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Electrophysiological properties of thalamic, subthalamic and nigral neurons during the anti-parkinsonian placebo response

Fabrizio Benedetti^{1,2}, Michele Lanotte¹, Luana Colloca^{1,2}, Alessandro Ducati¹, Maurizio Zibetti¹, Leonardo Lopiano¹

¹*Department of Neuroscience, University of Turin Medical School, Turin, Italy*

²*National Institute of Neuroscience, Turin, Italy*

Corresponding author

F. Benedetti: Dipartimento di Neuroscienze, Università di Torino, Corso Raffaello 30, 10125 Torino, Italy. Email: fabrizio.benedetti@unito.it

Abstract

Placebo administration to Parkinson patients is known to induce dopamine release in the striatum and to affect the activity of subthalamic nucleus (STN) neurons. By using intraoperative single-neuron recording techniques in awake patients, here we extend our previous study on STN recording, and characterize part of the neuronal circuit which is affected by placebos. In those patients who showed a clinical placebo response, there was a decrease in firing rate in STN neurons that was associated with a decrease in the substantia nigra pars reticulata (SNr) and an increase in the ventral anterior (VA) and anterior ventral lateral (VL_a) thalamus. These data show that placebo decreases STN and SNr activity whereas it increases VA/VL_a activity. By contrast, placebo non-responders showed either a lack of changes in this circuit or partial changes in the STN only. Thus, changes in activity in the whole basal ganglia–VA/VL_a circuit appear to be important in order to observe a clinical placebo improvement, although the involvement of other circuits, such as the direct pathway bypassing the STN, cannot be ruled out. The circuit we describe in the present study is likely to be a part of a more complex circuitry, including the striatum and the internal globus pallidus (GPi), that is modified by placebo administration. These findings indicate that a placebo treatment, which is basically characterized by verbal suggestions of benefit, can reverse the malfunction of a complex neuronal circuit, although these placebo-associated neuronal changes are short-lasting and occur only in some patients but not in others.

Abbreviations: GPe, external globus pallidus; GPi, internal globus pallidus; SNr, substantia nigra pars reticulata; STN, subthalamic nucleus; UPDRS, unified Parkinson's disease rating scale; VA, ventral anterior thalamus; VAdc, densicellular part of VA; VAmc, magnocellular part of VA; VAp_c, parvicellular part of VA; VL_a, anterior ventral lateral thalamus; Zi, zona incerta.

Placebos are known to affect the brain in different conditions and different systems, such as pain, motor disorders, depression, the immune and endocrine systems (Benedetti *et al.* 2005; Colloca & Benedetti, 2005; Pacheco-Lopez *et al.* 2006; Benedetti, 2008*a,b*). In recent years, the effects of placebos have been analysed with sophisticated neurobiological tools that have uncovered specific mechanisms at both the biochemical and cellular level, such as the activation of endogenous opioids (Levine *et al.* 1978; Amanzio & Benedetti, 1999; Petrovic *et al.* 2002; Zubieta *et al.* 2005; Wager *et al.* 2007), the decrease of pain transmission in some brain regions (Wager *et al.* 2004; Price *et al.* 2007), the release of dopamine in the striatum (de la Fuente-Fernandez *et al.* 2001; Strafella *et al.* 2006; Scott *et al.* 2007, 2008), and the modulation of the activity of single neurons in the subthalamic nucleus (STN) (Benedetti *et al.* 2004).

The placebo effect represents a complex psychobiological phenomenon whereby an inert treatment may induce a therapeutic benefit if the subject is made to believe that it is effective. This may occur through both expectation and conditioning mechanisms (Benedetti *et al.* 2003; Enck *et al.* 2008; Price *et al.* 2008). In this regard, Parkinson's disease shows substantial placebo responses (Shetty *et al.* 1999; Goetz *et al.* 2000, 2002, 2008; Pollo *et al.* 2002; Benedetti *et al.* 2003; Mercado *et al.* 2006), and a placebo-induced release of dopamine in the striatum has been found in Parkinson patients (de la Fuente-Fernandez *et al.* 2001, 2002; Strafella *et al.* 2006), along with a change in activity of STN neurons (Benedetti *et al.* 2004).

By considering the organization of the basal ganglia and the key role of STN in basal ganglia functioning (Albin *et al.* 1989; DeLong, 1990; Bolam *et al.* 2000; Magnin *et al.* 2000; Pollack, 2001; Francois *et al.* 2002; Garcia *et al.* 2005; DeLong & Wichmann, 2007; Hammond *et al.* 2007; Benarroch, 2008), these placebo-induced neuronal changes are likely to affect several output regions of the basal ganglia, for example the substantia nigra pars reticulata (SNr), the internal globus pallidus (GPi), and the motor thalamus that receives inputs from both SNr and GPi, such as the ventral anterior nucleus (VA) and the anterior ventral lateral nucleus (VLa). In fact, the basal ganglia exert an inhibitory control upon the thalamus which, in turn, projects to the motor cortex (Fig. 1A). For example, SNr, which receives a glutamatergic excitatory input from STN, exerts a GABAergic inhibitory control upon the motor thalamus, so that a reduced activity in STN and SNr leads to an increased output activity from the thalamus to the cortex (Benazzouz *et al.* 2000; Maurice *et al.* 2003; Tai *et al.* 2003; Shi *et al.* 2006; Maltete *et al.* 2007).

On the basis of these considerations, in the present study we recorded from single neurons of STN, SNr, VA and VLa (Fig. 1) during the placebo response in Parkinson patients who were undergoing electrode implantation for deep brain stimulation. In this way, we could characterize part of the neuronal circuitry that is involved in the anti-parkinsonian placebo response.

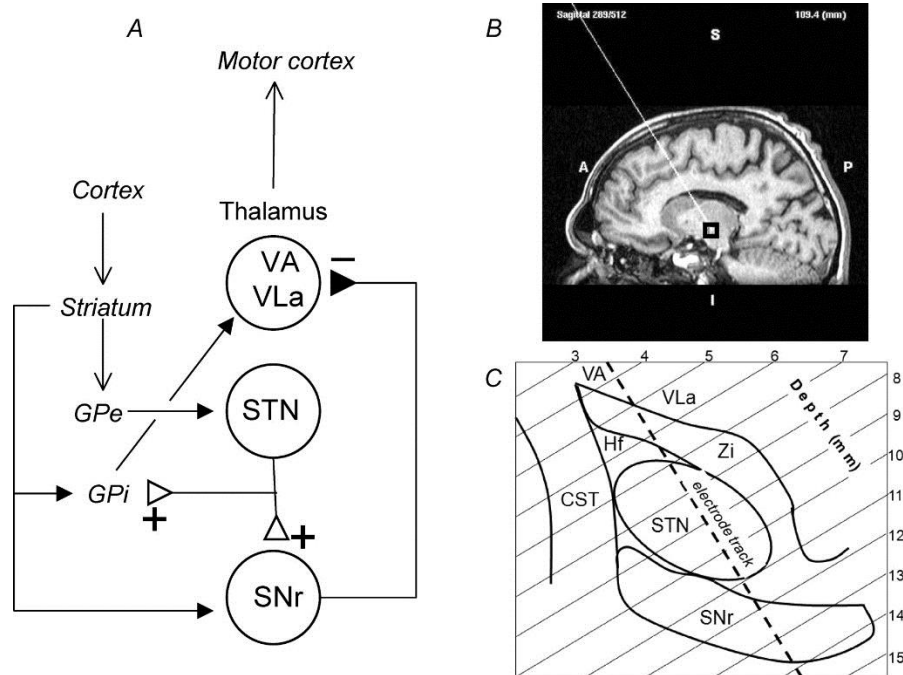


Figure 1. The neuronal circuit analysed in this study A, the circles represent the recorded neurons. The subthalamic nucleus (STN) neurons, which receive inputs from the cortex, the striatum, the external globus pallidus (GPe) as well as from other regions, send their output excitatory information to different regions, such as the substantia nigra pars reticulata (SNr) and the internal globus pallidus (GPi). SNr has an inhibitory connection with the thalamus, and the thalamus sends projections to the motor cortex. The striatum also sends projections to GPi, which in turn projects to the thalamus, and to SNr. B, magnetic resonance imaging of the electrode track with the electrode tip in the thalamic–subthalamic region. The square represents the region which is magnified in C. C, magnification of the square in B. It can be seen that the electrode track passes through VA, VLa, STN and SNr. We could record from all these regions during the placebo response, thus analysing the circuit shown in A.

Methods

Subjects

This study represents an extension of our previous study on single-neuron recording (Benedetti *et al.* 2004). Whereas in that study our analysis was performed in the STN only, in the present study we extended our analysis to VA/VLa and to SNr. As shown in the Supplemental material (available online only), one new patient was added to the placebo group. Therefore, a total of 24 patients participated in the study after written informed consent was obtained and after approval by the Ethics Committee of the University of Turin Medical School. All procedures conformed to the standards set by the *Declaration of Helsinki*. The patients were told that they participated in a study aimed at better understanding the mechanisms of deep brain stimulation, including the influence of some psychological factors. To do this, they were told that repeated administrations of apomorphine were necessary pre-operatively, and a similar injection might have been performed in the operating room. Thus, the reason that was given to the patients for the apomorphine administration pre-operatively was the need to better elicit some clinical and neurophysiological responses. The patients knew that a placebo could be given at one point in the course of the experiment; however, they did not know when. All the patients were diagnosed with idiopathic Parkinson's disease and clinical

evaluation was performed by means of the unified Parkinson's disease rating scale (UPDRS) (Fahn *et al.* 1987). The five stages of the disease, where stage 5 is the most severe, were also assessed (Hoehn & Yahr, 1967). The characteristics of each patient, the UPDRS scores before the surgical implantation of the electrodes, and the duration of the disease, as well as the drug therapy before surgery are shown in the online Supplemental material. Any pharmacological treatment was stopped the day before surgery. Atypical neuroleptics, like clozapine and quetiapine, were sometimes used to control either mild psychosis or dyskinesias (see Supplemental material). The patients were randomly subdivided into two groups (see below).

Surgical implantation of the electrodes

Before surgery a brain magnetic resonance imaging (MRI) scan (sequences of 2 mm contiguous slices) was obtained for each patient. At surgery, after positioning of a Cosman-Roberts-Wells stereotactic frame (CRW, Radionics, Burlington, MA, USA), a stereotactic computerized tomography (CT) scan was performed (2 mm contiguous slices). Then, the MRI and CT slices were fused by the Stereoplan system (Radionics) in order to obtain in the same images the spatial precision of CT and the better tissue definition of MRI. In this way, we assessed the anterior and posterior commissure coordinates and the length of the intercommissural line. The STN was anatomically localized 2.5 mm posterior and 4 mm inferior with respect to the midcommissural point and 12 mm from the midline. The electrode track was planned using a 58–63 anterior–posterior angle and 14–20 lateral angle (deg) (Fig. 1B). After local anaesthesia, a 14 mm pre-coronal burr hole was performed and the electrode lowered into the brain.

Electrical activity microrecording was performed starting from 10 mm above the anatomical target by using Microtargeting Electrodes (Type BP, FHC, Bowdoinham, ME, USA). The electrical signals were acquired by means of the Neurotrek system (NeuroTrek, Alpha Omega, Nazareth, Israel). The first activity corresponded to thalamic neurons in the VA and VL_a nuclei (Fig. 1C). After a low background activity corresponding to a region encompassing the zona incerta (Zi), the STN was identified by a background noise with a sustained and irregular pattern of discharge at a frequency of about 25–45 Hz, but also higher frequencies were considered. In addition, single units responsive to contralateral proprioceptive stimuli were sometimes identified and, in some cases, 'tremor neurons' were recorded with an oscillatory discharge of 4–6 Hz (parkinsonian tremor). When the microelectrode exited the STN (Fig. 1C), a low background noise was followed by a regular and high frequency discharge of units belonging to SNr. After the definition of the extension of the STN recording area, with its dorsal and ventral borders, the microstimulation procedures were started. In fact, further confirmation of good positioning of the electrode tip in the STN was obtained by means of microstimulation for the assessment of both clinical effects (reduction of rigidity, disappearance of tremor) and side effects (dyskinesias, muscle contractions, tingling sensations). Microstimulation was performed with a stimulus width of 60 μ s and a frequency of 130 Hz and an ascending stimulus intensity from 1 to 5 V.

Taking the microstimulation site with the best therapeutic effect as a reference, the anatomical location of the different recorded units was determined by projection on the Schaltenbrand and Wahren atlas (Schaltenbrand & Wahren, 1977) This procedure has been successfully adopted in one of our previous studies (Lanotte *et al.* 2005). In addition, in order to classify a neuron as a thalamic neuron, we considered only units at least 2 mm above the superior border of STN; thus we discarded some units which probably belonged to the Zi. The superior border of STN was identified by considering

the typical firing pattern of STN neurons (see above). As to SNr neurons, we considered a unit as belonging to SNr if at least 1 mm below the inferior border of STN, as assessed by means of electrophysiological criteria. In addition, SNr units fire with a typical pattern (see above) which helped us to identify them. There was a striking correlation between the electrophysiological criteria and the anatomical location, as assessed by measuring the distance from the best therapeutic stimulation site.

Procedure

Whereas the first group of patients ($n = 12$) did not receive any treatment, thus representing the no-treatment, or natural history, group, the second group ($n = 12$) received an intra-operative placebo treatment, along with verbal suggestions of motor improvement. In order to obtain robust placebo responses, these patients were given the anti-Parkinson agent, apomorphine, for 3 days before surgery. To do this, the patients (in the medication-off state) were given a 2–3 mg dose of apomorphine subcutaneously, along with domperidone to minimize nausea. The sequential steps of the entire procedure, both pre-operative and intra-operative, are shown in the online Supplemental material. Each time, a trained neurologist (who was not necessarily the same person who evaluated the patient intra-operatively) assessed the symptom improvement by using the UPDRS scores, with particular regard to muscle rigidity at the arm. We did not include those patients who developed dyskinesias after apomorphine injection, in order to avoid the possibility that the placebo could mimic the same dyskinetic effects produced by the pre-operative apomorphine.

On the day of surgery, during the implantation of the first electrode, neuronal activity was recorded from the first thalamus, STN and SNr, and rigidity of both arms was assessed several times. We limited our assessment to arm rigidity because of the following reasons. (1) Tremor is not a good measurement because of its fluctuations during surgery and because it is not present in all patients. (2) The changes of bradykinesia show a longer latency compared with rigidity. (3) A complete assessment of all the symptoms would require a longer time, thus prolonging the discomfort of the patient.

After the first electrode was implanted, the surgical procedures for the implantation of the second electrode began. The time interval between the first and the second implantation was about 1 h in all patients, and left and right implantation was randomized between subjects. During the second implantation, the tip of the electrode was stopped 10 mm above the STN. This was done in order to avoid any possible microlesion-induced effects in STN produced by passage of the microelectrode. At this point, after contralateral arm rigidity assessment, a subcutaneous injection of saline solution (placebo) was administered to Group 2 with the suggestion that it was the same anti-Parkinson drug given on the previous days, and that a motor improvement should be expected. More specifically, the patients were told that apomorphine was going to be injected and that a sensation of well-being should occur. In order to make the injection as equal as possible to the pre-operative apomorphine injection, the patients were also informed that an anti-nausea drug would be injected through one of the many intravenous lines. Then, arm rigidity was assessed after 5, 10 and 15 min by a blinded neurologist, who did not know anything about the subcutaneous injection. After 15 min, the electrode was lowered into VA, VL_a, STN and SNr, and neuronal recording began starting from VA and VL_a. A time interval of 15 min between the placebo injection and the beginning of the recording was chosen on the basis of the pharmacological action of apomorphine. In fact, the effect of apomorphine begins after

about this time lag. At the end of the recording, arm rigidity was assessed again by the same blinded neurologist. Fifteen minutes after placebo administration all the patients were asked to report any sensation of therapeutic benefit or, otherwise, of discomfort. In this way, we could correlate the subjective report of the patient with the objective evaluation of the blinded neurologist. It is important to point out that the blinded neurologist did not know anything about the purpose of the study and that the arm rigidity assessment was done without knowing the subjective report of the patient. In fact, in order to avoid any influence of the patients' reports of well-being on the blinded neurologist, the patients described their subjective sensations when the neurologist was out of the operating room.

The duration of each recording was in the range of 60–120 s. In particular, in the placebo condition, we did not want to record for more than 120 s because of the duration of the placebo response, which lasts about 30 min (see Fig. 2 and Benedetti *et al.* 2004). In this way, we could record from as many units as possible during the maximum response. After placebo administration, the mean recording time for each neuron was 93 s (range = 60–120 s) whereas the mean time between the first and last recording was 13.5 min (range = 2–23 min) from the maximum of the response. The investigator who made the recordings was blind regarding the assessment of muscle rigidity by the neurologist.

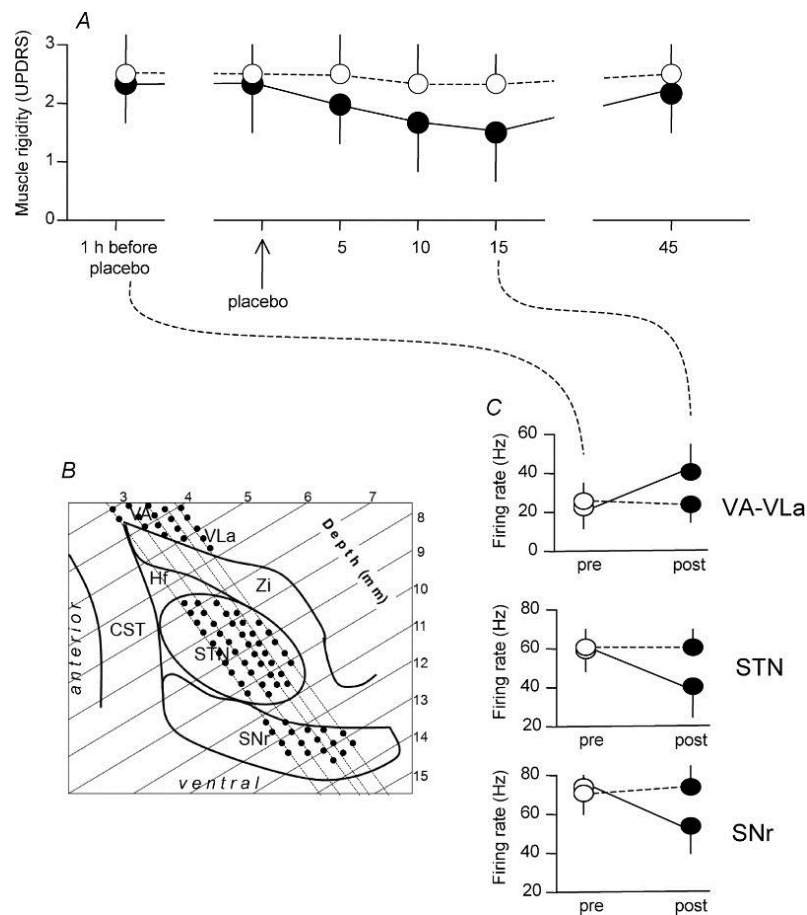


Figure 2. Data from all the patients who received the placebo treatment and from those who received no treatment (mean \pm S.D.) A, the clinical placebo response (filled circles) is compared with the no-treatment group (open circles). Pre-placebo recordings were performed 1 h before placebo treatment, whereas post-placebo recordings were carried out starting from 15 min (maximum of the response) after placebo administration. B, location of the recorded neurons on the

Schaltenbrand and Wahren atlas (Schaltenbrand & Wahren, 1977). It is important to note that many recording sites overlap, so that their number turns out to be smaller than the actual number of recorded units. C, neuronal firing rate in VA/VLa, STN and SNr, before (open circles) and after (filled circles) placebo (continuous lines). The dashed lines show the firing rate in the no-treatment group on the first side (open circles) and second side (filled circles) of recording. Note that during the maximum placebo response, VA/VLa neuronal activity increased whereas STN and SNr activity decreased.

Data analysis

The mean firing frequency of a neuron was assessed by means of an amplitude discriminator. For this reason, only those units with a stable background noise and spike amplitude, and spikes clearly distinguishable from the background, were analysed. Both single unit and multiunit recordings were considered. When more than one unit was present in the recording, the single spikes were separated by means of principal components analysis (AlphaSort, Alpha Omega Engineering, Nazareth, Israel), as described in detail in the online Supplemental material. In addition, we also performed bursting analysis to see whether bursting activity occurred in VA/VLa, STN and SNr (see Supplemental material for details). Statistical analysis of the clinical placebo response (muscle rigidity scores) was performed by using ANOVA followed by the *post hoc* Dunnett's test for multiple comparisons. Neuronal discharge was analysed by using ANOVA, with site as independent variable, treatment as within-group factor and firing rate as the dependent variable. This was followed by the *post hoc* Newman-Keuls test for multiple comparisons. The number of bursting and non-bursting neurons before and after placebo was compared by means of the χ^2 test. Linear regression analysis was performed in order to correlate neuronal firing rate with clinical improvement as well as the neuronal discharges in the different nuclei. Statistical significance was set at $P < 0.05$.

Results

Recording after placebo administration revealed a different pattern of neuronal discharge in STN, SNr, VA and VLa compared to pre-placebo baseline. The STN, SNr, VA and VLa on one side, during the implantation of the first electrode, were recorded before placebo and the same regions of the other side, during the implantation of the second electrode, were recorded after placebo (see Methods).

The data from all the patients of the placebo group are shown in Fig. 2. The clinical placebo response, as assessed by means of muscle rigidity at the wrist, in the placebo group ($n = 12$) is shown in Fig. 2A (filled circles) and compared to the no-treatment control group ($n = 12$) (open circles). ANOVA showed a significant decrease in muscle rigidity in the placebo group ($F(5,55) = 8.036$, $P < 0.001$), with a highly significant decrease at both 10 and 15 min after placebo compared to the pre-placebo baseline (*post hoc* Dunnett's test: $q(55) = 2.947$, $P < 0.01$ and $q(55) = 5.010$, $P < 0.01$, respectively). By contrast, no significant change was detected in the no-treatment group ($F(5,55) = 0.388$, $P = 0.855$). This rules out the possibility that the difference in muscle rigidity between the pre- and post-placebo condition was independent of the placebo treatment itself. In fact, in the no-treatment group the conditions were exactly

the same as those of the placebo group. The only difference was that these patients did not undergo any placebo treatment between the implantation of the first and second electrode.

In the placebo group, we recorded from a total of 98 neurons in VA/VLa (pre-placebo = 49, post-placebo = 49), 296 in STN (pre-placebo = 140, post-placebo = 156), and 91 in SNr (pre-placebo = 47, post-placebo = 44). The location of the recorded neurons, as measured on the Schaltenbrand and Wahren atlas (Schaltenbrand & Wahren, 1977), is shown in Fig. 2B, whereas the mean firing rate and standard deviations are shown in Fig. 2C for VA/VLa, STN and SNr. The difference between the pre-placebo and the post-placebo conditions was highly significant in all cases (continuous lines), with a significant interaction between recording site and treatment ($F(5,479) = 52.08$, $P < 0.001$), with an increase in firing rate in VA/VLa (pre-placebo mean firing rate = 24.3 ± 12.1 Hz, post-placebo mean firing rate = 40.6 ± 23.5 Hz; Newman-Keuls: $q(479) = 6.249$, $P < 0.01$), a decrease in STN (pre-placebo mean firing rate = 60.1 ± 16.8 Hz, post-placebo mean firing rate = 41.8 ± 20.8 Hz; Newman-Keuls: $q(479) = 11.483$, $P < 0.005$), and a decrease in SNr (pre-placebo mean firing rate = 76 ± 9.2 Hz, post-placebo mean firing rate = 56.2 ± 24.7 Hz; Newman-Keuls: $q(479) = 7.081$, $P < 0.01$).

In the no-treatment group, a total of 98 neurons were recorded from VA/VLa (pre-placebo = 48, post-placebo = 50), 298 from STN (pre-placebo = 148, post-placebo = 150), and 102 from SNr (pre-placebo = 50, post-placebo = 52). This group showed no significant interaction between recording site and treatment ($F(5,492) = 3.83$, $P = 0.512$), with no differences between the neuronal firing rates of the first and second side of electrode implantation (Fig. 2C, dashed lines) in VA/VLa (first side = 25.9 ± 12.7 Hz, second side = 23.6 ± 11.9 Hz), STN (first side = 60.8 ± 15.9 Hz, second side = 61.6 ± 16.8 Hz), and SNr (first side = 71.7 ± 13.7 Hz, second side = 74.6 ± 11.4 Hz), thus indicating that the difference in neuronal discharge between the first and the second side of implantation in the placebo group was due to the placebo intervention *per se*.

The distribution of the frequencies for all neurons in the placebo and no-treatment group can be seen in Fig. 3. Whereas the histograms on the left show the pre-placebo (shaded bars and dashed line) *versus* the post-placebo (filled bars and continuous line) condition at the level of VA/VLa (A), STN (B) and SNr (C), the histograms on the right show the first recording side (shaded bars and dashed line) *versus* the second recording side (filled bars and continuous line) in the no-treatment group. The almost complete overlapping of the histograms in the no-treatment group (right) compared to the histograms in the placebo group (left) can be seen. While there was an increase in the frequencies in VA/VLa, a decrease in both STN and SNr occurred.

We also found that the number of bursting neurons in STN decreased significantly from 99 before placebo to 52 after placebo administration ($\chi^2 = 39.775$, $P < 0.001$), whereas no difference was present between the pre- and post-placebo condition in VA and VLa (19 bursting units before placebo *versus* 15 bursting units after placebo; $\chi^2 = 0.405$, $P = 0.524$). In SNr, bursting neurons were present neither before nor after placebo administration. In the no-treatment group, no difference was present in bursting neurons between the first and second recording side (13 before and 16 after placebo in VA/VLa, 110 before and 102 after placebo in STN). No bursting units in the first and second recording side were found in SNr.

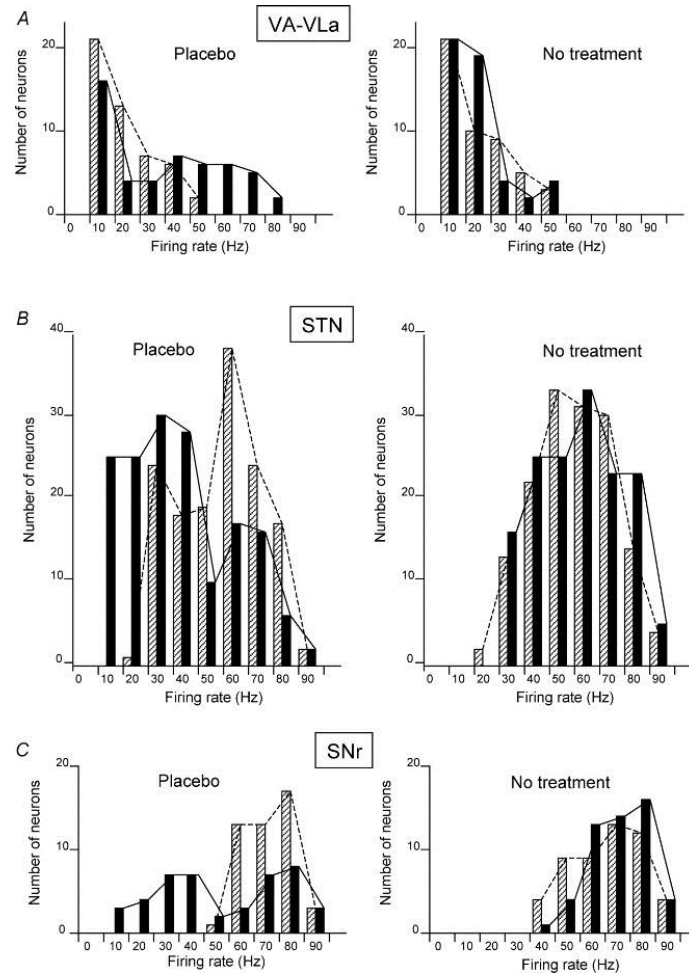


Figure 3. Distribution of the frequencies in the placebo group (left) and the no-treatment group (right) in VA/VLa (A), STN (B) and SNr (C) On the left, the shaded bars and dashed line show the pre-placebo condition whereas the black bars and the continuous line show the post-placebo condition. On the right, the shaded bars and the dashed line show the first recording side whereas the filled bars and the continuous line show the second recording side. Note the increased frequencies in VA/VLa and the decreased frequencies in STN and SNr after placebo. No changes are present in the no-treatment group.

By performing linear regression analysis between the percentage of clinical improvement after placebo and the percentage of neuronal firing rate change in VA/VLa, STN and SNr for each patient, we found that a significant correlation was present in all cases (Fig. 4), as shown by $r = -0.704$ ($t(10) = -3.136$, $P < 0.011$) for VA/VLa, $r = 0.715$ ($t(10) = 3.234$, $P < 0.009$) for STN, and $r = 0.835$ ($t(10) = 4.814$, $P < 0.001$) for SNr. Therefore, the higher the firing rate in VA and VLa, the larger the clinical placebo response, whereas the lower the firing rate in STN and SNr, the larger the clinical placebo response. In addition, the percentage of firing rate change in STN and SNr after placebo was negatively correlated with that of VA/VLa ($r = -0.904$, $t(10) = -6.690$, $P < 0.001$ and $r = -0.841$, $t(10) = -4.932$, $P < 0.001$, respectively) (Fig. 5A and C), whereas the percentage of firing rate change in STN was positively correlated with that of SNr ($r = 0.868$, $t(10) = 5.541$, $P < 0.001$) (Fig. 5B), which supports the excitatory and inhibitory connections of the neuronal circuit shown in Fig. 5D (see also Fig. 1A).

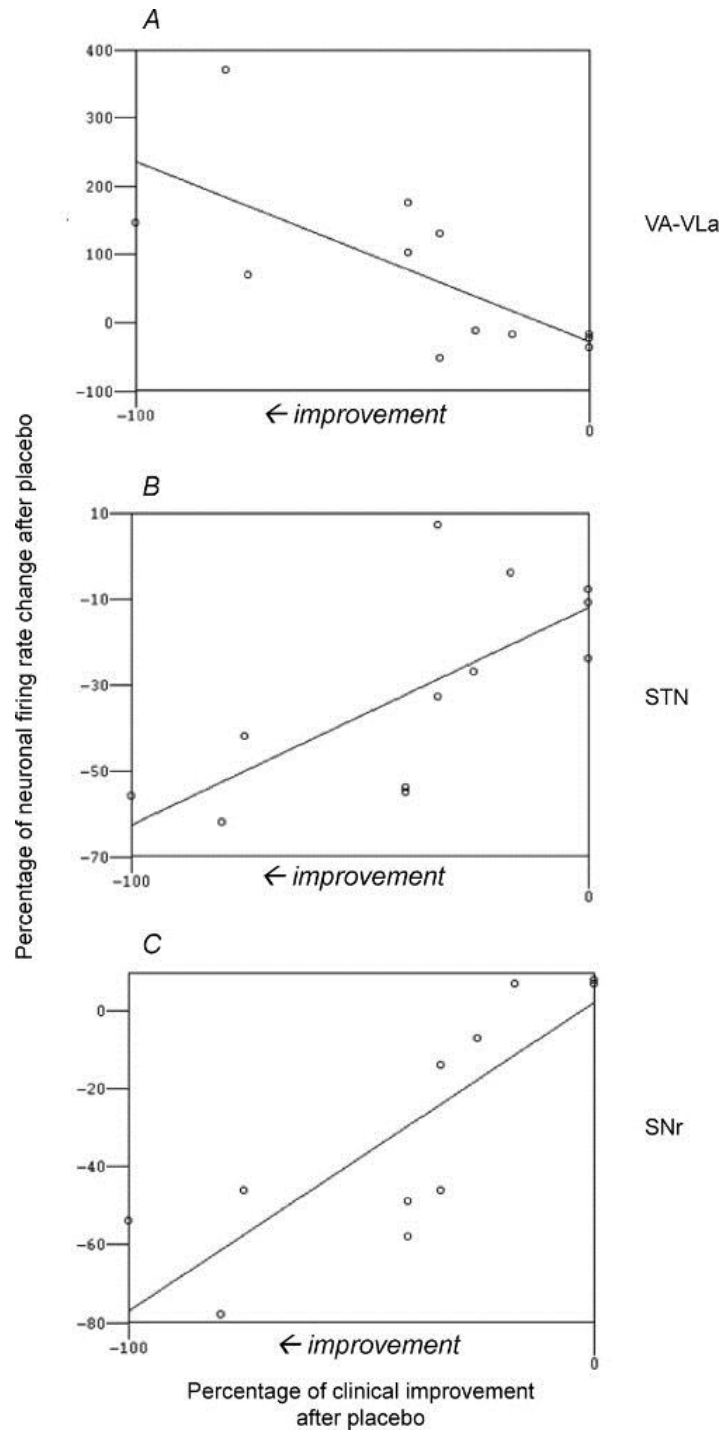


Figure 4. Correlation between percentage of clinical improvement and percentage of neuronal activity change of VA/VLa (A), STN (B) and SNr (C) In all cases there was a high correlation, according to the following rule: the larger the clinical improvement, the lower the firing rate in STN and SNr and the higher the firing rate in VA/VLa.

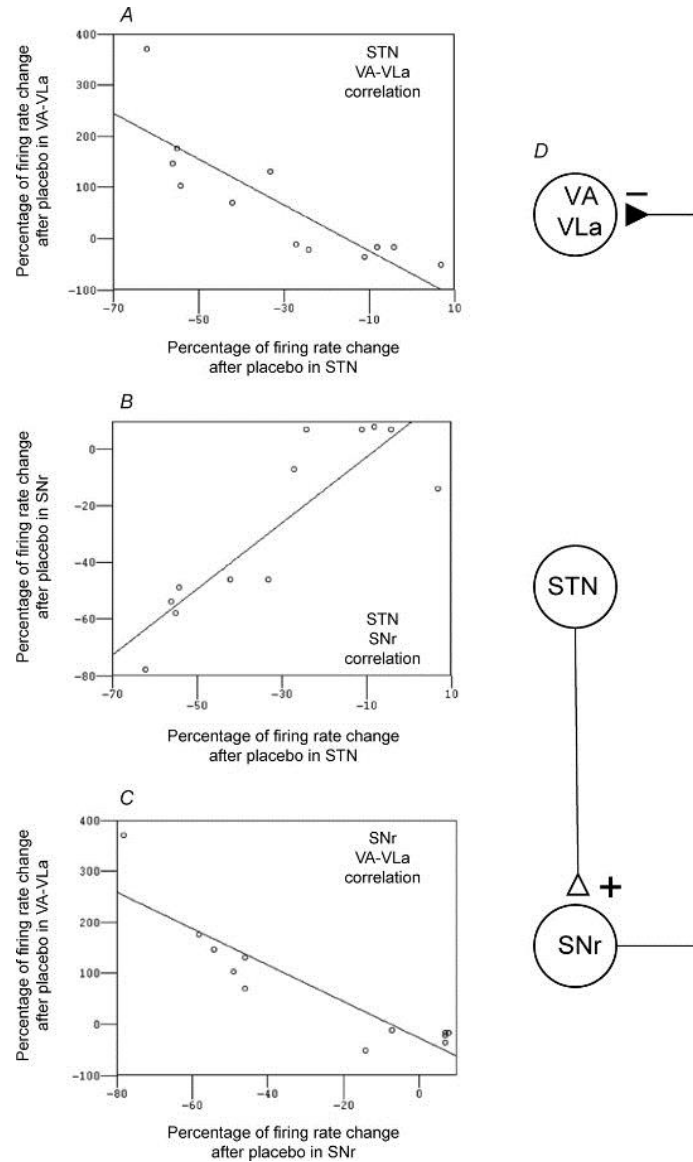


Figure 5. Correlation between the percentage of neuronal activity change of STN and that of VA/VLa (A), STN and SNr (B), SNr and VA/VLa (C) The pattern of correlation, positive in B and negative in A and C, supports the excitatory connection between STN and SNr, and the inhibitory connection between SNr and VA/VLa (D).

The data from individual subjects are shown and summarized in Fig. 6. By considering a placebo response as the decrease in muscle rigidity equal to or larger than 1 UPDRS, which represented the criterion of placebo responsiveness in our previous study (Benedetti *et al.* 2004), it can be seen that all placebo responders showed a significant deactivation (black) of STN that was invariably associated with a deactivation of SNr and activation (grey) of VA/VLa (subjects from 1 to 6 in Fig. 6). Conversely, placebo non-responders, i.e. with muscle rigidity reduction smaller than 1 UPDRS, showed no changes (white) in STN–SNr–VA/VLa circuit activity, with the exception of non-responders 8 and 10 (Fig. 6), who showed a significant STN deactivation but no changes in SNr and VA/VLa. Interestingly, the level of statistical significance in STN deactivation in non-responders 8 and 10 was much lower than that of the responders ($P < 0.03$ and $P < 0.05$, respectively), which indicates smaller STN changes after placebo. Thus,

according to both the clinical (muscle rigidity) and neurophysiological (neuron activity) data of Fig. 6, in our study there were six placebo responders and six non-responders. In the no-treatment group, significant differences were never found.

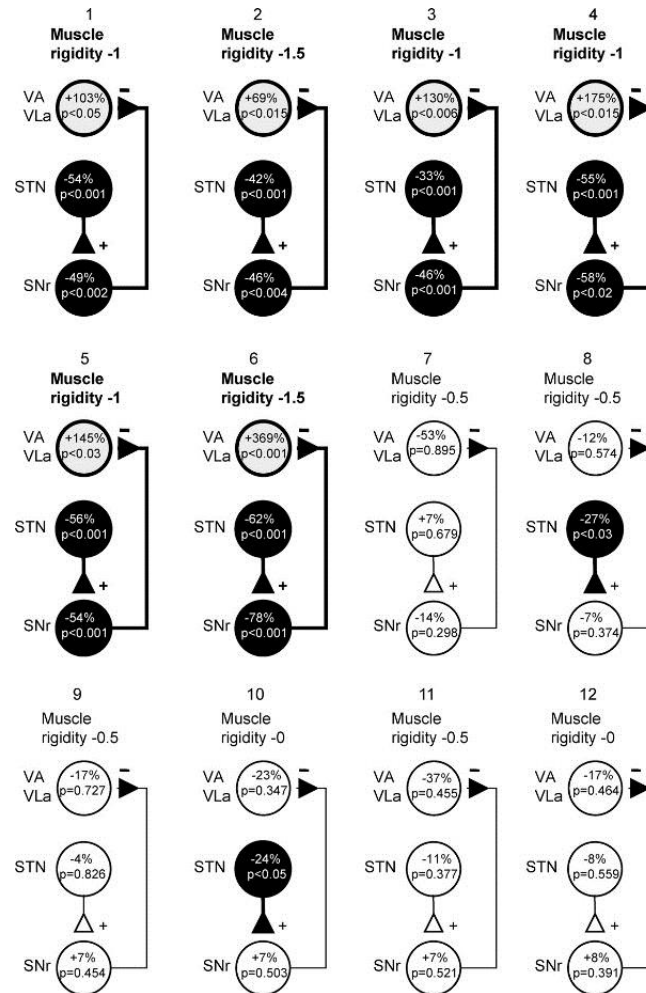


Figure 6. Deactivation (black) and activation (grey) pattern of the STN–SNr–VA/VLa circuit in placebo responders (subjects 1–6) and non-responders (subjects 7–12) The percentage decrease or increase in neuronal activity after placebo administration is shown along with statistical significance. The UPDRS decrease in muscle rigidity after placebo (clinical placebo response) is also shown. Note that STN and SNr are deactivated and VA/VLa is activated only in those subjects with a reduction in muscle rigidity equal to or larger than 1 UPDRS (responders). By contrast, no neuronal changes were present (white neurons) in those subjects with muscle rigidity reduction smaller than 1 UPDRS (non-responders). Also note that clinical non-responders 8 and 10 showed only partial changes, with a significant deactivation of STN but no changes in SNr and VA/VLa.

Discussion

In the present study, we considered only those patients where the electrode trajectory passed through the VA and VL of the thalamus, the STN and the SNr. In this way, we could investigate part of the neuronal circuit of the basal ganglia that is involved in

motor control, and whose impairment is known to induce the parkinsonian symptoms (Garcia *et al.* 2005; DeLong & Wichmann, 2007; Hammond *et al.* 2007). The neuronal circuit we recorded from has been investigated in detail both in animals and in humans (Albin *et al.* 1989; DeLong, 1990; Benazzouz *et al.* 2000; Bolam *et al.* 2000; Pollack, 2001; Maurice *et al.* 2003; Tai *et al.* 2003; Garcia *et al.* 2005; Shi *et al.* 2006; DeLong & Wichmann, 2007; Hammond *et al.* 2007; Maltete *et al.* 2007; Benarroch, 2008). It is characterized by STN, the major target for the surgical treatment of Parkinson's disease, which receives inputs from both the cortex and the GPe, and sends excitatory output pathways to both GPi and SNr (Fig. 1A). SNr and GPi are known to have connections with the thalamus (Fig. 1A), so that any modification of STN activity should be expected to affect SNr, GPi and the thalamus. Finally, the thalamus sends its projection to the motor cortex, thus its activity has an important influence on motor performance.

By considering our previous findings on the effects of a placebo treatment on the pattern of STN neuronal discharge (Benedetti *et al.* 2004), a substantial effect of placebo administration should also be expected in the STN output regions. In our previous STN recordings, we found significant neuronal changes for both firing rate and bursting activity after placebo administration. The present study shows that such STN changes affect the pattern of neuronal activity in both SNr and VA/VLa. In particular, we found a robust positive correlation between STN and SNr activity and a negative correlation between SNr and VA/VLa (Fig. 5), which suggests an excitatory and inhibitory connection, respectively. Thus, these placebo-induced neuronal changes support the model in which the thalamus receives inhibitory input from SNr, and SNr receives excitatory input from STN (Benazzouz *et al.* 2000; Maurice *et al.* 2003; Tai *et al.* 2003; Shi *et al.* 2006; Maltete *et al.* 2007).

One limitation of our study is that our recordings assess only part of the circuit that can be involved in the placebo response, for we had the possibility to record from STN, SNr and VA/VLa only. It should also be noted that anatomical studies in the monkey show that SNr projects to the magnocellular part of VA (VAmc), whereas GP projects to the parvocellular part of VA (VApc) and the densocellular part of VA (VAdc), which corresponds to VLa (Illinsky & Kultas-Illinsky, 2001). Therefore, our study cannot distinguish the thalamic neurons that receive the input from SNr from those that receive the input from GPi. In light of the projection from STN to GPi, which in turn projects to the thalamus, e.g. to VA and VLa (Magnin *et al.* 2000), there is the possibility that the increased thalamic activity was mediated by GPi and not by SNr. In other words, many thalamic neurons we recorded from were likely to be influenced by changes in GPi activity rather than SNr. However, this does not weaken the findings of our study because both SNr and GPi represent output nuclei of STN.

The possible involvement of other pathways and structures, such as GPi, is also suggested by at least two considerations. First, GPi stimulation is effective in alleviating motor symptoms, although its effects are smaller than STN stimulation (Deep Brain Stimulation Study Group, 2001), thus a change in GPi activity might also occur after placebo administration. Second, as shown in Fig. 1A, STN also projects to GPi, thus, if STN activity changes, a change in activity in both SNr and GPi should be expected. A future challenge will be to record from other regions, such as GPi, during the placebo response, so as to define the whole neuronal network involved in the anti-parkinsonian placebo response.

Another possible limitation of our study is related to the identification of the different neuronal populations. In fact, there is the possibility that some 'thalamic' neurons may be dorsal Zi neurons, and possibly some STN and SNr neurons may be incorrectly

identified, because there is overlap between distributions of STN and SNr neurons, and the border is not always clear.

Previous studies on the effects of apomorphine on basal ganglia have produced contrasting findings, with either no change in STN mean frequency discharge (Levy *et al.* 2001) or a pronounced decrease (Stefani *et al.* 2002) after the administration of apomorphine. The present study supports the idea that the relief of parkinsonian rigidity is associated with a decrease in neuronal firing rate, thereby favouring the pathophysiological model of Parkinson's disease whereby the hyperactivity of STN induces a hyperactivity in SNr which, in turn, increases its inhibition upon the thalamus (Bergman *et al.* 1994; Blandini *et al.* 2000). The decreased thalamic output to the motor cortex is believed to affect motor performance in Parkinson patients. According to this model, an anti-Parkinson treatment, such as deep brain stimulation, would restore a normal activity in STN (Limousin *et al.* 1998; Benazzouz & Hallett, 2000), and thus in SNr, with a decreased inhibition over the thalamus. The increased thalamic output would facilitate the control of movement by the motor cortex. In this regard, it is interesting that we found a correlation between the clinical improvement, as assessed by means of muscle rigidity at the wrist, and the firing rate in the circuit we analysed. In fact, muscle rigidity decreased along with the decrease of firing rate in STN and SNr and an increase in VA and VLa (Fig. 4). In addition, the data from the individual patients of Fig. 6 suggest that the involvement of the whole STN–SNr–VA/VLa circuit is a necessary condition for substantial clinical improvement. Interestingly, the significant but smaller changes in STN activity of non-responders 8 and 10 suggest that this smaller STN firing rate decrease did not produce significant effects on SNr and VA/VLa.

Although the firing rate of basal ganglia neurons seems to play a role in the motor parkinsonian symptoms, recent findings suggest that synchronized activity between different regions may be impaired in Parkinson's disease (Brown, 2003). For example, oscillations below 30 Hz have been described in experimental models of parkinsonism, such as in monkeys treated with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Nini *et al.* 1995). Likewise, intraoperative studies in Parkinson patients have shown synchronization of single neurons in both STN and GPi at 11–30 Hz (Levy *et al.* 2000, 2001, 2002). Oscillations greater than 60 Hz have also been described between STN, GPi and the cortex in Parkinson patients under treatment with levodopa (Brown *et al.* 2001; Williams *et al.* 2002). Overall, these data suggest that basal ganglia functioning is not mediated by neuronal firing rate only, but by different oscillatory activities as well (Brown, 2003).

Unfortunately, our study cannot resolve the issue of whether the firing rate model is more important than the oscillatory model, or vice versa, in the anti-parkinsonian placebo response, and this may represent a future challenge. Nor can it assess whether the neuronal changes we observed were the cause of the clinical improvement or, rather, they were merely associated with the improvement. Nonetheless, it is tempting to speculate that the placebo-induced release of dopamine in the striatum of Parkinson patients may be the cause of the changes we observed in STN, SNr and VA/VLa. In other words, the changes in firing rate in our study may be attributed to a downstream effect of placebo-induced dopamine release in the striatum (de la Fuente-Fernandez *et al.* 2001). In fact, the striatum projects to GPe which, in turn, projects to STN (Fig. 1A). This mechanism is not conclusive, however, as the placebo-induced dopamine release in the striatum and neuronal changes in STN were obtained in different studies (de la Fuente-Fernandez *et al.* 2001; Benedetti *et al.* 2004).

Besides the changes in firing rate in STN, we also found changes in bursting activity, whereby a placebo treatment turned a bursting pattern into a non-bursting activity, as

previously shown (Benedetti *et al.* 2004). We did not find similar changes in bursting activity in the thalamus. In fact, the number of bursting neurons before and after placebo administration were not different in VA and VL_a. Therefore, non-bursting activity seems to be more important for clinical improvement in STN than in VA and VL_a. We never found bursting neurons in SN_r, either before or after placebo.

It is worth noting that all these neuronal changes were observed after a preoperative pharmacological conditioning with apomorphine. Pharmacological and non-pharmacological conditioning is known to enhance placebo responsiveness in a number of experimental models, such as pain, immune responses and hormone secretion (Benedetti *et al.* 2003; Colloca & Benedetti, 2006; Pacheco-Lopez *et al.* 2006). In addition, robust placebo responses have been found after pharmacological conditioning in Parkinson's disease as well (Benedetti *et al.* 2004). In the present study, we performed preoperative apomorphine conditioning in order to increase placebo responsiveness. Therefore, we do not know whether the same changes would have been present without such pharmacological pre-conditioning, for example after verbal suggestions of improvement alone. Further studies are needed to answer this important question and to assess the role of learning in these effects.

It should also be pointed out that the assessment of the placebo response after 30–45 min showed a short-lasting effect. By considering the data in Fig. 2, it appears clear that the placebo effect lasted no longer than 45 min. Our experimental design does not allow us to precisely assess how long the placebo response lasted. This is mainly due to ethical constraints which limit our measurements intraoperatively. Within the context of learning mechanisms, it will be interesting to investigate whether the duration of the response can be increased by means of conditioning procedures.

Our study shows that a placebo treatment, which is mainly characterized by verbal suggestions of clinical benefit, be it a learning phenomenon or not, is capable of reversing, albeit for a short time, the malfunction of a complex neuronal circuit. This may have profound implications for both pharmacotherapy and psychotherapy. In the first case, the replacement of drugs with placebos can be used in therapeutic protocols aimed at reducing drug intake. In the second case, the enhancement of expectations through verbal suggestions may indeed induce specific changes in the brain, thus placing psychotherapy into a therapeutic context which *per se* is capable of modifying the patient's brain.

Author contributions

F.B. conceived, designed and performed the experiments, analysed the data, and wrote the paper. M.L., A.D. and L.L. conceived, designed and performed the experiments, and contributed to the final version of the paper. L.C. and M.Z. designed and performed the experiments, analysed the data, and revised the paper. The experiments were done in the Department of Neuroscience of the University of Turin Medical School.

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References

- Albin RL, Young AB & Penney JB (1989). The functional anatomy of basal ganglia disorders. *Trends Neurosci* **12**, 366–375.
- Amanzio M & Benedetti F (1999). Neuropharmacological dissection of placebo analgesia: expectation activated opioid systems *versus* conditioning-activated specific subsystems. *J Neurosci* **19**, 484–494.
- Benarroch EE (2008). Subthalamic nucleus and its connections: Anatomic substrate for the network effects of deep brain stimulation. *Neurology* **70**, 1991–1995.
- Benazzouz A, Gao DM, Ni ZG, Piallat B, Bouali-Benazzouz R & Benabid AL (2000). Effect of high-frequency stimulation of the subthalamic nucleus on the neuronal activities of the substantia nigra pars reticulata and ventrolateral nucleus of the thalamus in the rat. *Neuroscience* **99**, 289–295.
- Benazzouz A & Hallett M (2000). Mechanism of action of deep brain stimulation. *Neurology* **55**(Suppl. 6), S13–S17.
- Benedetti F (2008a). Mechanisms of placebo and placebo-related effects across diseases and treatments. *Annu Rev Pharmacol Toxicol* **48**, 33–60.
- Benedetti F (2008b). *Placebo Effects: Understanding the Mechanisms in Health and Disease*. Oxford University Press, Oxford.
- Benedetti F, Colloca L, Torre E, Lanotte M, Melcarne A, Pesare M, Bergamasco B & Lopiano L (2004). Placebo-responsive Parkinson patients show decreased activity in single neurons of subthalamic nucleus. *Nat Neurosci* **7**, 587–588.
- Benedetti F, Mayberg HS, Wager TD, Stohler CS & Zubieta JK (2005). Neurobiological mechanisms of the placebo effect. *J Neurosci* **25**, 10390–10402.
- Benedetti F, Pollo A, Lopiano L, Lanotte M, Vighetti S & Rainero I (2003). Conscious expectation and unconscious conditioning in analgesic, motor, and hormonal placebo/nocebo responses. *J Neurosci* **23**, 4315–4323.
- Bergman H, Wichmann T, Karmon B & DeLong MR (1994). The primate subthalamic nucleus. II. Neuronal activity in the MPTP model of parkinsonism. *J Neurophysiol* **72**, 507–520.
- Blandini F, Nappi G, Tassorelli C & Martignoni E (2000). Functional changes of the basal ganglia circuitry in Parkinson's disease. *Prog Neurobiol* **62**, 63–88.
- Bolam JP, Hanley JJ, Booth PAC & Bevan MD (2000). Synaptic organisation of the basal ganglia. *J Anat* **196**, 527–542.
- Brown P (2003). Oscillatory nature of human basal ganglia activity: relationship to the pathophysiology of Parkinson's disease. *Mov Disord* **18**, 357–363.
- Brown P, Oliviero A, Mazzone P, Insola A, Tonali P, Di Lazzaro V (2001). Dopamine dependency of oscillations between subthalamic nucleus and pallidum in Parkinson's disease. *J Neurosci* **21**, 1033–1038.
- Colloca L & Benedetti F (2005). Placebos and painkillers: is mind as real as matter? *Nat Rev Neurosci* **6**, 545–552.
- Colloca L & Benedetti F (2006). How prior experience shapes placebo analgesia. *Pain* **124**, 126–133.
- Deep Brain Stimulation for Parkinson's Disease Study Group (2001). Deep-brain stimulation of the subthalamic nucleus or the pars interna of the globus pallidus in Parkinson's disease. *New Engl J Med* **345**, 956–963.
- de la Fuente-Fernandez R, Phillips AG, Zamburlini M, Sossi V, Calne DB, Ruth TJ & Stoessl AJ (2002). Dopamine release in human ventral striatum and expectation of reward. *Behav Brain Res* **136**, 359–363.

- de la Fuente-Fernandez R, Ruth TJ, Sossi V, Schulzer M, Calne DB & Stoessl AJ (2001). Expectation and dopamine release: mechanism of the placebo effect in Parkinson's disease. *Science* **293**, 1164–1166.
- DeLong MR (1990). Primate models of movement disorders of basal ganglia origin. *Trends Neurosci* **13**, 281–285.
- DeLong MR & Wichmann T (2007). Circuits and circuit disorders of the basal ganglia. *Arch Neurol* **64**, 20–24.
- Enck P, Benedetti F & Schedlowski M (2008). New insights into the placebo and nocebo responses. *Neuron* **59**, 195–206.
- Fahn S, Elton RL, & the Members of the UPDRS Development Committee (1987). Unified Parkinson's disease rating scale. In *Recent Developments in Parkinson's Disease*, ed. Fahn S, Marsden CD & Calne DB, pp. 153–163. MacMillan, London.
- Francois C, Tande D, Yelnik J & Hirsch EC (2002). Distribution and morphology of nigral axons projecting to the thalamus in primates. *J Comp Neurol* **447**, 249–260.
- Garcia L, D'Alessandro G, Bioulac B & Hammond C (2005). High-frequency stimulation in Parkinson's disease: more or less? *Trends Neurosci* **28**, 209–216.
- Goetz CG, Laska E, Hickey C, Damier P, Müller T, Nutt J, Warren Olanow C, Rascol O & Russ H (2008). Placebo influences on dyskinesia in Parkinson's disease. *Mov Disord* **23**, 700–707.
- Goetz CG, Leurgans S & Raman R (2002). Placebo-associated improvements in motor function: comparison of subjective and objective sections of the UPDRS in early Parkinson's disease. *Mov Disord* **17**, 283–288.
- Goetz CG, Leurgans S, Raman R & Stebbins GT (2000). Objective changes in motor function during placebo treatment in PD. *Neurology* **54**, 710–714.
- Hammond C, Bergman H & Brown P (2007). Pathological synchronization in Parkinson's disease: networks, models and treatments. *Trends Neurosci* **30**, 357–364.
- Hoehn MW & Yahr MD (1967). Parkinsonism: onset, progression, and mortality. *Neurology* **17**, 427–442.
- Illinsky IA & Kultas-Illinsky K (2001). Neuroanatomical organization and connections of the motor thalamus in primates. In *Basal Ganglia and Thalamus in Health and Movement Disorders*, ed. Kultas-Illinsky K & Illinsky IA, pp. 267–274. Kluwer Academic/Plenum Publishers, Norwell, MA, USA.
- Lanotte M, Lopiano L, Torre E, Bergamasco B, Colloca L & Benedetti F (2005). Expectation enhances autonomic responses to stimulation of the human subthalamic limbic region. *Brain Behav Immun* **19**, 500–509.
- Levine JD, Gordon NC & Fields HL (1978). The mechanism of placebo analgesia. *Lancet* **2**, 654–657.
- Levy R, Dostrovsky JO, Lang AE, Sime E, Hutchinson WD & Lozano AM (2001). Effects of apomorphine on subthalamic nucleus and globus pallidus internus neurons in patients with Parkinson's disease. *J Neurophysiol* **86**, 249–260.
- Levy R, Hutchinson WD, Lozano AM & Dostrovsky JO (2000). High-frequency synchronization of neuronal activity in the subthalamic nucleus of parkinsonian patients. *J Neurosci* **20**, 7766–7775.
- Levy R, Hutchinson WD, Lozano AM & Dostrovsky JO (2002). Synchronised neuronal discharge in the basal ganglia of parkinsonian patients is limited to oscillatory activity. *J Neurosci* **22**, 2855–2861.
- Limousin P, Krack P, Pollak P, Benazzouz A, Ardouin C, Hoffmann D & Benabid AL (1998). Electrical stimulation of the subthalamic nucleus in advanced Parkinson's disease. *N Engl J Med* **339**, 1105–1111.
- Magnin M, Morel A & Jeanmonod D (2000). Single-unit analysis of the pallidum, thalamus and subthalamic nucleus in parkinsonian patients. *Neuroscience* **96**, 549–564.

- Maltete D, Jodoin N, Karachi C, Houeto JL, Navarro S, Cornu P, Agid Y & Welter ML (2007). Subthalamic stimulation and neuronal activity in the substantia nigra in Parkinson's disease. *J Neurophysiol* **97**, 4017–4022.
- Maurice N, Thierry AM, Glowinski J & Deniau JM (2003). Spontaneous and evoked activity of substantia nigra pars reticulata neurons during high-frequency stimulation of the subthalamic nucleus. *J Neurosci* **23**, 9929–9936.
- Mercado R, Constantoyannis C, Mandat T, Kumar A, Schulzer M, Stoessl AJ & Honey CR (2006). Expectation and the placebo effect in Parkinson's disease patients with subthalamic nucleus deep brain stimulation. *Mov Disord* **21**, 1457–1461.
- Nini A, Feingold A, Sloviter H & Bergman H (1995). Neurons in the globus pallidus do not show correlated activity in the normal monkey, but phase-locked oscillations appear in the MPTP model of parkinsonism. *J Neurophysiol* **74**, 1800–1805.
- Pacheco-Lopez G, Engler H, Niemi MB & Schedlowski M (2006). Expectations and associations that heal: Immunomodulatory placebo effects and its neurobiology. *Brain Behav Immun* **20**, 430–446.
- Petrovic P, Kalso E, Petersson KM & Ingvar M (2002). Placebo and opioid analgesia – imaging a shared neuronal network. *Science* **295**, 1737–1740.
- Pollack AE (2001). Anatomy, physiology, and pharmacology of the basal ganglia. *Neurol Clin* **19**, 523–534.
- Pollo A, Torre E, Lopiano L, Rizzone M, Lanotte M, Cavanna A, Bergamasco B & Benedetti F (2002). Expectation modulates the response to subthalamic nucleus stimulation in Parkinsonian patients. *Neuroreport* **13**, 1383–1386.
- Price DD, Craggs J, Verne GN, Perlstein WM & Robinson ME (2007). Placebo analgesia is accompanied by large reductions in pain-related brain activity in irritable bowel syndrome patients. *Pain* **127**, 63–72.
- Price DD, Finniss DG & Benedetti F (2008). A comprehensive review of the placebo effect: Recent advances and current thought. *Annu Rev Psychol* **59**, 565–590.
- Schaltenbrand G & Wahren W (1977). *Atlas for Stereotaxy of the Human Brain*. Thieme, New York.
- Scott DJ, Stohler CS, Egnatuk CM, Wang H, Koeppe RA & Zubieta JK (2007). Individual differences in reward responding explain placebo-induced expectations and effects. *Neuron* **55**, 325–336.
- Scott DJ, Stohler CS, Egnatuk CM, Wang H, Koeppe RA & Zubieta JK (2008). Placebo and nocebo effects are defined by opposite opioid and dopaminergic responses. *Arch Gen Psychiat* **65**, 220–231.
- Shetty N, Friedman JH, Kieburtz K, Marshall FJ & Oakes D (1999). The placebo response in Parkinson's disease. Parkinson Study Group. *Clin Neuropharmacol* **22**, 207–212.
- Shi LH, Luo F, Woodward D & Chang JY (2006). Basal ganglia neural responses during behaviorally effective deep brain stimulation of the subthalamic nucleus in rats performing a treadmill locomotion test. *Synapse* **59**, 445–457.
- Stefani A, Bassi A, Mazzone P, Pierantozzi M, Gattoni G, Altibrandi MG, Giacomini P, Peppe A, Bernardi G & Stanzione P (2002). Subdyskinetic apomorphine responses in globus pallidus and subthalamus of parkinsonian patients: lack of clear evidence for the 'indirect pathway'. *Clin Neurophysiol* **113**, 91–100.
- Strafella AP, Ko JH & Monchi O (2006). Therapeutic application of transcranial magnetic stimulation in Parkinson's disease: the contribution of expectation. *Neuroimage* **31**, 1666–1672.
- Tai CH, Boraud T, Bezard E, Bioulac B, Gross C & Benazzouz A (2003). Electrophysiological and metabolic evidence that high-frequency stimulation of the subthalamic nucleus tames neuronal activity in the subthalamic nucleus and the substantia nigra reticulata. *FASEB J* **17**, 1820–1830.

- Wager TD, Rilling JK, Smith EE, Sokolik A, Casey KL, Davidson RJ, Kosslyn SM, Rose RM & Cohen JD (2004). Placebo-induced changes in fMRI in the anticipation and experience of pain. *Science* **303**, 1162-1167.
- Wager TD, Scott DJ & Zubieta JK (2007). Placebo effects on human μ -opioid activity during pain. *Proc Natl Acad Sci U S A* **104**, 11056-11061.
- Williams D, Tijssen M, Van Bruggen G, Bosch A, Insola A, Di Lazzaro V, Mazzone P, Oliviero A, Quartarone A, Speelman H & Brown P (2002). Dopamine dependent changes in the functional connectivity between basal ganglia and cerebral cortex in the human. *Brain* **125**, 1558-1569.
- Zubieta JK, Bueller JA, Jackson LR, Scott DJ, Xu Y, Koeppe RA, Nichols TE & Stohler CS (2005). Placebo effects mediated by endogenous opioid activity on mu-opioid receptors. *J Neurosci* **25**, 7754-7762.