Neurobiology of deep brain stimulation

TITLE: Cholinergic mechanisms of deep brain stimulation in entopeduncular nucleus

Running title: DBS in EP

Authors: Feng Luo, Zelma HT Kiss

Affiliations: Department of Clinical Neurosciences and Hotchkiss Brain Institute
University of Calgary, Calgary AB CANADA

Corresponding author:
Zelma Kiss, MD PhD
Room 1AC58, HRIC, 3280 Hospital Dr. NW
Calgary, Alberta CANADA T2N4N1
Tel: 403-220-4839
FAX: 403-210-9550
E-mail: zkiss@ucalgary.ca

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ABSTRACT: Chronic, high frequency (>100 Hz) electrical stimulation, known as deep brain stimulation (DBS), of the internal segment of the globus pallidus (GPi) is a highly effective therapy for Parkinson disease (PD) and dystonia. Despite some understanding of how it works acutely in PD models, there remain questions about its mechanisms of action. Several hypotheses have been proposed, such as depolarization blockade, activation of inhibitory synapses, depletion of neurotransmitters, and/or disruption/alteration of network oscillations. Here we investigated the cellular mechanisms of simulated DBS (sDBS) in entopeduncular nucleus (EP, rat equivalent of GPi) neurons using whole-cell patch clamp recordings. We found that sDBS applied inside the EP nucleus induced a prolonged afterdepolarization that was dependent on stimulation frequency, pulse duration, and current amplitude. The high frequencies (>100 Hz) and pulse widths (>0.15 ms) used clinically for dystonia DBS could reliably induce these afterdepolarizations which persisted under blockade of ionotropic glutamate (kynurenic acid, 2 mM), GABAa (picrotoxin, 50 µM), GABAb (CGP 55845, 1 µM) and acetylcholine nicotinic (DHβE, 2 µM) receptors. However, this effect was blocked by atropine (2 µM, non-selective muscarinic antagonist) or TTX (0.5 µM). Finally, the muscarinic-dependent afterdepolarizations were sensitive to calcium-sensitive nonspecific cationic (CAN) channel blockade. Hence, these data suggest that muscarinic receptor activation during sDBS can lead to feedforward excitation through the opening of CAN channels. This study for the first time describes a cholinergic mechanism of sDBS in EP neurons and provides new insight into the underlying mechanisms of DBS.
Introduction

Chronic, high frequency (>100 Hz) electrical stimulation, known as deep brain stimulation (DBS), through surgically implanted electrodes in the thalamus and basal ganglia has revolutionized the treatment of movement disorders (Kringelbach et al., 2007). Thalamic DBS alleviates tremor (Benabid et al., 1996), while STN and GPi DBS relieve Parkinson's disease (PD) (Follett et al., 2010). DBS targeting the GPi is also effective for multiple types of dystonia (Kiss et al., 2007; Vidailhet et al., 2005). How DBS works to achieve clinical benefit remains under investigation.

There are several general hypotheses proposed to explain the mechanism(s) of action of DBS: depolarization blockade (Beurrier et al., 2001), synaptic inhibition (Dostrovsky et al., 2000), inhibition of the soma and activation of efferent axons (McIntyre and Grill, 2002), and stimulation-induced disruption of pathological network activity (Montgomery, Jr. and Baker, 2000). Our previous work suggested that DBS rapidly depleted glutamatergic synaptic afferents to thalamus where glutamate is the predominant neurotransmitter (Anderson et al., 2004; 2006). Clinically DBS suppresses tremor immediately within the same time scale as these changes in slice experiments (Anderson et al., 2006). DBS applied in the GPi, however, has a slower time scale of clinical benefit (Reese et al., 2011), suggesting that other mechanisms may also be involved.

Cholinergics are ubiquitous in the basal ganglia, play a neuromodulatory role, and have been largely neglected by those studying DBS mechanisms. GPi receives significant cholinergic
inputs from brainstem pedunculopontine tegmental nucleus (PPN) in rodents (Woolf and Butcher, 1986) and primates (Lavoie and Parent, 1994). To date, no previous study has reported or investigated cholinergic mechanisms of DBS.

In rodents, the entopeduncular nucleus (EP) is analogous to the GPi in primates and located within the fibers of the internal capsule (Nakanishi et al., 1990). It, along with the substantia nigra pars reticulata, is the major output nucleus of the basal ganglia, placing it in a key location where it could influence the entire basal ganglia thalamocortical circuit (McCracken and Kiss, 2014). Previous studies on EP DBS applied stimulation in the adjacent internal capsule, and suggested that DBS depressed neuronal activity in EP via elevation of extracellular K⁺ (Shin et al., 2007), mediated by enhancement of hyperpolarization-activated nonspecific cation channels (Shin and Carlen, 2008). Because DBS is applied clinically inside the GPi, not the internal capsule, and the time frame of clinical improvement is not immediate for dystonia (Reese et al., 2011), we postulated that DBS may involve other longer lasting mechanisms. Using whole cell patch clamp, we tested the hypothesis that DBS applied within EP will activate cholinergic fibers, and these will modulate neural responses to DBS in EP neurons. DBS induced prolonged afterdepolarizations (ADP) in EP neurons, and these were dependent on activation of muscarinic receptors.

Material and methods

Animals and brain slice preparation
49 Sprague-Dawley rats (Charles River, Canada) of either sex (P11-P20) were used for this study. All the experiments were performed according to protocols approved by the University of Calgary Animal Care Committee in accordance with the guidelines established by the Canadian Council on Animal Care. Under deep isoflurane anesthesia, rats were decapitated and the brains quickly removed and immersed in ice-cold carbogen-bubbled (95% O₂, 5% CO₂) slicing solution (in mM, 207 sucrose, 2.5 KCl, 26 NaHCO₃, 1.0 CaCl₂, 2.0 MgCl₂, 1.2 NaH₂PO₄, and 10 D-glucose; pH 7.4). 300-µm coronal slices were cut with a vibratome (VT1000S, Leica) and transferred to a holding chamber that contained carbogen-bubbled artificial cerebrospinal fluid (aCSF) solution (in mM, 126 NaCl, 2.5 KCl, 26 NaHCO₃, 2.5 CaCl₂, 1.5 MgCl₂, 1.25 NaH₂PO₄, and 10 D-glucose; pH 7.4). EP slices were incubated at 32-33 ºC for 60 min and then kept at room temperature.

Electrophysiology

For whole cell recording, a slice was placed in a recording chamber, and continually superfused with 32-33 ºC carbogen-bubbled aCSF solution at a rate of 2-3 mL/min. Amplified current or voltage signals obtained from a borosilicate glass electrode (1.5 mm outer diameter; tip resistance of 4-6 MΩ; King Precision Glass Inc., USA) filled with intracellular solution containing (in mM): 108 K-gluconate, 8 Na-gluconate, 8 KCl, 2 MgCl₂, 1 EGTA, 10 HEPES, 4 K-ATP, 0.35 Na-GTP, pH 7.2 (corrected with KOH), osmolarity 286. The EP was visually recognized within the internal capsule and individual neurons identified using an upright microscope (Olympus BX51W, Japan) fitted with differential interference contrast optics.
All recordings were obtained using a Digidata 1440A acquisition system connected to a 200B Axopatch amplifier (Molecular Devices, USA). The recordings were low-pass filtered at 1 kHz, digitized at 10 kHz on-line with Clampex 10.3 and analyzed off-line using Clampfit 10.3 (Molecular Devices). The data were accepted for off-line analysis only if changes in access resistance were <20%. No series resistance compensation was performed. The liquid junction potential was 14 mV and reported voltage data were not corrected.

Resting membrane potential and input resistance were measured immediately after establishing stable whole-cell configuration. Constant 30 pA current steps, 1 s in duration, were injected to obtain a current-voltage (I-V) curve to measure input resistance and the action potential (AP). AP threshold was calculated as the membrane voltage potential at which point the slope of the first action potential was > 10 V/s. The slope of the I-V plot represented the input resistance. For neurons displaying spontaneous spikes, the resting membrane potential was defined as the average membrane potential halfway between the most repolarized potential after an AP and the threshold potential (Shin and Carlen, 2008). For neurons without spontaneous spikes, the resting membrane potential was defined as the average membrane potential over 2 minutes after establishing stable whole-cell recording.

EP neurons were identified by the following electrophysiological properties (Nakanishi et al., 1990): anomalous rectification at hyperpolarizing potentials, rebound spikes at the offset of membrane hyperpolarization and high, and non-adapting repetitive firing at depolarizing potentials.
Electrical stimulation and "blanking" operation

A bipolar concentric electrode (NEX-100, Rhodes Medical Instruments, Woodland Hills, CA) was placed within EP. Stimulation was delivered with a constant-current stimulus isolator (A365, World Precision Instruments, USA) connected to stimulus generator (Model 2100, A-M System, USA). We refer to the electrical stimulation applied in slice as simulated DBS (sDBS).

The stimulating electrode in EP was positioned such that single pulses could reliably evoke postsynaptic currents (PSCs) under voltage clamp (clamped at -60 mV). To minimize the sDBS trains applied while determining stimulation thresholds, we started sDBS using a current that produced 30% of a maximum PSCs. Stimulation trains consisted of either 60, 150 or 400-µs pulse width, square-wave monophasic pulses. We tested sDBS frequencies of 10 Hz or 125 Hz and train durations of 10 s. For each pulse width, current was increased in 50 µA increments until an ADP response was successfully induced. If response to the previous sDBS train did not return to baseline, data were not included in analysis.

The high frequency stimulation used for sDBS produces an artifact that can obscure membrane responses. As described previously (Anderson et al., 2006), to eliminate the artifact during sDBS, "blanking" pulses (0.1-1.0 ms) were triggered with each stimulation pulse (A-M Systems 2100; A-M Systems). These voltage pulses initiated a blanking operation of the Axopatch 200B amplifier, which prevented the membrane voltage from updating during the blanking pulse, thus significantly reducing the stimulus artifacts (Fig. 1A).

Chemicals
Chemicals were diluted in aCSF from aliquots and applied by continuous bath-perfusion to the slices. Some recordings were performed during full blockade of ionotropic glutamatergic and GABAergic neurotransmission with a cocktail consisting of either kynurenic acid (2 mM) and picrotoxin (100 µM), or a mixture of 6, 7 - Dinitroquinoxaline - 2, 3 - dione (DNQX, 10 µM), DL - 2 - amino - 5 - phosphonopentanoic acid (AP5, 50 µM) and picrotoxin (100 µM), tetrodotoxin (TTX, 1 µM), atropine (4 µM), oxotremorine M (OXO, 10 µM, muscarinic receptor agonist) and dihydro-β-erythroidine hydrobromide (DHβE, 2 µM, competitive nicotinic acetylcholine receptor antagonist) were applied from stock solution made in double distilled water. Flufenamic acid (FA, 50 µM, non-specific inhibitor of calcium-activated chloride channels) and CGP 55845 (1 µM, selective GABAb receptor antagonist), were applied from stock solutions made in dimethyl sulfoxide (DMSO). The final concentration of DMSO in aCSF was ≤ 0.1%, which showed no effect on neuronal activities in control experiments. After drug application of TTX, atropine or FA, slices were washed with aCSF for 10-40 mins until recovery of ADP responses was obtained. Picrotoxin, DNQX, TTX and FA were purchased from Tocris, and all other chemicals were purchased from Sigma.

Data analysis

ADP amplitude was measured as voltage difference between resting membrane potential and the peak ADP membrane potential. ADP duration was measured as time difference between offset point of electrical stimulation and the time point when the membrane potential dropped to resting. To evaluate the effect of different agonists and blockers on sDBS, we compared the ADP
responses before and at least 5 min after application of the drugs.

All group data were expressed as mean ± standard error of the mean (SEM). Data were analyzed using Student's paired t-test comparing firing and depolarization induced by sDBS applied under aCSF vs. bath application of drugs.

Results

Properties of EP neurons

Electrophysiological properties were recorded from 102 EP neurons of which 73 had spontaneous spikes at resting membrane potential. The input resistance was $369.6 \pm 15.6 \text{ M\Ohm}$ and resting membrane potential was $-53.2 \pm 0.8 \text{ mV}$. Spontaneously active EP neurons had more depolarized resting membrane potentials ($-51.5 \pm 0.9 \text{ mV}$) than non-spontaneously active cells ($-59.2 \pm 1.8 \text{ mV}$, $p < 0.01$). When 1-s current steps were injected into the recorded neurons, the responses resembled those described by Nakanishi et al. (1990). Recorded neurons did not show spike adaption at depolarizing potentials, and the maximum spike rate induced by a 110 pA (1 s) current step was $30.0 \pm 1.8 \text{ Hz}$ (Fig.1B). At hyperpolarizing potentials, the neurons displayed anomalous rectification and a strong rebound activation at the offset of membrane hyperpolarization (Fig. 1C, insets). The rectification sag was measured as the membrane potential difference between the maximum potential at the rectification sag and the potential at the offset of the hyperpolarization. This sag increased with more hyperpolarized current injection (Fig. 1C) and resembled that described in previous studies (Nakanishi et al., 1990; Shin et al.,
After obtaining stable whole cell current clamp recording for at least 5 min, sDBS was
applied with neurons at resting membrane potential (n = 71 neurons, 55 with and 16 without
spontaneous activity). The stimulation electrode was placed 229.6 ± 9.9 µm from 54 recorded
cells (in which this distance was measured). A 10-s train of sDBS induced responses after the
offset of stimulation in all neurons: afterdepolarizations (ADP) that were either short lasting
(ADP-S) or prolonged (ADP-Pro). Neurons with ADP-S responses showed depolarization right
after the end of sDBS, which was accompanied by increased spiking (Fig. 2A). The membrane
potential of ADP-S neurons gradually returned to resting membrane potential after the ADP
reached a peak. Neurons with ADP-Pro responses showed an initial ADP-S at the offset of sDBS
and then gradually depolarized again (Fig. 2B). Spontaneous spikes increased during this
prolonged depolarization. Typically, the ADP-S responses lasted less than a minute while the
ADP-Pro response duration ranged from several minutes to as long as the neurons were recorded.
Neurons with (55/71) and without (16/71) spontaneous spikes at resting membrane potential both
produced ADP-S (spontaneous, 25/55; non-spontaneous, 6/16) and ADP-Pro (spontaneous,
30/55; non-spontaneous, 10/16) responses to sDBS. There were no differences in resting
membrane potential between ADP-S and ADP-Pro responding neurons of either spontaneous
(-51.4 ± 1.2 mV vs. -51.8 ± 1.3 mV) or non-spontaneous (-62.3 ± 3.8 mV vs. -57.9 ± 1.6 mV)
firing type.
As discussed in the following sections, the effects of drugs on both ADP-S and ADP-Pro responses were similar, indicating that both responses share the same cellular mechanisms. Here we focus on the neurons displaying ADP-S responses.

**ADP responses were related to frequency and pulse width of sDBS applied**

High frequency DBS is required to achieve clinical benefit. Benefit for dystonia usually requires longer pulse widths (Krauss et al., 2004; Cacciola et al., 2010). Thus, we investigated whether the ADP responses were related to stimulus parameters of frequency and pulse width. During and after a 10 s train of 10 Hz sDBS, the resting membrane potential and spontaneous spike frequency did not show significant changes (Fig. 2B, top figure, n = 4). High frequency (125 Hz) stimulation resulted in dramatic change in membrane potential, spike frequency, as well as an ADP-S response, i.e. depolarization at the offset of sDBS in which the spike frequency also increased (Fig. 2B, bottom figure). Spike frequency and membrane potential returned to baseline within 1 min.

Next, we tested the effects of different pulse widths on ADP-S responses. Three pulse widths were used to determine the minimal current required to induce an ADP-S (n = 8): short (60 µm), medium (150 µm) or long (400 µm). The amplitude required to elicit ADP-S using long pulse width (181.3 ± 29.8 µA, *p* < 0.01) was significantly lower than that required using short (759.4 ± 150.5 µA) and medium pulse widths (387.5 ± 77.6 µA) (Fig. 2C). The amplitude and duration of the ADP-S responses at long pulse width (10.8 ± 1.9 mV, *p* < 0.01; 14.3 ± 2.2 s, *p* < 0.05) were significantly higher and longer than those obtained using short (4.6 ± 0.8 mV, 9.2 ±
1.1 s) and medium pulse width (6.2 ± 1.4 mV, 11.2 ± 2.1 s) (Fig. 2D and E). A 10-s sDBS train
with long pulse width induced a consistent and reliable ADP, therefore to investigate the cellular
mechanisms of the ADP in further experiments, we used only high frequency stimulation (125
Hz) with long pulse width (400 µs).

**ADP responses are dependent on synaptic transmission**

In one group of experiments, we blocked ionotropic glutamate receptors (2 mM kynurenic
acid), GABAa (50 µM picrotoxin), GABAb (1 µM CGP 55845) and nicotinic acetylcholine
receptors (2 µM DHβE) and applied sDBS to our slice. Of 4 neurons tested, sDBS induced
ADP-S in 1 cell and 3 cells showed ADP-Pro responses (Fig. 3A).

To rule out the possibility that the ADP responses were induced via direct activation of
recorded EP neurons, we blocked all synaptic transmission with TTX (0.5 µM). Figure 3B shows
an example of the ADP-Pro response being abolished after 5-min application of TTX. Both the
amplitude and duration of ADP significantly decreased from 8.4 ± 3.3 mV to 1.7 ± 1.0 mV, and
7.1 ± 1.3 s to 2.3 ± 1.4 s, respectively (Fig. 3C and D, n = 4).

In another set of experiments we moved the stimulating electrode around the EP neuron and
delivered single pulses until a spike was initiated, indicating direct antidromic activation of the
recorded neuron. In this case sDBS failed to induce ADP responses from these neurons at a
stimulus intensity of 300 µA above the threshold shown in Figure 2C (n = 2, data not shown).

**ADP responses are dependent on activation of muscarinic receptor**
Because of the time course of ADP responses and the existence of a cholinergic projection from brainstem to EP (Clarke et al., 1997), we hypothesized that cholinergic synapses may be involved. ADP responses in EP neurons significantly decreased after 4 µM atropine application to aCSF (Fig 4A). The average ADP amplitude was reduced from 13.2 ± 2.5 mV in aCSF to 4.5 ± 1.8 mV (Fig. 4B), while the ADP duration was diminished from 32.4 ± 4.6 s in aCSF to 12.4 ± 4.5 s under muscarinic blockade (Fig. 4C) (n = 7, p < 0.01, paired t test). No ADP response was obtained in 3 of 7 cells (42.9%) and in the remaining 4 cells ADP response was almost eliminated after atropine blockade. In another group of experiments, cholinergic involvement in generating the ADP responses was tested by applying the muscarinic receptor agonist OXO and a 10-s current injection into the cell (n=8). In aCSF, a 10-s 100 pA current simulating synaptic responses of DBS did not change the membrane potential (Fig. 4D, top). However, when the muscarinic receptor was activated by OXO in the bath, the same stimulation induced dramatic depolarization of the membrane potential leading to ADP response (Fig. 4D, bottom). These data together suggest that sDBS induced ADP responses were highly dependent on the activation of muscarinic receptors.

ADP requires activation of calcium-sensitive nonspecific cationic (CAN) channels

Activation of CAN channels through muscarinic receptors is thought to underlie the ADP and persistent activity in entorhinal cortex (Egorov et al., 2002) and primary visual and motor cortex (Rahman and Berger, 2011). Hence, we applied 50 µM of the general CAN channel blocker flufenamic acid (FA) during sDBS. In one cell (Fig. 5A), the amplitude and duration of
the ADP response induced by sDBS disappeared entirely in aCSF plus FA. The average ADP amplitude was diminished from 13.9 ± 1.8 mV in aCSF, to 4.1 ± 1.1 mV (Fig. 5B), and ADP duration decreased from 15.0 ± 2.8 s in aCSF to 2.9 ± 0.8 s with FA (Fig. 5C, $p < 0.01$, paired t test). In 2 of 8 cells (25%) ADP was entirely eliminated after blockade of CAN channels. These data suggested that CAN channels are involved in sDBS-induced ADP responses.

Discussion

DBS effects depend on the physiological properties of the target neurons, the stimulus parameters (frequency, pulse width, duration of DBS applied) and the anatomic connectivity of the target nucleus (Kringelbach et al., 2007). Not only can DBS inhibit or stimulate neural activity in the target nucleus (Vitek, 2002), but also alter downstream connected nuclei and cortical regions (McCracken and Kiss, 2014). Most previous studies have focused on how DBS alters GABA or glutamate transmission (Chiken and Nambu, 2013; Lee et al., 2004) or membrane properties (Beurrier et al., 2001; Shin et al., 2007). Despite most of the basal ganglia being innervated by cholinergic fibers from the PPN, the effects of DBS on such fibers have not been reported. Here we found that sDBS exerted post-stimulation effects, an ADP dependent on frequency and current density applied, and mediated by muscarinic receptors.

Post-sDBS responses were either short (ADP-S) or prolonged (ADP-Pro), based on the duration of the ADP. Both ADP-S and ADP-Pro responses were equally prevented by TTX, atropine and FA, indicating that they shared the same mechanisms. In fact, ADP-S may be a
subthreshold form of ADP-Pro, because ADPs are usually considered a subthreshold form of
long lasting depolarizations (Egorov et al., 2002). TTX, atropine and FA virtually eliminated the
ADP, supporting the involvement of cholinergic synaptic transmission and CAN channels. The
excitatory effects of acetylcholine are probably mediated via either blockade of a
G-protein-coupled inwardly rectifying potassium channel (Krnjevic, 2004), or activation of the
phospholipase C pathway which activates CAN channels (Schaefer et al., 2000). CAN channels
are permeable to Na⁺ and Ca²⁺, and the increased intracellular Ca²⁺ could in turn amplify the
CAN channel responses and lead to long lasting depolarization (Rahman and Berger, 2011). The
intracellular Ca²⁺ is linked to a Ca²⁺ activated K⁺ conductance, which may return the membrane
potential from depolarized to resting. We recorded all cells at resting membrane potential to test
the effects of sDBS in physiological conditions. We suspect that the ADP-S response occurred
from the Ca²⁺ activated outward K⁺ conductance overcoming the CAN channel activated inward
conductance in some cells; whereas in others, it did not and resulted in the ADP-Pro response.

Our previous work in thalamus indicated that the primary target of DBS is the axon and its
synaptic terminals with high frequency stimulation activating and depleting glutamatergic
terminals of neurotransmitter (Anderson et al., 2006;Iremonger et al., 2006). In EP, sDBS may
also activate the robust cholinergic afferents from PPN (Woolf and Butcher, 1986). This is
comparable to previous studies in which activation of muscarinic receptors with carbachol paired
with extracellular stimulation could induce ADP and persistent activities (Egorov et al., 2002).

There have been very few studies investigating the effects of DBS on EP neurons. Shin et al.
(2007) reported that sDBS-induced an elevation of extracellular potassium resulting in reduction in EP neural activity. This occurred through the enhancement of hyperpolarization-activated cyclic nucleotide-gated (HCN) channels (Shin and Carlen, 2008). Our study differed in two respects. First, Shin et al. (2007) placed their stimulating electrode in the internal capsule while we placed ours in the EP cellular mass. High frequency stimulation of the internal capsule is expected to activate a large number of axons and result in a massive efflux of intracellular K⁺ at the same time. The increased K⁺ conductance associated with abundant neuronal activity and restricted extracellular space would lead to accumulation of extracellular K⁺, which amplifies the accumulation of extracellular K⁺ (Nicholson, 1980). Also, Shin et al. applied 150 Hz, 100-300 µA and 60-µs pulse width DBS to their EP slices. We applied a variety of pulse widths, frequencies and amplitudes. Both long and short pulses could induce the ADP responses, but longer pulses (400 µs) had the lowest thresholds. While our results are similar to those reported by Shin et al. (2007) with respect to depolarization post-sDBS, the differences may be due to the distance and orientation of the EP neurons to the current applied, including voltage gradient, stimulation parameters and the properties of synaptic transmission on the target neurons (Kringelbach et al., 2007).

The EP receives major inputs from striatum, GP and STN in the basal ganglia and sends major output to the thalamus (Ogura and Kita, 2002). The striatal axons projecting to EP contain GABA, dynorphin and substance P (Penny et al., 1986; Reiner et al., 1999) and the STN neurons projecting to EP contain glutamate (Nakanishi et al., 1991). Dynorphin may control the activity
of EP neurons by regulating striatal terminal GABA release and also by hyperpolarizing the postsynaptic membrane (Ogura and Kita, 2002). It is possible that neurotransmitters other than acetylcholine also play a role in generating ADP responses during EP sDBS, but our data suggests that the majority of this effect is cholinergic mediated.

Clinical implications of sDBS induced ADP in EP

DBS is applied to GPi for PD and dystonia (Kringelbach et al., 2007). In contrast to the immediate effectiveness of DBS for PD, the clinical benefits for dystonia are gradual (Krauss et al., 2004). This suggests that different mechanisms of action may be at play with DBS for PD and dystonia.

The benefits of DBS for PD are thought to involve an altering of abnormal basal ganglia oscillations in the beta frequency band (Kuhn et al., 2008). Recent in vivo work suggests that EP DBS induces both synchronizing and desynchronizing effects by modulating local as well as distant neural activities and altering oscillations (McCracken and Kiss, 2014). Similarly in EP slice, sDBS modulated responses of local EP neurons via activation of cholinergic fibers. Disruption of neural responses in EP by induction of ADP may provide longer effects on pathological network activities.

Dopamine (DA) reduction in PD is accompanied by a rise in cholinergic signaling in striatum (DeBoer et al., 1996). This is thought to be involved in PD symptoms and has motivated the use of anticholinergic drugs acting on muscarinic acetylcholine receptors (mAChR) in treatment (Pisani et al., 2007). While we did not examine a DA-depleted PD model, we found
that sDBS induced cholinergic ADP responses via the activation of muscarinic receptors.

Because of its dependence on high frequency and longer pulse width stimulation, cholinergic activation may actually contribute to the therapeutic effects of DBS in GPi.

How dystonia is improved gradually by GPi DBS may be related to a gradual change in abnormal sensorimotor cortical maps (Chen and Hallett, 1998; Ruge et al., 2011). Our data suggest that GPi DBS could activate cholinergic terminals in GPi, which depolarizes GPi GABAergic neurons and enhances inhibition on thalamus (Anderson et al., 2003).

Simultaneously, DBS may drive cholinergics antidromically to activate the PPN. Neurons in PPN densely innervate thalamus, motor areas of brainstem and spinal cord (Garcia-Rill, 1991). Activation of PPN paired with a stimulus to the sensory system induces auditory plasticity in both thalamus (Luo et al., 2011) and cortex (Luo and Yan, 2013). Therefore, GPi DBS may drive both the GPi GABAergic and PPN cholinergic inputs to thalamus at the same time. Due to the modulatory nature of cholinergic action, it may need weeks or months to induce thalamic and eventually cortical plasticity through the GPi action on the motor system.

Because anticholinergic drugs are used to treat PD and dystonia, a possible beneficial effect of cholinergic activation appears paradoxical. However, the receptor subtypes are likely responsible for different effects in different nuclei. The anticholinergics approved for treating PD clinically are non-selective and therefore limited in utility by central and peripheral adverse effects (Langmead et al., 2008). There are 5 subtypes of muscarinic cholinergic receptors (mACHRs) widely distributed in the central nervous system and divided into 2 groups based on...
These receptors have been best studied in the striatum (Goldberg et al., 2012): presynaptic mAChRs (M2 group) are largely inhibitory and act as inhibitory autoreceptors with M4 receptors predominating. Postsynaptic mAChRs can be either inhibitory (M2 group) or excitatory (M1 group) (Wess et al., 2003). Other than in striatum and substantia nigra (Weiner et al., 1990), cholinergic receptor subtypes have not been characterized in the GPi/EP or other basal ganglia nuclei. A better understanding of the role of cholinergic modulation in the basal ganglia circuit may allow development of more specific treatment strategies for movement disorders.

Finally, DBS is also being experimentally applied for Alzheimer dementia (Laxton et al., 2010) and memory enhancement (Suthana and Fried, 2014). Some have suggested that the mechanism for such memory improvement is neurogenesis in hippocampus (Stone et al., 2011). Our data on cholinergic mechanisms may provide alternative or additional possibilities.

In conclusion, our results demonstrate that muscarinic receptor activation by sDBS can generate post-stimulation effects, which are mediated by feedforward excitation through the opening of CAN channels. Therefore cholinergic afferents play a significant role in the effects of sDBS on EP neurons and provides new insight into possible mechanisms of clinical DBS in humans.
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Figure 1. Schematic representation of whole-cell patch-clamp recording setup in entopeduncular nucleus (EP) slice. Recordings were made from EP neurons and sDBS was delivered within the EP (A). EP neurons did not show spike adaption when currents up to 110 pA were injected into the cell (B). Typically, these neurons also showed rectification at hyperpolarizing potentials and rebound spikes at the offset of membrane hyperpolarization. The rectification sag (voltage differences between the two arrows) was plotted as a function of the membrane potential (C).

Figure 2. ADP induced by sDBS in EP neurons at resting membrane potential. According to ADP duration, the responses were classified as short (ADP-S) or prolonged (ADP-Pro) (A). High frequency (125 Hz) but not low frequency (10 Hz) stimulation evoked ADP in the same neuron (B). The pulse width, duration and intensity of the sDBS were 400 µs, 10 s and 300 µA. Longer pulse widths were more effective than shorter pulse widths in evoking ADPs. The threshold amplitude required to evoke an ADP was significantly lower (C) while the corresponding amplitude (D) and duration (E) of evoked ADP was higher at 400 µs pulse width than that obtained with 60 µs and 150 µs pulse width. Grey spheres indicate individual neuronal responses and bar graphs the means.

Figure 3. sDBS induced ADP depends on synaptic transmission. Blockade of glutamatergic, GABAergic and cholinergic nicotinic transmission did not eliminate ADP (A). TTX abolished the sDBS induced ADP (B). Individual cell responses (in grey) and group data (bar graphs) show
that both the amplitude (C) and duration (D) of the ADP response were significantly decreased in
0.5 µM TTX.

**Figure 4.** sDBS induced ADP was dependent on activation of muscarinic acetylcholine receptors. Blockade of muscarinic receptors decreased the sDBS induced ADP response (A). Group data show that both the amplitude (B) and duration (C) of the ADP were significantly reduced after bath application of 4 µM atropine. The muscarinic agonist, oxotremorine, together with a 10-s 100 pA current injection into the recorded cell (line below voltage trace) replicated the ADP responses seen with sDBS (D).

**Figure 5.** sDBS induced ADP was dependent on activation of calcium-sensitive nonspecific cationic (CAN) channels. Blockade of CAN channels with 50 µM flunamic acid (FA) prevented ADP (A). Group data show that both the amplitude (B) and duration (C) of the ADP were significantly reduced after bath application FA.
**, p<0.01, *, p < 0.05, RM-ANOVA test
A

ADP

2 mM Kynurenic acid
50 μM Picrotoxin
2 μM DHβE
1 μM CGP55845

10 s

B

ACSF

0.5 μM TTX

20 mV

10 s

C

D

***, p<0.01, *, p < 0.05, paired t test; same as following figures