to sham stimulation (95).

There is rising interest in the use of transcranial direct current stimulation as a treatment modality in neurodegenerative disease. Although a Cochrane review did not support benefit for motor symptoms in Parkinson's disease (96), there may be mechanistic justification for efficacy in targeting cognitive impairment. Transcranial direct current stimulation is thought to modulate cortical excitability in regions such as the dorsolateral prefrontal cortex, influenced by but not necessarily dependent on cholinergic frontoparietal neural network dysfunction (97). A recent systematic review and meta-analysis suggested positive (though overall modest) effects of transcranial direct current stimulation on executive cognitive functions but highlighted the need for further research due to the lack of clear cause-effect relationship between the intervention and enhanced cognition (98).

Novel deep brain stimulation targets of the cholinergic system

Although deep brain stimulation of the subthalamic nucleus and globus pallidus internus is effective in improving cardinal motor signs in Parkinson's disease, effects on freezing of gait and postural instability are unsatisfactory (99). Results from several studies in animals and humans have supported the pivotal role of the pedunculopontine nucleus in modulating gait (100). The pedunculopontine nucleus includes cholinergic, glutamatergic, GABAergic and glycinergic neurons, and it is deeply connected with the basal ganglia and the spinal cord. Small double-blind, pilot clinical trials reported significant reduction of freezing and falls (101, 102). Due to differences in methodology and stereotactic coordinates localizing the pedunculopontine nucleus, the small number of subjects involved. and short follow-ups, results are inconsistent (100). Only one study with long-term (4 years) follow-up after unilateral pedunculopontine nucleus deep brain stimulation is available, reporting sustained benefit in freezing of gait in four out of six Parkinson's disease subjects (103). With the most recent technological advances, such as adaptive stimulation and MRI tractography, a well-designed clinical trial critically evaluating pedunculopontine nucleus deep brain stimulation for gait and balance issues in Parkinson's disease may be warranted.

Though spinal cord stimulation is a well-recognized treatment of neuropathic pain, this technique has been recently investigated to treat gait issues in Parkinson's disease but with variable and generally modest results (104, 105). The pedunculopontine nucleus sends cholinergic efferents to the medullary reticular formation that is connected directly to the spinal cord. Therefore, spinal cord stimulation might be beneficial on gait through indirect involvement of the pedunculopontine nucleus (106). However, effects may not

necessarily involve cholinergic mechanisms. Like pedunculopontine nucleus deep brain stimulation studies, there is lack of homogeneous and adequate patient populations, and different spinal cord stimulation levels and stimulation parameters were used in different studies. Harmonized patients samples and new stimulation techniques using burst stimulation or intermittent current delivery might help to improve future spinal cord stimulation studies in Parkinson's disease (107).

The visual system is a non-motor network affected in early-stage Parkinson's disease. A recent study has shown early vulnerability of the superior colliculus - which receives multimodal sensory inputs and is critical for orientation to relevant stimuli - in newly diagnosed patients with Parkinson's disease (108). Superior colliculus neurons send efferents to the thalamus and basal ganglia nuclei, receiving cholinergic afferents from the pedunculopontine nucleus and the parabigeminal nucleus (109). Abnormal superior colliculus function might be an early biomarker of Parkinson's disease, perhaps reflecting cholinergic systems dysfunctions. Finally, there is increased interest in studying deep brain stimulation of the nucleus basalis of Meynert to ameliorate cognitive deficits in Parkinson's disease (110). Data coming from studies in Lewy body dementia may show a possible signal (111), and there are ongoing clinical trials to investigate the efficacy of this more invasive approach to improve cognitive functions in patients with Parkinson's disease and dementia (see for recent systematic review (110)).

Conclusions and future directions

Our review of the recent literature points to distinct regional patterns of brain cholinergic deficits in Parkinson's disease. This is in contrast to the prevailing impression of diffuse cholinergic losses. These observations parallel the evolving conceptualization of cholinergic systems from diffuse neuromodulators to regionally specific deterministic functions (6). The topography of cholinergic vulnerability underlying cognitive decline in Parkinson's disease includes a rather symmetric opercular-insular and peri-central cortical, cingulum, thalamic complex (especially the lateral geniculate nucleus) and striatal regions. These regions include hubs of higher-order cognitive control networks such as the salience and cingulo-opercular networks (41). This pattern of cholinergic deficits related to cognitive impairments is common to deficits across cognitive domains, including memory, attention and executive functions (41). This shared topography of predominantly midline and peri-central cortical regions suggests that the cholinergic system is involved in rapid exchange of information within and between the hemispheres, allowing 'cross-talk' functions between various large-scale neural networks (112).

Postural instability and gait difficulties show spatially distinct cholinergic deficits. Key hubs of the topography associated with non-episodic axial motor impairments include the medial geniculate nucleus and the entorhinal cortex, regions involved in multisensory processing and spatial navigation, respectively (55). Distinct regional vulnerability patterns were seen between patients with falls (right lateral geniculate nucleus, right caudate nucleus) and freezing of gait (similar regions as fallers but with additional prominent bilateral striatal, limbic and prefrontal reductions) (51). These observations suggest a temporal profile of progressive cholinergic system changes from non-episodic balance and gait changes, to falls and then with most severe cholinergic losses present in patients with freezing of gait.

An interesting feature of the recent literature is preliminary evidence of bidirectional cholinergic system changes in Parkinson's disease, suggesting disease-related regulation of cholinergic neurotransmission and the presence of possible compensatory mechanisms. Regionally specific vulnerabilities and the presence of bidirectional cholinergic system changes may pose challenges for systemic cholinergic pharmacotherapy. Regions differ in the relative presence of specific nicotinic or muscarinic receptor subtypes. These challenges may be overcome by research showing preferential clinical effects of subtype specific nicotinic or muscarinic receptor agents.

Regional cholinergic deficits associated with specific clinical morbidities support a system-level disorder framework of Parkinson's disease and provide novel therapeutic targets to treat motor and cognitive symptoms in Parkinson's disease. A systems neuroscience strategy also emphasizes the importance of neural circuitry rather than pathology limited to single brain regions or single pathways to better explain the mechanisms underlying dementia and axial motor impairments in Parkinson's disease. The brainstem is an important confluence for multiple pathways and circuitries interconnecting the basal ganglia, thalamus, and the cerebellum. Therapeutic modalities activating some brainstem targets may also activate connecting circuitry, as shown for caloric vestibular stimulation (113).

Deep brain stimulation of the pedunculopontine nucleus, superior colliculus, and nucleus basalis of Meynert, and repurposing of vagus nerve, caloric vestibular, and transcranial direct current non-invasive neurostimulation approaches may allow targeting of hypocholinergic brain regions in Parkinson's disease. Research in this field so far has been limited by the small number of randomized controlled clinical trials reflecting many hurdles, such as difficulties randomizing and/or blinding of neurostimulation targets. However, recent research on cholinergic deficits suggests multiple directions for potential interventions to ameliorate treatment refractory features of Parkinson's disease. It will be important to demonstrate that proposed interventions are mediated by significant cholinergic mechanisms. This may be achieved by specifically targeting brain regions originating cholinergic projections, such as the nucleus basalis of Meynert or the pedunculopontine nucleus. For more widespread brain targets, the use of molecular cholinergic imaging studies before, after, or during targeted invasive or non-invasive neurostimulation interventions may allow the assessment of significant cholinergic mechanistic effects. Another approach is to use the cholinergic denervation topography in individual patients determined by cholinergic molecular imaging to inform montage frames for therapeutic non-invasive neurostimulation as currently performed for research purposes at our center (*e.g.*, ClinicalTrials.gov Identifier: NCT04817891). This individualized approach could be applied to other interventions. Cholinergic vulnerability of the metathalamus (lateral and medial geniculate nuclei) - which associates not only with cognitive but also with axial motor impairments - may be a target for novel sensory augmentation rehabilitation approaches, such as the use of virtual reality to enhance the perception of the visual and auditory environment. Such a strategy used during treadmill walking has already been successfully applied to reduce falls and improve postural stability in patients with Parkinson's disease (114). We conclude that recent research on cholinergic deficits have opened the door to multiple innovative and rational therapeutic strategies in Parkinson's disease.

Search strategy and selection criteria

References to be included for this review were identified by searching PubMed from January 1, 1980 to July 2021 using the search terms "Parkinson", "cholinergic", "acetylcholine", "cognitive", "motor", "gut", "autonomous", "sleep", "REM sleep", "prodromal", "vagus", "auricular", "vestibular", "stimulation", "imbalance", "falls", "freezing of gait", and "sensory integration". Additional references were extracted from the references of the selected articles. We restricted the search to articles published in English. The final reference list was generated on the basis of relevance to this Review, with priority given to publications within the last 5 years (total search of 724 papers for Parkinson's disease and cholinergic/acetylcholine with combinations of the other search terms), particularly cholinergic molecular imaging studies, reviews and randomized controlled trials.

Contributors

NB screened the results of the literature searches for relevant articles. All authors equally contributed to the writing of the manuscript. Figure 1 was created by RA and NB, figure 2 by PB and MAB and figure 3 by MM. All authors reviewed the manuscript and are in agreement with regard to the contents.

Declaration of interests

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***Figure** 1:* **Anatomic cholinergic cell groups a projections**. Cholinergic in focus (A) and brainstem s (B). Abbreviations: MDN, rsal motor nucleus of the vagus; hDB, horizontal imb nucleus of the diagonal band of Broca; MS, medial septal nuclei; MVN, medial vestibular nucleus; nBM, nucleus Bas Meynert; OB, Olfactory Bu LDT, pedunculopontine nu laterodorsal tegmental co substantia nigra; STN, sub nucleus; vDB, vertical limb .

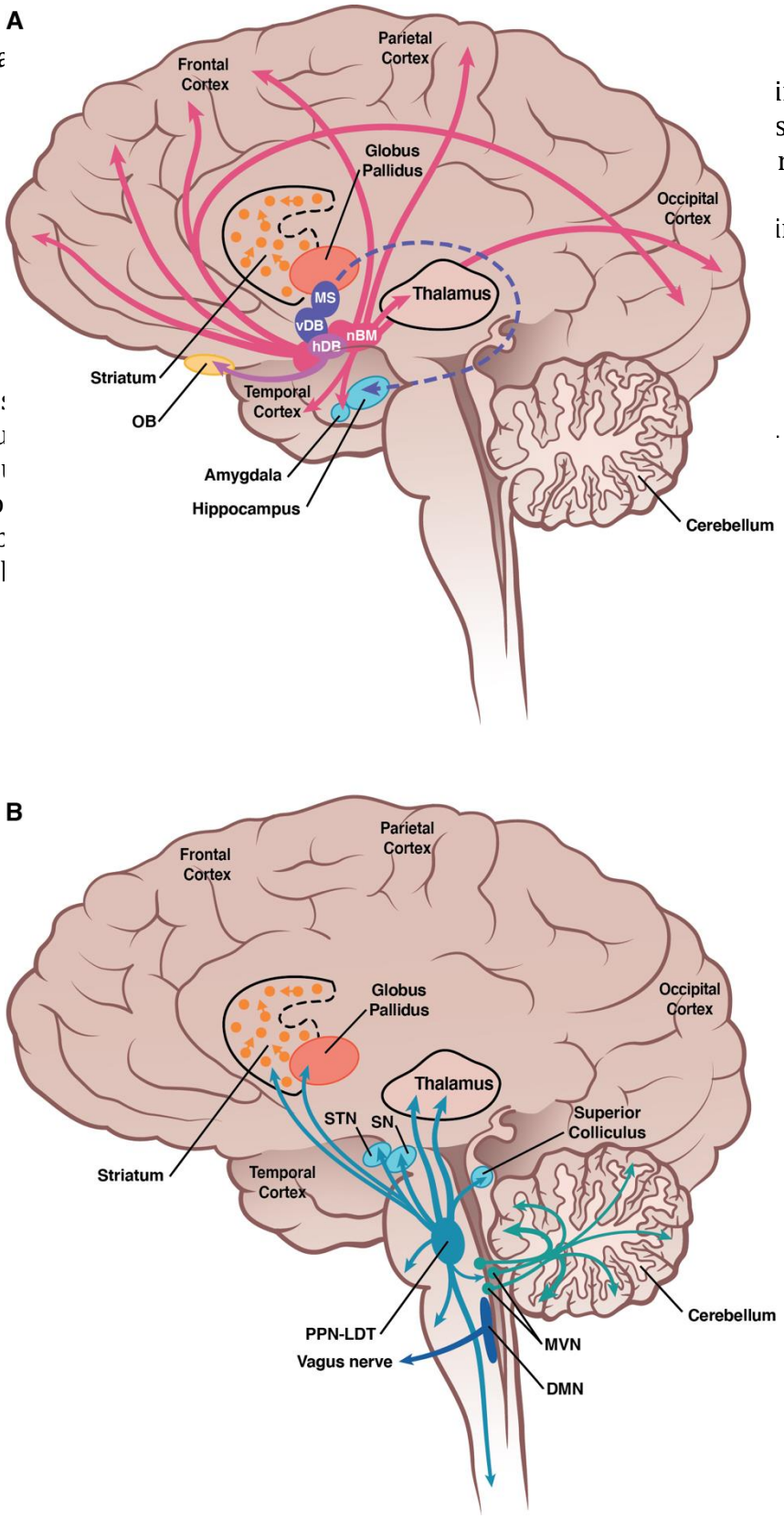

Figure 2: **Cholinergic PET imaging in patients with Parkinson's disease (PD) and isolated REM sleep behavior disorder (iRBD)**. (A) Decreased [^{11}C]-donepezil colon signals in isolated REM sleep behavior disorder (RBD) and Parkinson's disease with RBD (A); Increased [^{18}F]-FEOBV PET binding in isolated RBD patients (B).

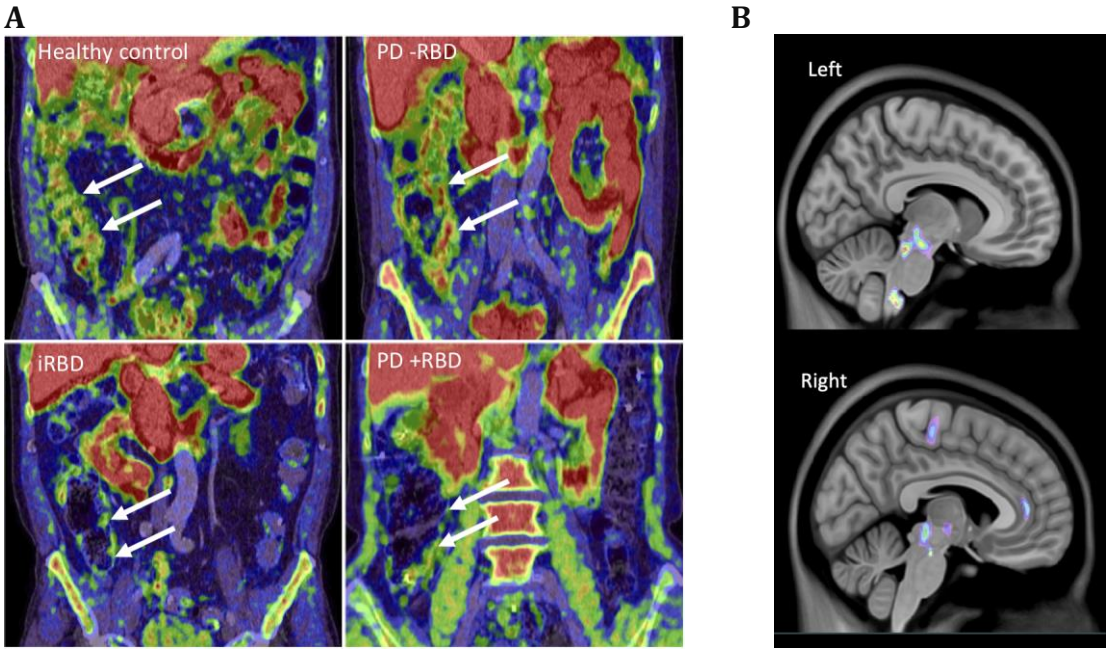

Figure 3: **Expression pattern of muscarinic and nicotinic acetylcholine receptors relevant to PD**. (A) Muscarinic and nicotinic acetylcholine receptors have a diverse expression profile throughout the striatum with several mAChRs and nAChRs expressed on pre-synaptic inputs from the cortex, thalamus, cholinergic interneurons, and dopaminergic terminals as well as post-synaptically on direct and indirect pathway spiny projection neurons. (B) Relative to the striatum, expression of mAChR and nAChR subtypes in the substantia nigra pars reticulata are less diverse. Abbreviations: GPe, globus pallidus pars externa; mAChR, muscarinic acetylcholine receptor; nAChR, nicotinic acetylcholine receptor; PPN, pedunculopontine nucleus; SPN, spiny projection neuron.