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Deranged NMDAergic cortico-subthalamic transmission underlies parkinsonian motor deficits

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Parkinson's disease (PD) is the most prevalent hypokinetic movement disorder, and symptomatic PD pathogenesis has been ascribed to imbalances between the direct and indirect pathways in the basal ganglia circuitry. Here, we applied glutamate receptor blockers to the subthalamic nucleus (STN) of parkinsonian rats and evaluated locomotor behaviors via single-unit and local-field recordings. Using this model, we found that inhibition of NMDAergic cortico-subthalamic transmission ameliorates parkinsonian motor deficits without eliciting any vivid turning behavior and abolishes electrophysiological abnormalities, including excessive subthalamic bursts, cortico-subthalamic synchronization, and *in situ* beta synchronization in both the motor cortex and STN. Premotor cortex stimulation revealed that cortico-subthalamic transmission is deranged in PD and directly responsible for the excessive stimulation-dependent bursts and time-locked spikes in the STN, explaining the genesis of PD-associated pathological bursts and synchronization, respectively. Moreover, application of a dopaminergic agent via a microinfusion cannula localized the therapeutic effect to the STN, without correcting striatal dopamine deficiency. Finally, optogenetic overactivation and synchronization of cortico-subthalamic transmission alone sufficiently and instantaneously induced parkinsonian-associated locomotor dysfunction in normal mice. In addition to the classic theory emphasizing the direct-indirect pathways, our data suggest that deranged cortico-subthalamic transmission via the NMDA receptor also plays a central role in the pathophysiology of parkinsonian motor deficits.

Introduction

According to the classic view, the basal ganglia circuitry is composed of 2 major pathways (1, 2). The excitatory direct pathway and the inhibitory indirect pathway are counterbalanced to make a principle control mechanism of motor behavior. Optogenetic activation of the striatal median spiny projection neurons (MSNs) containing dopamine receptor 1 (D1R) from the direct pathway facilitates motor behavior in normal mice, while activation of MSNs containing dopamine receptor 2 (D2R) from the indirect pathway increases freezing and motor inhibition (3). The classic view has evolved into the widely accepted concept that the symptomatic pathogenesis of Parkinson's disease (PD) is ascribable to a hypoactive direct pathway and a hyperactive indirect pathway due to the loss of dopaminergic neurons in the substantia nigra (1, 2). However, it is noted that striatal dopaminergic stimulation with D1R or D2R agonists, which presumably perturbs the balance between the direct and indirect pathways, fails to change MSN firing in mice (3) or locomotor behavior in rats (4). In this regard, it is interesting to note that the therapeutic effects of levodopa and dopamine agonists in PD are mainly investigated by systemic application (2, 5–7) rather than striatal infusion. Acute administration of dopamine antagonists rarely causes acute parkinsonism in humans (8) and fails to cause parkinsonian electrophysiological abnormalities in animals (9, 10). While striatal changes related to the dopaminergic denervation are undoubtedly prominent in PD (11–15), it remains

unclear whether this pathology alone is sufficient to cause the parkinsonian hypokinetic movements. In addition to the direct and indirect pathways in the cortico-basal ganglia circuitry, there is a relatively neglected cortico-subthalamic (or “hyperdirect”) pathway that bypasses the striatum and sends cortical commands directly to the subthalamic nucleus (STN) (16, 17). Moreover, a growing body of data indicates a central role of the STN in the genesis of parkinsonian symptoms. Excessive subthalamic bursts (9, 18–20) and abnormal cortico-subthalamic beta synchronization (10, 21–24) are 2 specific landmarks directly linked to PD in both patients (10, 19, 23, 24) and rodent models (9, 18, 20–22). An increase in subthalamic burst discharges has been shown to play a causal role in hypokinetic symptoms (25, 26). Injection of T-type calcium channel antagonists or depolarizing currents into the STN readily decreases subthalamic burst discharges and remedies parkinsonian locomotor deficits (25, 26). Hyperpolarization of subthalamic neurons, on the other hand, increases burst discharges and renders a normal animal hypokinetic (26). Cortico-subthalamic beta synchronization, another electrophysiological landmark in PD, is also correlated with hypokinetic movements and can be suppressed by dopaminergic agents (21, 23). With the establishment of the STN's key role in the symptomatic pathogenesis of PD, it is desirable to investigate the effect of cortico-subthalamic transmission on electrophysiological and behavioral deficits in PD as well as on normal motor processing.

Results

Subthalamic NMDA receptor blocker remedies hypokinetic movements. In light of the glutamatergic nature of the cortico-subthalamic

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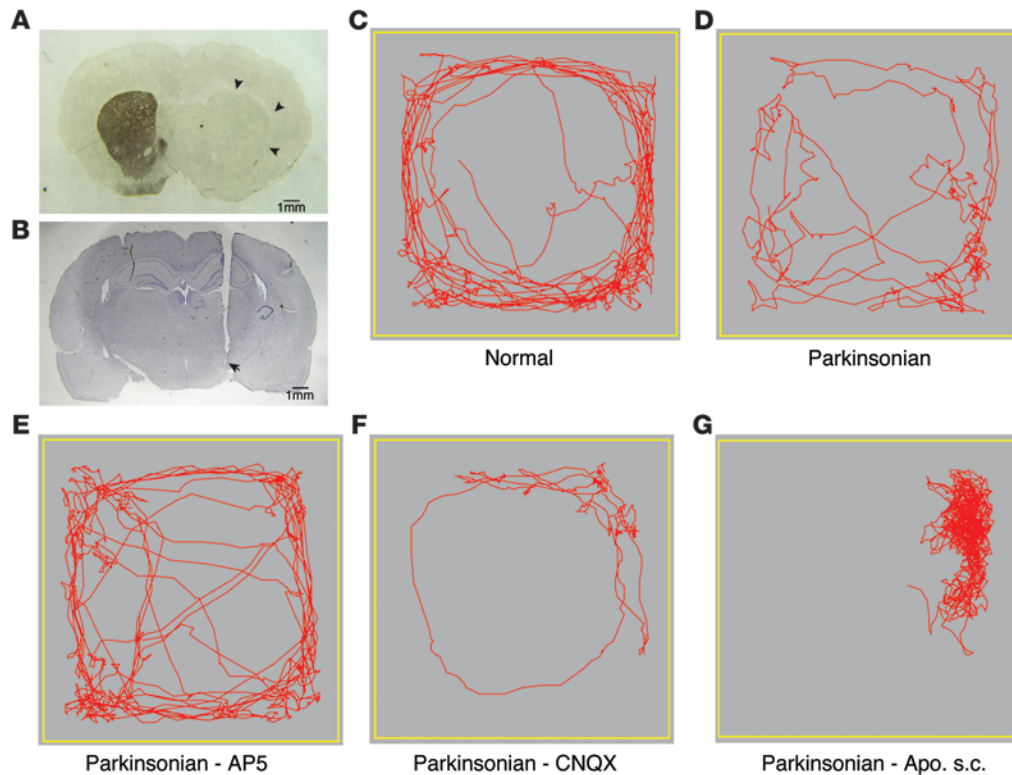


Figure 1. The effect of glutamate receptor antagonists and apomorphine on locomotor activities in hemiparkinsonian rats. (A) Immunohistochemical staining of tyrosine hydroxylase in parkinsonian rats with unilateral 6-OHDA lesions. Note the unilateral loss of dopaminergic innervation in the right striatum (arrowheads). (B) Location of the inserted cannula (arrow) in the STN ipsilateral to the 6-OHDA lesion site. See also Supplemental Figure 1 for the entire collection. (C) Sample 5-minute trace of free movements of a normal rat in a square arena. (D–G) Sample traces of movements in parkinsonian rats with microinfusion of vehicle aCSF (D), AP5 (E), and CNQX (F) into the STN and systemic application of apomorphine (G) by s.c. injection (Apo. s.c.). Motor deficits in panel (D) were corrected by AP5, while CNQX had no discernible effect. Note that the trace of systemic apomorphine was restricted to the corner due to unilateral rotations of the rat in a small area. Scale bars: 1 mm.

pathway, we first locally delivered the specific NMDA receptor antagonists D-(–)-2-amino-5-phosphonopentanoic acid (AP5) and *cis*-4-[phosphomethyl]-piperidine-2-carboxylic acid (CGS) and the α -amino-3-hydroxy-5-methylisoxalone-4-propionic acid (AMPA) receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione sodium (CNQX) to the STN. Subthalamic microinfusion of AP5 and CGS, but not CNQX, readily remedied locomotor deficits and asymmetrical movements in hemiparkinsonian rats (Figures 1 and 2). While NMDA (27, 28) and AMPA receptors (29, 30) are both involved in cortico-subthalamic transmissions, our study demonstrates that only NMDA receptors are essential for the genesis of motor deficits in PD. Subcutaneous application of apomorphine, a nonselective dopamine receptor agonist typically used for PD treatment, could also reverse most hypokinetic movements. However, there were evident paradoxical turning behaviors as well as head tilting and climbing abnormalities when the rats were exposed to systemic apomorphine but not subthalamic NMDA receptor antagonists (Figure 2, D–F). Like the striatum, the STN is full of dopamine receptors (31) and is innervated by the dopaminergic neurons of the substantia nigra (32–34). Interestingly, local injection of apomorphine into the STN, which did not change the dopamine balance in the striatum, also effectively ameliorated the hypokinetic parkinsonian symptoms without vivid turning or other induced behaviors (Figure 2). These findings lend strong support for the pivotal and

specific role of the STN in the symptomatic pathogenesis of hypokinetic movements in PD and in the therapeutics to treat this disease. We also examined the effect of NMDA receptor antagonists in normal animals. Although much better than parkinsonian rats, the locomotor activities were significantly compromised (Supplemental Figure 2; supplemental material available online with this article; doi:10.1172/JCI175587DS1), implicating an imperative role of the cortico-subthalamic pathway in normal motor physiology as well.

NMDA receptor blocker suppresses subthalamic burst firing. Consistent with the behavioral profiles, single-unit recordings in the STN revealed that NMDA receptor antagonists selectively suppressed burst firing but left spike-firing units unaffected (Figure 3 and Supplemental Figure 3). In contrast, the AMPA receptor antagonist CNQX did not show any discernible effect (Supplemental Figure 4). The burst-suppressing effect of NMDA receptor antagonists was present in both normal and parkinsonian rats, suggesting the essential role of cortical input in the generation of burst discharges in both conditions. Of note, the much higher burst counts in parkinsonian rats were markedly reduced by NMDA receptor antagonists to a level approximating the counts in the normal rats. The intraburst paradigms (Figure 3E and Supplemental Figure 3C), on the other hand, were mostly unchanged by AP5 or CGS. These findings indicate that the NMDA receptor mainly serves as a burst initiator, but not a burst maintainer.

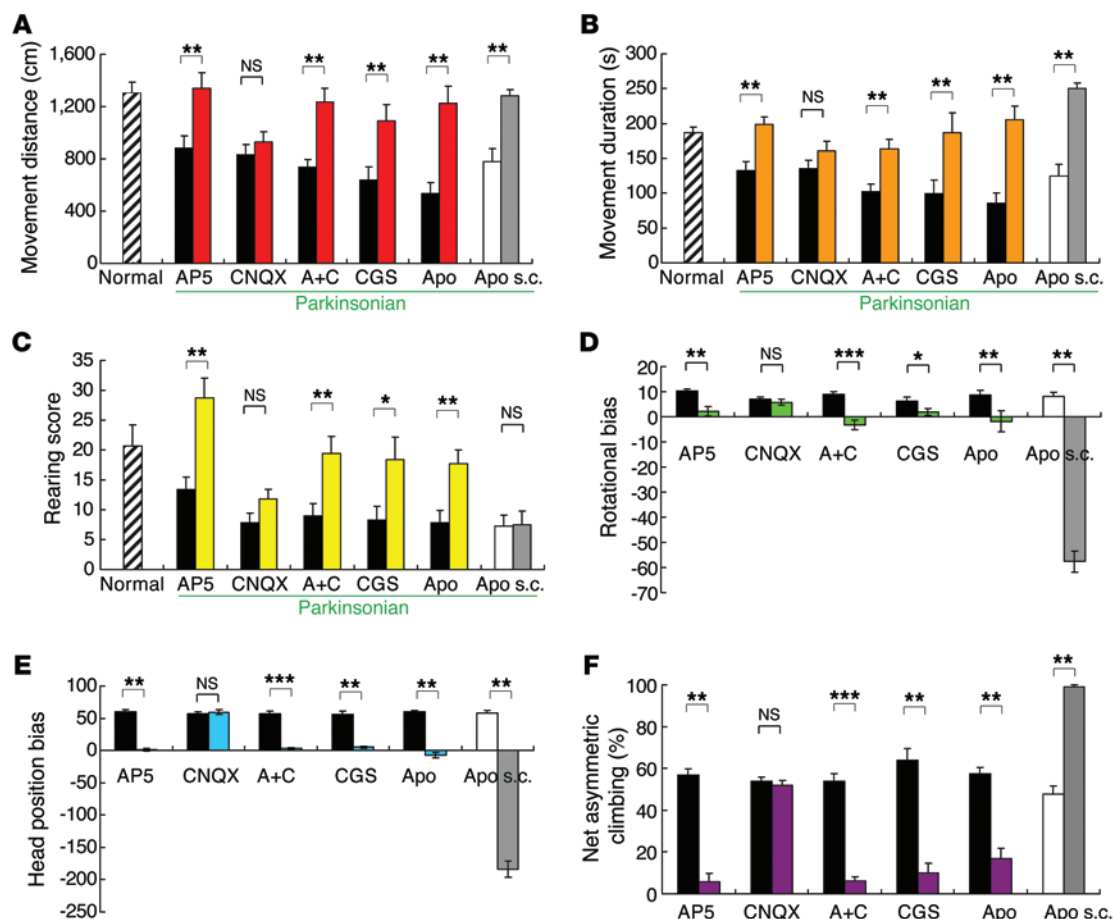


Figure 2. Quantitative parameters of locomotor activities and asymmetrical movements in hemiparkinsonian rats. NMDA receptor blockers AP5 ($n = 13$ different rats) and CGS ($n = 9$), AMPA receptor blocker CNQX ($n = 21$), a mixture of AP5 and CNQX (A + C, $n = 18$), and nonselective dopamine receptor agonist apomorphine (Apo, $n = 13$) were locally delivered to the STN. Each drug (colored bars) had a paired control with aCSF microinfusion (black bars). Movement distance (A), movement duration (B), and rearing score (C) demonstrate movement ability. Performance of the normal rats ($n = 12$) is also shown for reference. Rotational bias (D), head position bias (E), and net asymmetrical climbing (F) indicate asymmetrical movements due to unilateral 6-OHDA lesions. The same parameters were also documented for systemic s.c. injection of apomorphine (Apo s.c., $n = 11$, gray bars) and a paired control with s.c. injection of saline (white bars). Note that NMDA, but not AMPA, receptor antagonists improved all locomotor parameters and ameliorated asymmetrical motor deficits. Subthalamic apomorphine had an effect similar to that of NMDA receptor blockers. However, systemic apomorphine induced strong paradoxical movements, which included unilateral rotation (D), paradoxical head deviation (E), and worsening of asymmetrical climbing (F). Statistical analyses were performed using a nonparametric Wilcoxon signed-rank test. Data represent the mean \pm SEM. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

NMDA receptor blocker suppresses cortico-subthalamic synchronization. To explore whether glutamatergic transmission is also involved in the genesis of cortico-subthalamic beta synchronization, another electrophysiological phenomenon specific to PD, we recorded premotor cortical and STN local field potentials (LFPs) simultaneously in parkinsonian rats, while slowly infusing NMDA or AMPA receptor blockers into the STN. The interactions of electrical activities between the 2 cerebral regions can be illustrated by frequency-dependent synchronization. We found cortico-subthalamic synchronization of beta frequencies (20–40 Hz) in parkinsonian, but not in normal, rats, and NMDA receptor blockers readily abolished this abnormality in a real-time fashion (Figure 4). In contrast, AMPA receptor blockers had no noticeable effect (Figure 4, D and G).

NMDA receptor blocker suppresses synchronization in situ. While cortico-subthalamic beta synchronization represents long-range coupling of electrical activities between different cortical

regions, it is also important to evaluate this synchronization in situ, which involves synchronization of electrical activity between nearby neurons. In normal rats, we observed no intrasubthalamic synchronization in the low-frequency range (Figure 5, A–C). In contrast, abnormal synchronization in situ was evident in beta frequencies in parkinsonian rats. Intrasubthalamic infusion of NMDA receptor blockers not only dissociated the long-range (cortico-subthalamic) coupling, but also abolished in situ synchronization in the STN (Figure 5, A–C and E). Intrasubthalamic synchronization thus depends on cortical inputs via NMDA receptors. It is worth noting that abnormal in situ synchronization was also evident in the premotor cortex of parkinsonian rats, and direct injection of NMDA receptor blockers into the STN could also remedy the abnormal cortical synchronization (Figure 5, A, B, D, and F). The results indicate that in situ synchronization in either the cortex or the STN is not a local event, but requires long-range interactions in the cortico-basal ganglia circuitry. AMPA

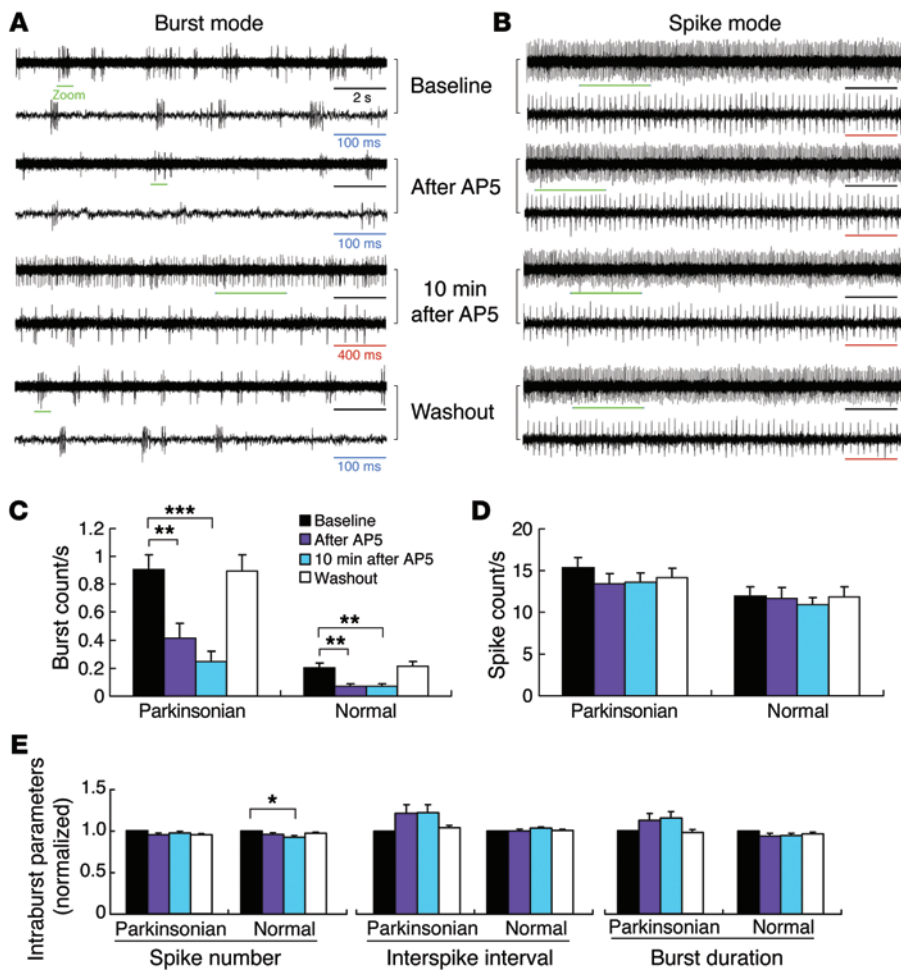


Figure 3. The effect of AP5 on subthalamic discharges. AP5 was slowly infused into the STN while subthalamic single units were recorded. **(A)** Typical sweeps from a burst-firing unit. Raw sweeps are presented with a 2-second scale (black scale bar) and are enlarged (blue scale bar, 100 ms; red bar, 400 ms). Green line indicates where the raw sweeps are enlarged. Note that the bursts (firing clusters) are highly clustered (clustering of the clusters) at baseline but markedly reduced after AP5 microinfusion (After AP5). The firing pattern then shifted to spike mode 10 minutes later (10 min after AP5) and returned to baseline after AP5 washout. **(B)** Typical sweeps from a spike-firing unit under AP5 microinfusion. AP5 apparently did not change subthalamic firing in spike mode. **(C and D)** Quantitative measurement of burst-firing (in burst mode) and spike-firing (in spike mode) frequencies. The burst count was significantly reduced by AP5 in both parkinsonian and normal rats (parkinsonian group, $n = 15$ units in 6 rats; normal group, $n = 12$ units in 5 rats). In contrast, spike count remained unchanged (parkinsonian group, $n = 16$ in 5 rats; normal group, $n = 14$ in 5 rats). **(E)** Intraburst parameters (of the units in panel C) remained mostly unchanged, except for the slightly reduced spike numbers in normal rats ($P = 0.013$). Statistical analyses were performed by 1-way ANOVA with Dunnett's post-hoc correction. Data represent the mean \pm SEM. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

receptor blockers, which failed to modulate cortical-subthalamic beta synchronization, also failed to suppress in situ synchronization in the premotor cortex or STN (Supplemental Figure 5).

Cortico-subthalamic transmission modulates subthalamic firing. We have shown that subthalamic bursts and beta synchronization are controlled by an NMDA receptor-dependent mechanism. It would be desirable to explore whether the mechanism is pathway dependent. We therefore evoked single-unit firing in the STN by electrical stimulation of the premotor cortex (Figure 6A). The population of neurons showing evoked bursts (Figure 6B) and the burst counts in each neuron were both significantly increased in parkinsonian animals (Figure 6C), once again supporting the view that the cortico-subthalamic pathway is functionally deranged in PD. The stimulation-dependent bursts were suppressed by NMDA receptor blockers, indicating that the NMDA receptor is directly responsible for cortico-subthalamic transmission (Figure 6D). In addition to the firing patterns, we also characterized the distribution of evoked subthalamic firing (Figure 7). Normal rats showed a typical 2-peak distribution in the spike histogram (Figure 7C). The first and second peaks, which are located at 15 to 25 ms and 55 to 65 ms, are related to cortico-subthalamic input (29, 30, 35) and separated by an interpeak suppression period ascribable to the indirect pathway (30, 36). In contrast, parkinsonian rats showed only a single, strictly time-locked peak at approximately 9 ms

(Figure 7D), a latency very close to the lower margin of subthalamic firing triggered by cortico-subthalamic transmission based on intracellular and single-unit recordings in normal rats (29, 30, 35) and much faster than the modulation traveling via the indirect pathway (30, 36–38). The time-dependent spike distribution was also abolished by local infusion of AP5 into the STN (Figure 7, C and D), lending strong support to the notion that the cortico-subthalamic pathway profoundly alters STN firing via the NMDA receptor. In addition to its burst-generating propensity, cortico-subthalamic transmission is also deranged in parkinsonian rats in the form of more effective and rapid driving of erroneously timed subthalamic discharges. Moreover, the strictly time-locked firing between the premotor cortex and the STN via NMDA receptors may constitute a mathematical basis for cortico-subthalamic beta synchronization (see Discussion).

Cortico-subthalamic overactivation triggers parkinsonian hypokinetic movements. While the existence of cortico-subthalamic transmission is required for parkinsonian hypokinetic movements and the corresponding electrophysiological abnormalities, it is important to evaluate whether the derangement of this transmission alone is sufficient to generate hypokinetic movements in the absence of deficits in the dopaminergic system. In PD, cortico-subthalamic transmission generates excessive bursts in each subthalamic neuron (Figure 6C) as well as an increase in

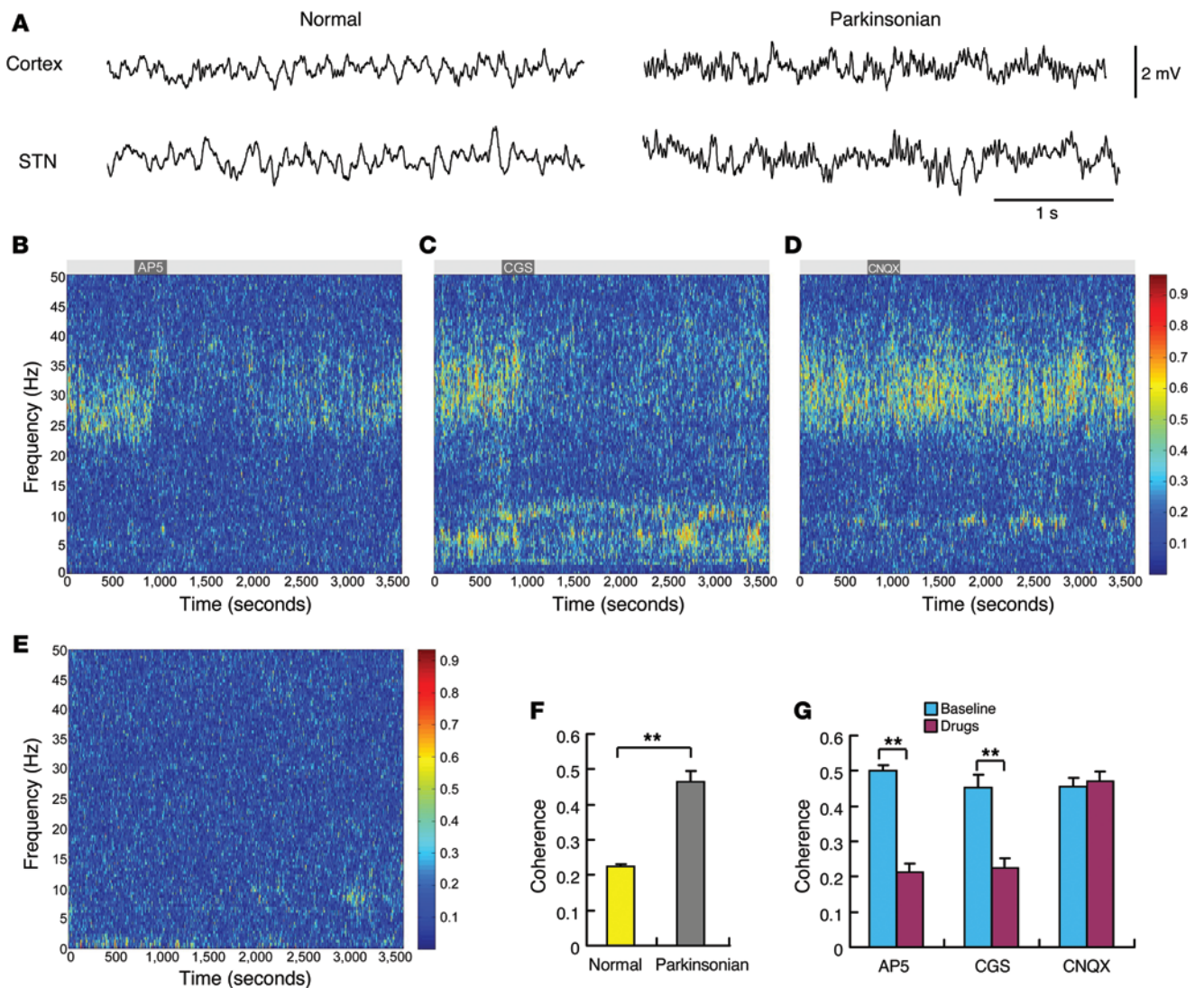


Figure 4. Effect of glutamate receptor antagonists on cortico-subthalamic beta synchronization. Electrical activity in the low-frequency range was simultaneously recorded in the premotor cortex and STN, and frequency-dependent synchronizations were quantified by coherence analysis. **(A)** Raw sweeps of electrical activity simultaneously recorded in the premotor cortex and STN. **(B)** Time-frequency coherence plot of cortico-subthalamic synchronization. Subthalamic AP5 microinfusion started at 780 seconds (13 minutes) and lasted 300 seconds (5 minutes; gray bar at top of the plot). The total recording lasted 1 hour. Note the band of synchronization at beta frequencies (20–40 Hz) at baseline and that AP5 readily abolished beta synchronization. **(C)** CGS has an effect similar to that of AP5. **(D)** CNQX did not change the coherence in beta frequencies. **(E)** There was no synchronization of beta frequencies in a normal rat. **(F)** Quantitative measurement of coherence of beta frequencies. Coherence was significantly different between normal and parkinsonian rats ($n = 13$ different rats for each group; $P = 0.001$). **(G)** Quantitative measurement of the drug effect in parkinsonian rats. Coherence of beta frequencies was significantly suppressed by AP5 ($n = 12$, $P = 0.002$) and CGS ($n = 9$, $P = 0.008$), while CNQX ($n = 8$, $P = 0.575$) did not have such an effect. Statistical analyses were performed using a nonparametric Wilcoxon signed-rank test. Data represent the mean \pm SEM. $**P < 0.01$.

the population of bursting neurons (Figure 6B) and augments the temporal responsiveness of subthalamic firing (Figure 7, B and D). We also observed abnormal in situ synchronization of the premotor cortex and STN (Figure 5). We therefore investigated the behavioral consequences in normal animals via overactivation and synchronization of cortico-subthalamic transmission. To activate the cortico-subthalamic axons in a more specific and synchronous way, we used an optogenetic method in Thy1::ChR2-EYFP transgenic mice, which have been validated for movement evaluations and cortico-subthalamic manipulation (39). Thy1::ChR2 mice express channelrhodopsin 2 (ChR2) in afferent axons from cortical pyramidal neurons to the STN,

but not in subthalamic neurons themselves or GABAergic neurons afferent to the STN (39–41). Illuminating the STN with blue light (473 nm) opens ChR2 and thus selectively activates the set of cortico-subthalamic axons in a synchronous manner (39). We implanted a fiberoptic cannula into the STN and introduced a blue-light laser to stimulate the cortico-subthalamic pathway. Figure 8 shows that optogenetic overactivation of the cortico-subthalamic fiber alone can instantaneously and reversibly turn a normal mouse into a hemiparkinsonian one (see also Supplemental Video 1 for a demonstration). It is evident that an overexpressed and synchronized cortico-subthalamic transmission itself is sufficient to cause parkinsonian hypokinetic movements.

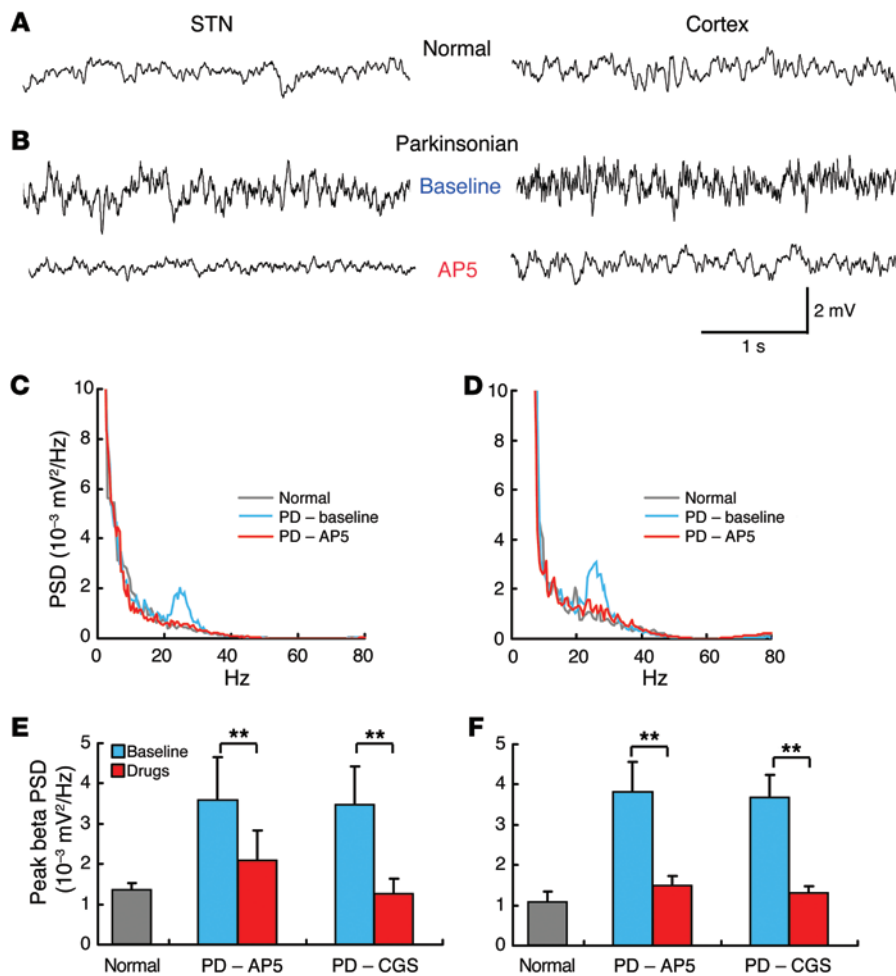


Figure 5. Effect of NMDA receptor antagonists on in situ synchronization of the STN and premotor cortex. Results from the STN are shown on the left, and those from the premotor cortex are shown on the right. **(A)** Typical sweep of LFPs in a normal rat. **(B)** LFP sweeps in the parkinsonian condition. As compared with a normal condition, high-frequency components were significantly increased at baseline and could be reversed by subthalamic infusion of AP5 into both the STN and cortex. **(C and D)** Representative PSD distribution in the STN **(C)** and premotor cortex **(D)**. There is an abnormal peak in beta frequencies (20–40 Hz) in parkinsonian rats (PD – baseline), but not in normal rats. The beta peak could be suppressed by subthalamic infusion of AP5 (PD – AP5). **(E and F)** Quantitative measurement of peak PSDs in beta frequencies. As compared with normal rats ($n = 8$ different rats), the peak beta PSD in the STN **(E)** was much higher in the parkinsonian rats and could be suppressed by AP5 ($n = 12, P = 0.009$) and CGS ($n = 9, P = 0.008$). **(F)** Results simultaneously recorded in the premotor cortex ($P = 0.003$ and $P = 0.008$ for the AP5 and CGS groups, respectively) are similar to those in the STN. Note that abnormal synchronization in the premotor cortex could also be abolished by local infusion of NMDA receptor blockers into the STN. Statistical analyses were performed using a nonparametric Wilcoxon signed-rank test. Data represent the mean \pm SEM. ****** $P < 0.01$.

Discussion

In addition to the classic direct-indirect theory, we demonstrate that the cortico-subthalamic pathway via the NMDA receptor may also participate in the central mechanism of parkinsonian hypokinetic movements (see Figure 9 for a schematic summary). Our results underscore the importance of glutamatergic inputs from the premotor cortex in the treatment of PD. Subthalamic neurons show automaticity in vitro (42, 43), and it has been hypothesized that with autonomous pacemakers, inhibitory GABAergic modulations are sufficient in the basal ganglia circuitry, and excitatory inputs are not required (44). However, our study demonstrates that STN firing in vivo, especially the timing and pattern of discharges and, consequently, in situ synchronization, are very much dependent on glutamatergic cortical input. Moreover, cortico-subthalamic intervention also corrects the electrophysiological abnormalities at the cortical level (Figure 5), further strengthening the role of this pathway in the symptomatic pathogenesis of PD. In this regard, it is interesting that the underlying molecular mechanism is related to the NMDA, but not the AMPA, receptor. The excitatory postsynaptic potentials (EPSPs) driven by NMDA receptors are much longer than those driven by AMPA receptors. The dependence of NMDA receptors may implicate an intrinsic inadequacy of the h-currents, and thus longer inward currents are necessary to bring the STN neuronal membrane potential to the firing threshold. The dependence on glutamatergic cortical input

would therefore be more pronounced in PD, a condition in which subthalamic neurons are more hyperpolarized due to the overexpression of NMDA receptor-dependent K-ATP channels under chronic dopamine depletion (45, 46). The hyperpolarized state could result in increased availability of the T-type calcium channel (25, 42) and cause a much higher prevalence of cortically driven subthalamic bursts (26). The full development of the foregoing serial changes requires chronic dopamine depletion (45), which explains why subthalamic bursts (18, 20), cortico-subthalamic synchronization (10), and intrasubthalamic synchronization (10) all take several days or longer to develop. By the same token, acute administration of dopamine receptor blockers, which readily compromises the balance between direct and indirect pathways in the classic pathophysiology of PD but lacks NMDA receptor-associated changes, fails to show these pathognomonic phenomena (9, 10). Consistently, short-term use of neuroleptics rarely causes hypokinetic movements in humans, but prolonged exposure is usually associated with a markedly increased risk of parkinsonism (8). It is also worth noting that the responsiveness of the STN to cortical input is faster and more synchronized in PD (Figure 7). Such a functional manifestation may explain why MK-801, a pore blocker specific to the open NMDA channel, does not have behavioral (ref. 47 and see also Supplemental Figure 6) or electrophysiological effects (Supplemental Figure 7) in parkinsonian rats. Because it takes only 9 ms to start the whole cortico-subthalamic

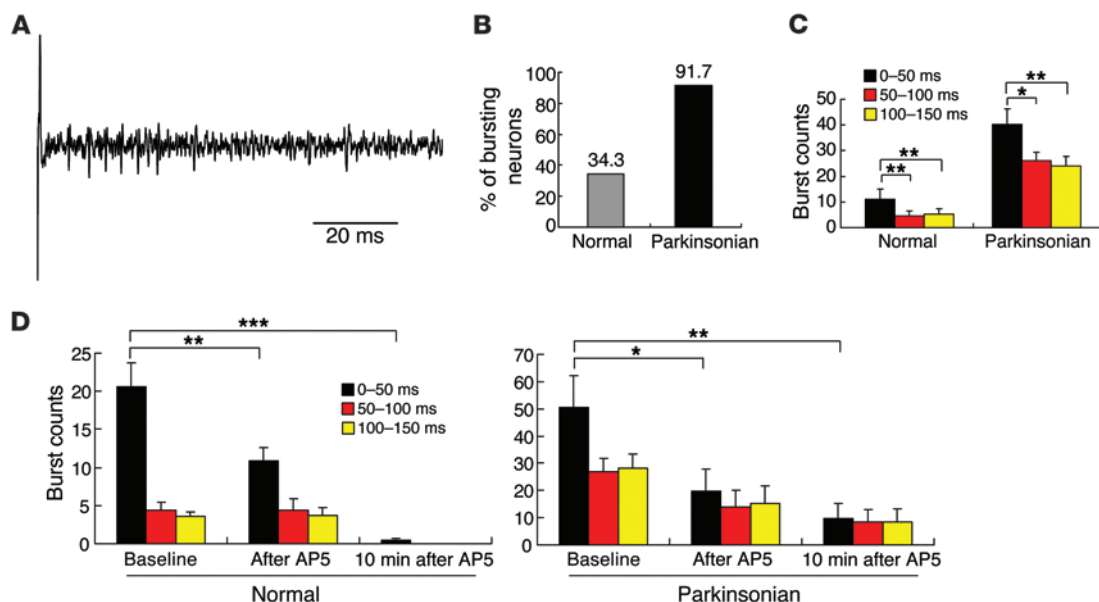


Figure 6. Burst firing of subthalamic single units evoked by electrical stimulation of the premotor cortex. To avoid a fixed-frequency effect, the stimulation frequencies were pseudorandomized between 1 and 2 Hz for a total of 400 stimuli. For each stimulus, the evoked subthalamic firing activity for 150 ms was analyzed. **(A)** Typical sweep of a bursting unit evoked by cortical stimulation. Note the stimulation artifact at the beginning of the sweep and the following single-unit firing in a cluster. **(B)** Population of stimulation-responsive bursting units in parkinsonian and normal rats. 91.7% (11 of 12 units in 3 rats) of subthalamic single units in parkinsonian rats were burst generating under cortical stimulation, much higher than the 34.3% (11 of 32 units in 10 rats) seen in normal rats. **(C)** Time-dependent profiles of evoked subthalamic bursts in parkinsonian and normal rats. Cortical stimulation evoked excessive bursts mainly during the initial 50 ms in both parkinsonian ($n = 11$) and normal rats ($n = 11$), illustrating the stimulation-dependent nature of subthalamic bursts. **(D)** Evoked subthalamic bursts (at 0–50 ms) were suppressed by subthalamic infusion of AP5 into both normal ($n = 7$ units in 6 rats) and parkinsonian rats ($n = 9$ units in 5 rats). Note that the burst counts in the 50–100 ms and 100–150 ms periods were also reduced. Thus, the spontaneous cortico-subthalamic transmissions, which were not time-locked by electrical stimulation, were also suppressed by AP5. Statistics were calculated by 1-way ANOVA with Dunnett's post-hoc correction. Data represent the mean \pm SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

process in PD (Figure 7D), the very slow binding rate of MK-801 (48, 49) would make it impossible to block the open NMDA channels in time. On the other hand, NMDA receptor blockers targeting the glutamate-binding domain, including AP5 and CGS, readily bind to the receptor in its closed state and prevent further channel activation from the very beginning.

Based on the discovery of cortical subthalamic beta synchronization in PD, rate-dependent modulation has been the conceptual basis underlying the design and refinement of deep brain stimulation (DBS). Subthalamic DBS in beta frequencies may worsen parkinsonian motor behaviors (50, 51), while high-frequency stimulation is an effective therapy for PD (2, 52). However, our study reveals a different possibility, as we investigated the mechanism underlying the genesis of cortico-subthalamic beta synchronization. The spike histogram in parkinsonian rats revealed strictly time-locked firing of the STN in response to cortical events (Figure 7, B and D). Interlocking of firing activity between the cortex and STN in the time domain spontaneously caused a fixed time delay of downstream synaptic potentials, which can be mathematically transformed into phase lock in the frequency domain and represents frequency-dependent synchronization. Moreover, the fast kinetics of EPSPs from NMDA receptors falls into beta frequency range (53), and NMDA receptor antagonists in the STN readily abolished beta synchronization (Figure 4G) as well as time-dependent, cortically triggered firing (Figure 7D). The interlocked firing activity between the cortex and STN could therefore con-

tribute to the basis of cortico-subthalamic beta synchronization. Based on this mechanism, cortico-subthalamic synchronization would require only the temporal relationship of neuronal activity between the premotor cortex and the STN rather than any specific firing rate, consistent with the view that beta synchronization is related to neuronal synchrony over the firing rate (22). The beta frequencies may actually reflect the spectral distribution of NMDA receptor currents rather than firing rates in the beta range. Therefore, neuronal firing or electrical stimulation at any specific rate is not necessarily required to modulate cortico-subthalamic beta synchronization. In fact, we have documented that DBS can generate very different or even opposite effects with different pulse durations but fixed stimulation frequencies (25). Moreover, direct-current stimulation of the STN, obviously a rate-independent process, has a very strong therapeutic effect in parkinsonian rats (26). These findings suggest that the rate-independent mechanisms of stimulation, such as modulation of membrane potential and suppression of subthalamic bursts (25, 26), may play a major role in the therapeutic basis of DBS. While there is a wide range of electrical properties implicating how DBS affects neuronal circuits (25, 26, 39, 54, 55), understanding the pathophysiological mechanism of hypokinetic movements helps to explain why DBS works in PD and provides the conceptual guidance to optimize the settings.

In this study, we have addressed the central role of cortico-subthalamic transmission in parkinsonian motor deficits. Such

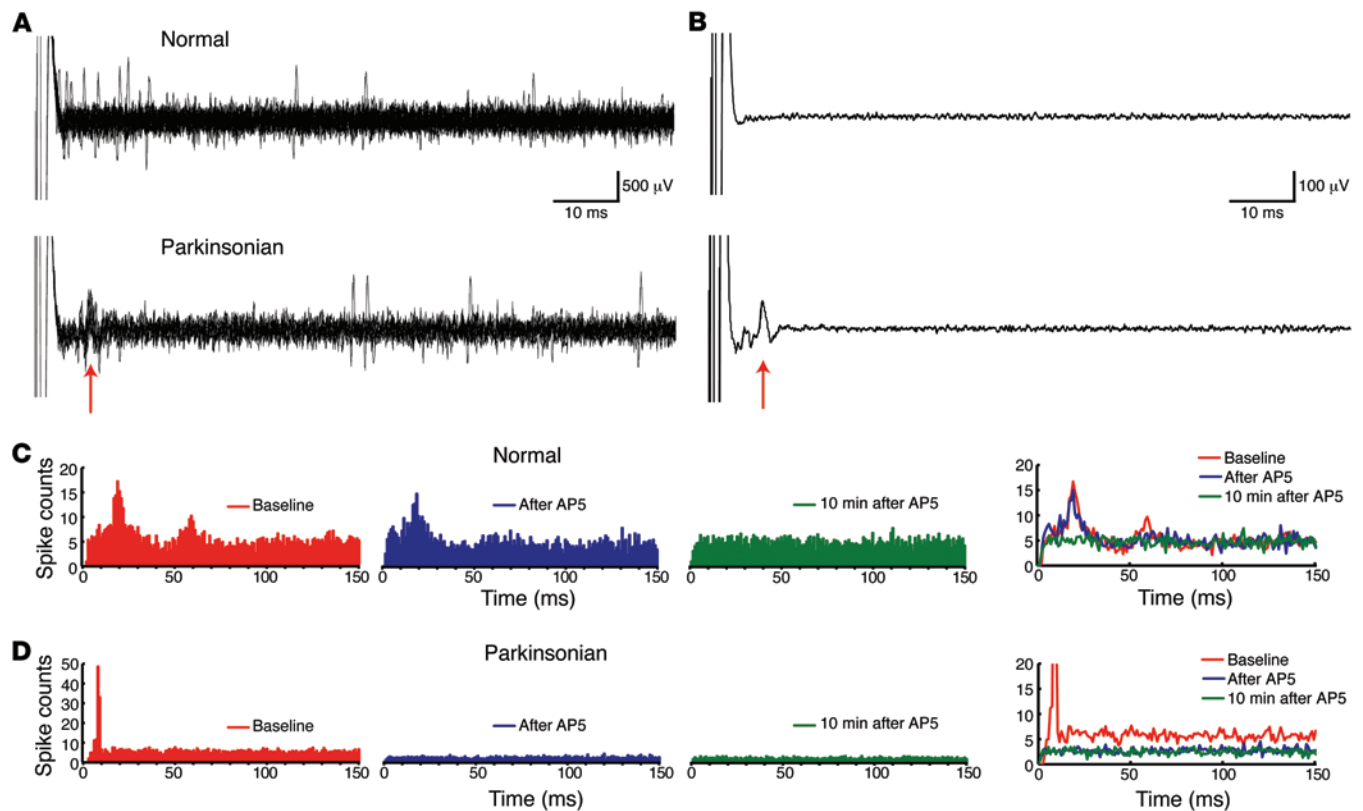


Figure 7. Temporal distribution of subthalamic single units evoked by electrical stimulation of the premotor cortex. In each test, 400 cortical stimuli were delivered while evoked single-unit discharges were simultaneously recorded in the STN. **(A)** Ten consecutive sweeps superimposed in both normal and parkinsonian conditions. Time-locked discharges (arrow) were noted in parkinsonian, but not in normal, rats. **(B)** An average of 400 traces were evoked by cortical stimuli. The single-unit discharges were averaged out in normal rats, but were still visible in the parkinsonian condition (arrow), indicating that the evoked subthalamic firing was much more time-locked, with fewer jitters. **(C)** Spike histogram of evoked subthalamic firing in normal rats ($n = 7$ units in 6 rats). Cortical stimulation evoked 2 peaks at around 15 to 25 ms and 55 to 65 ms under baseline conditions. Both peaks could be suppressed by subthalamic infusion of AP5. **(D)** Spike histogram pattern in parkinsonian rats ($n = 9$ units in 5 rats) revealed highly time-locked firing at 8–9 ms at baseline. This stimulation-dependent firing was also abolished by subthalamic infusion of AP5.

results may significantly contribute to therapeutic strategies in PD. Systemic dopamine replacement therapy, which reverses dopamine deficiency and presumably modulates the balance between direct and indirect pathways, has long been the standard treatment of parkinsonian motor deficits, with the price being motor complications after long-term use (2). Consistently, direct intervention in these pathways induces various forms of paradoxical movements in both normal and parkinsonian conditions (56). In contrast, cortico-subthalamic intervention with NMDA receptor blockers readily ameliorated the hypokinetic motor deficits without the turning behaviors (Figure 2, D–F) related to dopamine receptor supersensitivity (57, 58). Local manipulation of the STN or cortico-subthalamic inputs, thus, may have the potential to avoid motor complications related to the systemic use of dopaminergic agents. In fact, amantadine, a well-known nondopaminergic and nondyskinesogenic agent widely prescribed for the treatment of parkinsonian hypokinetic movements (59), is a weak NMDA receptor antagonist (60). Also, metabotropic glutamate receptor 5 (mGluR5) was shown to colocalize with NMDA receptors in the STN and potentiate NMDA receptor currents (61). Infusion of mGluR5 antagonist into the STN can remedy motor deficits in parkinsonian rats (62). In addition, we localized

the therapeutic effect of dopaminergic agents to the STN, and observed no apparent paradoxical turning behaviors (Figure 2, D–F). Dopamine could depolarize subthalamic neurons and suppress STN bursts (63), very consistent with the view that it also targets the burst-generating cortico-subthalamic cascade. It was intriguing to see that the genesis of parkinsonian motor deficits (Figure 8) and the therapeutic effect of dopamine (Figure 2) could both work through the cortico-subthalamic pathway without the need to directly modulate the balance of dopamine in the striatum, making the systemic delivery of dopaminergic agents less beneficial and more risky in terms of motor complications. Together with our previous arguments regarding the mechanism underlying the burst-suppressing effect of DBS (25, 26), the hypokinetic-specific mechanism in this study further strengthens the observations that early DBS can be superior to medical treatment in PD patients with early motor complications (64) and offers an imperative rationale for optimizing cortical stimulation as well as other nondopaminergic therapies for PD.

In addition to the parkinsonian condition, our study also illustrates the pivotal role of the cortico-subthalamic pathway and presents novel perspectives on normal motor control in the basal ganglia circuitry. Inhibition of this glutamatergic pathway

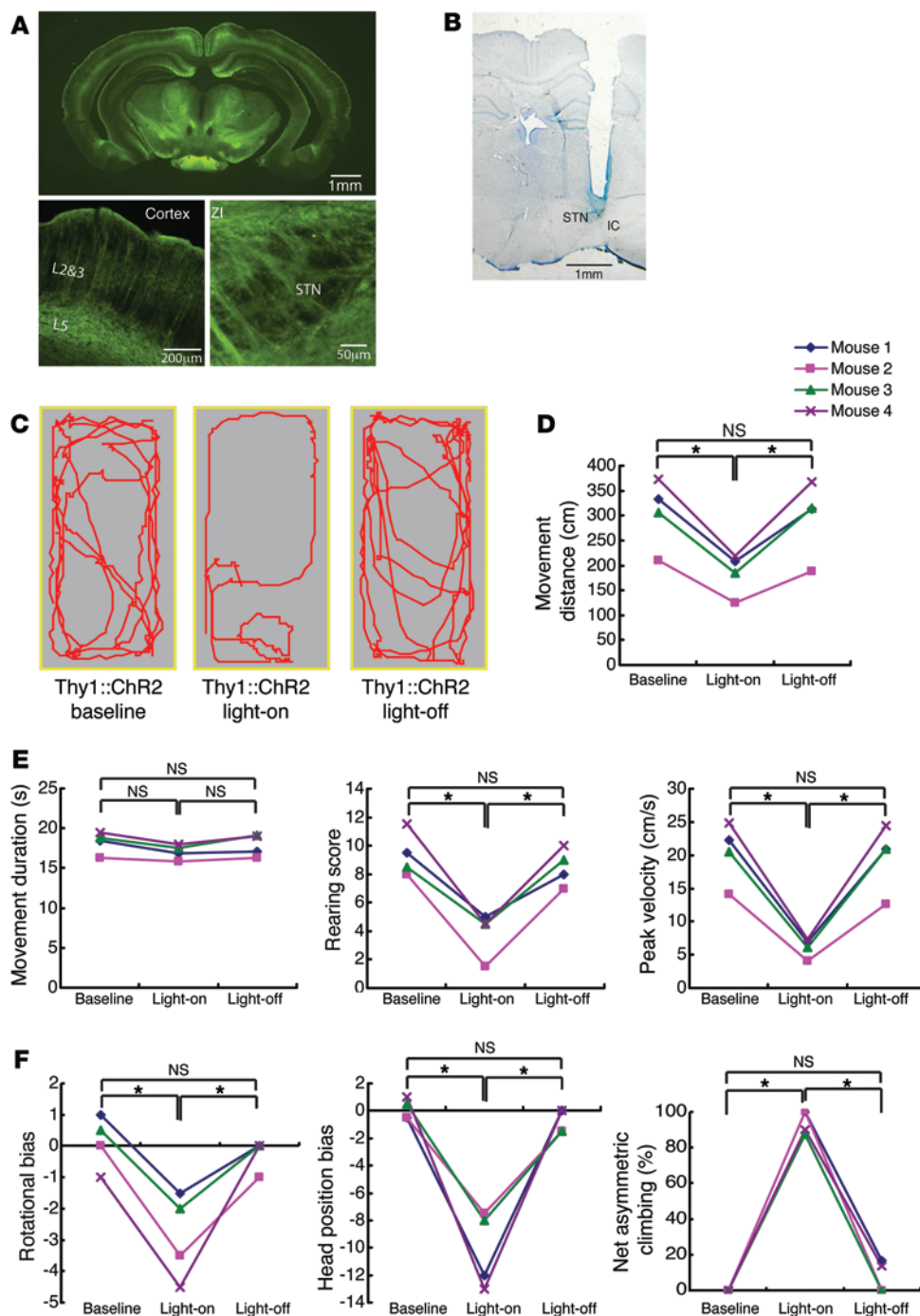


Figure 8. Effect of optogenetic activation of the cortico-subthalamic pathway on locomotor activities in Thy1::ChR2 mice. To avoid a fixed-frequency effect, the STN was illuminated at frequencies between 8 and 20 Hz in a pseudorandomized order. **(A)** ChR2-EYFP fluorescent expression. Note that the layer V pyramidal neurons and the projecting axons both expressed ChR2. The cortical afferents into the STN also expressed ChR2. **(B)** Location of fiberoptic cannula. **(C)** Representative traces of 30-second free movements before, during, and after illumination in a mouse. Locomotor activity was significantly suppressed by cortico-subthalamic stimulation. **(D and E)** Quantitative parameters of movement ability. Except for the movement duration, which did not reveal significant changes ($n = 4$ mice; experiments were repeated twice in each mouse), all of the other locomotor parameters including movement distance, rearing score, and peak velocity were significantly reduced during illumination (light-on versus baseline or light-off; $*P < 0.017$ under all conditions). **(F)** Quantitative parameters of asymmetrical movements. Stimulation also caused contralateral motor deficits in all parameters ($*P < 0.017$ under all conditions). Motor behaviors readily returned to baseline in the light-off state (baseline versus light-off; $P > 0.1$ under all conditions). All statistical analyses were performed using a non-parametric Wilcoxon signed-rank test with Bonferroni's post-hoc correction for multiple comparisons. ZI, zona inserta; IC, internal capsule. Refer also to Supplemental Video 1.

readily abolished the cortico-triggered, time-dependent firing (Figure 7) and burst firing (Figure 6) of the STN in normal rats. The glutamatergic cortico-subthalamic pathway probably works by “turning on” the basal ganglia circuitry, so the GABAergic modulations can function appropriately for motor processing. It is intuitive that the globus pallidus externa (GPe), the major GABAergic afferent of the STN, could no longer adequately address the inhibitory effects in an already suppressed STN, in which both cortical-triggered excitation phases have been abolished. Consistently, the early and late excitation phases of the globus pallidus interna (GPi) neurons (the immediate target innervated by glutamatergic subthalamic neurons) by cortical stimulation can be

suppressed by subthalamic application of NMDA receptor blockers in healthy monkeys (28). Once again, inhibitory GABAergic modulation from the striatum is less likely to influence firing rates of the already suppressed GPi neurons. Consequently, GABAergic modulations from the direct and indirect pathways are significantly compromised without adequate cortico-subthalamic transmission. This novel perspective explains the deoptimization of normal motor functions by cortico-subthalamic inhibition (Supplemental Figure 2). The same concept also applies to the parkinsonian condition, in which hyperresponsive and hypersynchronous cortico-subthalamic transmission may provide more room for GABAergic modulation from the affected direct

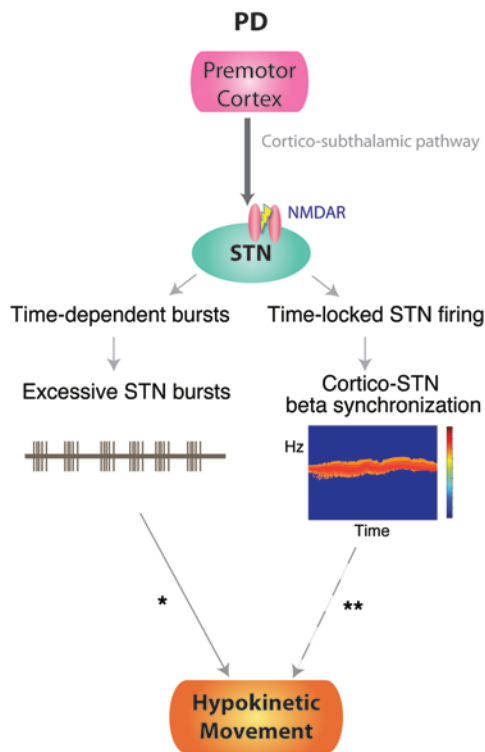


Figure 9. Simplified scheme of cortico-subthalamic transmission and relevant manifestations in PD. In PD, cortico-subthalamic transmission via the NMDA receptor generates deranged STN responses, namely hyper-responsive, time-dependent STN bursts and time-locked STN firing. The former constitutes the basis of excessive STN bursts, which directly cause the locomotor symptoms of PD (*) (26). The latter generates cortico-subthalamic beta synchronization, whose causal relations with PD symptoms remain unclear (**). The cortical activity itself may also be deranged, so that the in situ synchronization is increased in the premotor cortex of parkinsonian rats (Figure 5, B, D, and F). In this regard, the hypokinetic motor deficits can also be generated in normal animals with overdriven and synchronized cortico-subthalamic transmission (Figure 8).

dopamine (6-OHDA; Sigma-Aldrich) to produce hemiparkinsonian rats (see Supplemental Methods). For optogenetic experiments, we used adult Thy1::ChR2-EYFP line 18 transgenic mice (catalog 007612; The Jackson Laboratory). This animal expresses channelrhodopsin-2 in pyramidal neurons of the cortical layer V, but not in the STN, and has been validated as an ideal animal model for selective stimulation of cortico-subthalamic axons (39). We used only normal transgenic mice without 6-OHDA injection.

Implantation of a microinfusion cannula. To assess rat behavior during local infusion of neurotransmitter blockers, a microinfusion cannula was implanted into the STN. Both naive and parkinsonian rats were anesthetized with chloral hydrate and remained fully anesthetized throughout the procedure. Each rat was mounted onto a stereotactic instrument (David Kopf Instruments) with the skull exposed. A stainless-steel cannula (C315G; Plastics One) was inserted perpendicularly into the STN (anteroposterior [AP] -3.8 mm, lateral [L] 2.4 mm, depth [D] 7.5 mm from bregma). The cannula was fixed with bone cement on the skull, and the wound was closed with 4-0 nylon monofilament. The experiments and recordings were performed 1–2 weeks after the surgery, when body weight and health condition had returned to the presurgical state.

Implantation of the fiberoptic cannula. To assess mouse behavior during selective stimulation of the cortico-subthalamic fiber optogenetically, we implanted the fiberoptic cannula (catalog CFMC12L05; Thorlabs) into the STN (AP -1.9 mm, L 1.7 mm, D 4.2 mm from bregma). The animal was anesthetized with isoflurane, mounted onto the stereotactic instrument, and remained fully anesthetized throughout the procedure. The surgical procedures were the same as those performed in rats, except for the STN coordinates and the implantation devices (fiberoptic cannula rather than microinfusion cannula).

Open-field test of animal behaviors. The animals were brought into a quiet room with dim light 2 hours before the test during the dark cycle. For conditioning, each rat or mouse was placed into a square arena (45 cm × 45 cm for rats and 40 cm × 20 cm for mice) made of Plexiglass for 5 minutes every other day until their behavioral profiles were stabilized several days later. For the neurotransmitter experiment, aCSF or neurotransmitter receptor blocker was infused at a rate of 0.4 μ l/minute for 8 minutes (infused at 3 minutes before and 5 minutes during the behavior recording). The animal was placed into the arena, above which was installed a video recorder and tracking system (EthoVision 3.0; Noldus), and recorded for 5 minutes. For each set of experiments, the animal received either aCSF or the neurotransmitter blocker randomly in the first run, and the other agent in the second run. The 2 runs were separated by at least 1.5 hours to avoid a carryover effect. For the experiments with s.c. saline or apo-

and indirect pathways. Moreover, we found that synchronized cortico-subthalamic overactivation by itself could apparently induce parkinsonian symptoms in normal mice. In summary, the glutamatergic cortico-subthalamic pathway could create a platform to optimize the modulatory function of other pathways in the basal ganglia circuitry. Derangement of cortico-subthalamic transmission may therefore compromise the flow of information in the circuitry and consequently affect the cortex itself via the cortico-subcortical reentrant loop. We believe that elucidation of the true essential elements of the basal ganglia circuitry and their electrophysiological attributes will significantly contribute to a more rational evaluation of the basic principles of motor control and lead to better therapeutic strategies for movement disorders.

Methods

Neurotransmitter receptor modulators. The selective NMDA receptor blockers AP5 (2 mM; Tocris) and CGS (1 mM; Tocris) and the AMPA receptor blocker CNQX (2 mM; Tocris) were used as glutamate receptor blockers in this study. MK-801 (6 mM; Tocris), a pore blocker of the open NMDA receptor, was also used. These drugs were dissolved in artificial cerebrospinal fluid (aCSF), with the pH adjusted to 7.4. The nonselective dopamine receptor agonist apomorphine (Sigma-Aldrich) was used locally in the STN (3 μ g/ μ l) and systemically by s.c. injection (0.05 mg/kg). Apomorphine was dissolved in saline solution, with the pH adjusted to 7.4.

Animal materials and preparation of the parkinsonian model. We used adult male Wistar rats weighing between 250 and 400 g. The animals (2–3 rats/cage) were housed in an environmentally controlled vivarium under a 12-hour day/12-hour night cycle and had free access to food and water. The rats were randomly assigned to a normal or parkinsonian group. For the parkinsonian rat model, we used 6-hydroxy-

morphine, the animals were placed into the arena 20 minutes after the injection. Three parameters, including movement distance, movement duration, and rearing score, were automatically recorded by the EthoVision (Noldus) system to demonstrate the movement abilities. These parameters indicated the total movement distance, total nonfreezing time, and the number of times the rat reared freely in a 5-minute recording period. Rotation bias, head position bias, and net asymmetrical climbing were selected to quantify the asymmetrical movements (see Supplemental Methods for the definition of each parameter). For optogenetic experiments, a 473-nm diode laser (CrystaLaser) was connected to the fiberoptic cannula through an optic fiber cord, and the light intensity was adjusted to 4 mW. During the experiments, the fiber cord was attached to the cannula mounted on the mouse's skull, and the mouse was placed into the 40 cm × 20 cm arena for 90 seconds. The laser was turned on in the middle 30 seconds, while the first and the last 30 seconds were self-controls in the light-off state. During the light-on state, the duty cycle was 50%, and the illumination was set in pseudorandomized order between 8 and 20 Hz to avoid a rate-dependent effect (see also Supplemental Methods). The recording system and behavioral parameters were the same as those used in the rat experiments, except for the additional parameter of peak velocity, which can also be reliably measured in an arena appropriately sized for a mouse.

Continuous *in vivo* single-unit recording. We recorded single-unit firing activity of the STN in normal or apomorphine-validated parkinsonian rats. The rat was anesthetized with urethane (1.2 g/kg *i.p.*; Sigma-Aldrich), and the skull was exposed on the stereotaxic instrument. For microinfusion of the tested drug, a 30-gauge brain-lesioning cannula was placed into the STN (AP -3.8 mm, L 2.4 mm, D 7.5 mm from bregma) with a 20-degree tilt in the sagittal plane and then withdrawn 0.1 mm from the target. For single-unit recordings, a bundle of 8 insulated stainless steel electrodes (0.002 inch diameter, no. 304; California Fine Wire) was inserted perpendicularly into the STN and recorded single-unit firing with and without drug effect (see Supplemental Methods). At the end of the recording, negative currents of 1 mA were injected through the recording electrodes for 10 seconds for localization purposes. The rats were subsequently transfused intracardially with 10% formalin, and the brain was removed for histological verification and electrode localization by Prussian blue staining.

Evoked single-unit recordings with premotor electrical stimulation. Subthalamic single-unit discharges were also recorded with electrical stimulation of the premotor cortex. The surgical procedure, cannula, electrode insertions, and amplifier settings were the same as those for the continuous single-unit recordings. In addition, a concentric bipolar stimulation electrode was placed into the premotor cortex (AP -2.7 mm, L 2.0 mm, D 0.1 mm from bregma). For each set of stimulations, 400 electrical pulses were delivered over a 4-minute period at 1 to 2 Hz in a pseudorandomized order to avoid rate-dependent effects. The negative electrical pulses were generated in 200- μ A, 600- μ s square-wave shapes by an isolated pulse stimulator (model 2100; A-M Systems). Each sweep of the single-unit recordings was triggered by a single cortical stimulus and lasted 400 ms, and 400 sweeps were recorded in each stimulation set. For those recordings designed to evaluate the effect of the neurotransmitter blockers, stimulations were first applied under baseline conditions and repeated immediately and 10 minutes after completion of the drug infusion (0.2 μ l/minute for 5 minutes).

Implantation of electrodes for LFPs. For analysis of cortico-subthalamic synchronization, epidural electrodes were implanted into the premotor cortex and deep brain electrodes into the STN of both naive and parkinsonian rats. For those rats designed to evaluate the effect of glutamate receptor blockers, another non-metallic microinfusion cannula (C315G/PK/SC; PlasticsOne) was placed into the STN at a 30-degree tilt in the sagittal plane (see Supplemental Methods).

Recordings of cortical and subthalamic LFPs. LFPs from epidural and subthalamic electrodes were simultaneously recorded. The rat was placed into an open-field arena in dim light, and a wired headstage was connected to the connector on the head. The animal was allowed free movement, while electrical signals from the premotor cortex and STN were simultaneously recorded for 1 hour. For those rats designed to evaluate the effect of neurotransmitter blockers, the drug was locally infused into the STN (0.2 μ l/minute) for 5 minutes in the middle (at 13 to 18 minutes) of the 1-hour recording period (see Supplemental Methods for details).

Data processing of continuous and evoked single-unit recordings. For the electrical signals recorded from either continuous or evoked single-unit recordings, we used the same procedures as those described in our previous work for quality control, spike sorting, and burst selection (ref. 25 and see also Supplemental Methods). Single units, which included at least 1 burst, were defined as the burst mode, and those that did not reveal any bursts were defined as the spike mode. We quantified the burst-firing rates of units in the burst mode and the spike-firing rates of units in the spike mode. We also quantified 3 intraburst parameters including spike count in each burst (spike number), intraburst interspike interval, and burst duration for the bursting units. We analyzed these profiles with protocols of continuous or evoked single-unit recordings and made a comparison before and after the delivery of different glutamate receptor antagonists. For the burst analysis for evoked single-unit recordings, the first 150 ms in the 400-ms sweep were further divided into 3 segments of 50 ms each. The burst was counted in a segment if the burst initiated in the time window of that segment (see Supplemental Methods for details).

Data processing of LFPs. The recorded LFPs from the premotor cortex and STN were post-processed by MATLAB 7.4 software (MathWorks). LFP signals were characterized in the frequency domain by power spectrum density (PSD) function. Coherence analysis of the cortex and STN is a function of the frequency, defined by the square of the cross-spectrum between the 2 signals, divided by the auto-spectrum of each signal. The value of coherence is between 0 and 1, where 1 indicates that one signal can be linearly transformed into the other one in the given frequency, and zero denotes a complete lack of linear relationship. The data were organized in the time-frequency plot for direct visualization. The peak PSD and maximal mean coherence were calculated in the beta frequency range of 20 to 40 Hz at baseline (sampled from minutes 5 to 10). For the group designed to evaluate the effect of neurotransmitter blockers, another set of coherence data were sampled 10 minutes after the drug infusion was completed (e.g., sampled from minutes 28 to 33). The detailed paradigms for PSD and coherence are described in the Supplemental Methods.

Statistics. We only analyzed data from validated recording sites (see Supplemental Methods for details). The statistical analyses were performed using SPSS 13.0 statistical software (SPSS) and plotted with Excel 2003 (Microsoft). A nonparametric Wilcoxon signed-rank test was used to analyze paired data including behavioral profiles, coher-

ence analysis, and peak beta PSD comparisons (Figure 2; Figure 4, F and G; Figure 5, E and F; and Figure 8, D–F; Supplemental Figure 2, B–E; Supplemental Figure 5, D and E; Supplemental Figure 6, C–F; and Supplemental Figure 7, D–F). We performed 1-way ANOVA with Dunnett's correction for data requiring multiple post-hoc comparisons including continuous and evoked single-unit recordings (Figure 3, C–E; Figure 6, C and D; and Supplemental Figures 3 and 4). For all data analyses, a *P* value of less than 0.05 was considered significant.

Study approval. All rat and mice studies were approved by the IACUC of the National Taiwan University College of Medicine and College of Public Health.

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