JPn Neuroscience
The Journal of Physiology
J Physiol 594.19 (2016) pp 5647–5660

Teaching neurons to respond to placebos

Fabrizio Benedetti[1,2], Elisa Frisaldi[1], Elisa Carlino[1], Lucia Giudetti[3], Alan Pampallona[3], Maurizio Zibetti[1], Michele Lanotte[1] and Leonardo Lopiano[1]

[1] *University of Turin Medical School, Neuroscience Department, Turin, Italy*
[2] *Plateau Rosa Labs, Breuil-Cervinia, Italy, Zermatt, Switzerland*
[3] *Giancarlo Quarta Foundation, Milan, Italy*

Key points

- We analysed the placebo response at the single-neuron level in the thalamus of Parkinson patients to see the differences between first-time administration of placebo and administration after pharmacological pre-conditioning.
- When the placebo was given for the first time, it induced neither clinical improvement, as assessed through muscle rigidity reduction at the wrist, nor neuronal changes in thalamic neurons.
- However, if placebo was given after two, three or four prior administrations of an anti-Parkinson drug, apomorphine, it produced both clinical and neuronal responses.
- Both the magnitude and the duration of these placebo responses depended on the number of prior exposures to apomorphine, according to the rule: the greater the number of previous apomorphine administrations, the larger the magnitude and the longer the duration of the clinical and neuronal placebo responses.
- These findings show that learning plays a crucial role in the placebo response and suggest that placebo non-responders can be turned into placebo responders, with important clinical implications.

Abstract Placebos have been found to affect the patient's brain in several conditions, such as pain and motor disorders. For example, in Parkinson's disease, a placebo treatment induces a release of dopamine in the striatum and changes the activity of neurons in both thalamic and subthalamic nuclei. The present study shows that placebo administration for the first time induces neither clinical nor neuronal improvement in Parkinson patients who undergo implantation of electrodes for deep brain stimulation. However, this lack of placebo responsiveness can be turned into substantial placebo responses following previous exposure to repeated administrations of the anti-Parkinson agent apomorphine. As the number of apomorphine administrations increased from one to four, both the clinical response and the neuronal activity in the ventral anterior and anterior ventrolateral thalamus increased. In fact, after four apomorphine exposures, placebo administration induced clinical responses that were as large as those to apomorphine, along with long-lasting neuronal changes. These clinical placebo responses following four apomorphine administrations were again elicited after a re-exposure to a placebo 24 h after surgery, but not after 48 h. These data indicate that learning plays a crucial role in placebo responsiveness and suggest that placebo non-responders can be turned into responders, with important implications in the clinical setting.

(Received 20 July 2015; accepted after revision 14 January 2016; first published online 10 February 2016)
Corresponding author F. Benedetti: Department of Neuroscience, University of Turin Medical School, Corso Raffaello 30, 10125 Turin, Italy. Email: fabrizio.benedetti@unito.it

DOI: 10.1113/JP271322

Abbreviations GPi, internal globus pallidus; SNr, substantia nigra pars reticulata; STN, subthalamic nucleus; UPDRS, Unified Parkinson's Disease Rating Scale; VA, ventral anterior thalamus; VLa, anterior ventrolateral thalamus; Zi, zona incerta.

Introduction

A placebo can be defined as the administration of an inert treatment along with a positive psychosocial context inducing positive expectations of clinical improvement. Thus, the crucial element of a placebo is the psychosocial context around the patient and the therapy (Benedetti, 2013, 2014). Although various mechanisms have been identified across different medical conditions, there is today compelling evidence that learning plays an important role across all conditions, such as pain (Amanzio & Benedetti, 1999; Colloca & Benedetti, 2006; Guo *et al.* 2010; Benedetti *et al.* 2011) and the immune (Goebel *et al.* 2002; Pacheco-Lopez *et al.* 2006) and endocrine systems (Stockhorst *et al.* 2000; Benedetti *et al.* 2003). For example, previous exposure to pharmacological agents and subsequent replacement of the drug with a placebo leads to substantial placebo responses, whereby the placebo is capable of mimicking the pharmacological action of the previously administered drug. Several important translational implications emerge from these findings, such as the occurrence of learning in the clinical trial setting as well as the possibility to exploit the drug-mimicking action of placebos in routine clinical practice (Benedetti, 2014).

In recent years, Parkinson's disease has emerged as an interesting model to study these placebo mechanisms, for at least three reasons. First, the placebo responses in Parkinson's disease have been widely observed and are robust and substantial (Shetty *et al.* 1999; Goetz *et al.*, 2000, 2002, 2008*a*,*b*; McRae *et al.* 2004; Mercado *et al.* 2006; Diederich & Goetz, 2008; Keitel *et al.* 2013). Second, a placebo treatment induces the release of dopamine in the striatum, and this release can be quantified (de la Fuente-Fernandez *et al.* 2001, 2002; Strafella *et al.* 2006; Lidstone *et al.* 2010). Third, the recording from single neurons during implantation of electrodes for deep brain stimulation allows us to investigate placebo responses at the level of single neurons (Benedetti *et al.* 2004, 2009; Frisaldi *et al.* 2014).

In the present study we combined these two elements, i.e. learning on the one hand and single-neuron recording in Parkinson's disease on the other, to see whether neurons can learn to respond to placebos. To do this, we compared the effects of first-time placebo administration to placebo administration after several previous exposures to the anti-Parkinson agent apomorphine. A crucial point of this approach is the possibility to investigate if placebo non-responders can be turned into responders and to analyse the correlation between the clinical and the neuronal responses.

Methods

Ethical approval

The study followed the standards set by the latest revision of the *Declaration of Helsinki*. Written informed consent was obtained by all patients after approval by the Ethics Committee of the University of Turin Medical School and Medical Centre.

Subjects

A total of 42 patients participated in the study. They were told that they would participate in a study aimed at better understanding the mechanisms of deep brain stimulation, including the influence of some psychological factors. They were informed that apomorphine would be administered pre-operatively and that the same medication would be given in the operating room. The patients were also informed that a placebo could be given during the whole procedure, yet they did not know when. All patients suffered from idiopathic Parkinson's disease, and this was assessed through a modified Unified Parkinson's Disease Rating Scale (UPDRS) (Fahn *et al.* 1987), whereby rigidity scores in steps of 0.5 were adopted. The patients' characteristics, along with the UPDRS scores in the medication-off state, the duration of the disease, drug therapy before surgery and levodopa equivalent daily dose (LEDD) are shown in Table 1. All the patients suffered from rigidity. On the day before surgery all pharmacological treatments were stopped and any medication was interrupted until termination of the study. Note that atypical neuroleptics, such as clozapine and quetiapine, were used in some patients to control either mild psychosis or dyskinesias. The patients were randomly subdivided into six groups (see below).

Surgical implantation of electrodes

The neuroanatomical localization of the subthalamic nucleus (STN) by means of magnetic resonance imaging (MRI) is described in detail in our previous studies (e.g. Benedetti *et al.* 2009). Briefly, we assessed the anterior and posterior commisurae coordinates and the length of the intercommissural line, and the STN was localized 2.5 mm posterior and 4 mm inferior with respect to the mid-commissural point and 12 mm from the midline. After local anaesthesia, a 14 mm pre-coronal burr hole was made and the electrode waslowered into the brain with a 58–63 deg anterior–posterior angle and 14–20 deg lateral angle (Fig. 1).

Table 1. Patient characteristics

Patient	Age (years)	Sex	Duration of Parkinson's disease (years)	UPDRS before surgery (medication off)	Therapy before surgery*	Levodopa equivalent daily dose (mg)
No treatment						
1	61	M	11	63	s, ro, ap, q	408
2	63	F	15	54.5	m, ro, v	456
3	54	F	18	50.5	m, pr, ca, d, ci, re, ama	670
4	70	F	20	65	s, pr, q	485
5	71	M	21	61.5	m, s, ca, am, ap, ama	646
6	52	M	18	44.5	s, pe, ap	550
7	63	F	14	59	m, cl, b	536
Placebo 0						
1	62	F	12	60.5	m, ro, am, ama	468
2	68	M	12	55	s, ro, ap, q	505
3	70	M	17	47.5	m, pe, b, am, ama	605
4	51	M	20	59	m, ro, v	480
5	59	F	19	67.5	m, ro, am	490
6	55	F	16	44	m, pe, ama	540
7	68	F	20	70	m, s, ca, am, ap, ama	710
Placebo 1						
1	66	M	19	48.5	s, pr, q	460
2	60	M	23	59.5	m, s, pe	543
3	70	F	17	66	m, cl, b	578
4	56	F	13	57	m, ca, d, ci, re	420
5	53	F	22	66	m, pr, ca, ci, re, ama	579
6	72	M	16	71.5	m, s, ca, am, ap, ama	615
7	74	M	15	58	m, ro, am	558
Placebo 2						
1	54	F	24	70	s, pr, q	521
2	61	M	18	40	m, ca, am, ap, ama	560
3	54	F	11	56.5	m, pr, ca, d, ci, ama	495
4	69	F	15	67.5	s, ro, ap, q	568
5	67	F	16	60.5	m, ro, am, ama	643
6	72	M	20	61	m, s, ca, am, ap, ama	788
7	67	M	20	48	m, cl, b	458
Placebo 3						
1	58	M	14	58	m, ro, am	651
2	75	F	13	49	m, ro, am, ama	700
3	70	M	15	45	m, pr, ca, ci, re	608
4	54	M	20	64.5	s, ro, ap, q	555
5	65	F	20	61.5	m, pr, ca, ci, re, ama	786
6	69	M	14	53	m, ca, d, ci, re	540
7	60	F	17	67.5	s, pr, q	510
Placebo 4						
1	66	F	16	43.5	m, pr, ca, d, ci, re, ama	808
2	60	F	14	70.5	s, pr, q	596
3	51	M	23	57	m, s, ca, am, ap, ama	765
4	59	M	20	61	m, cl, b	518
5	73	F	11	69.5	m, ro, am	537
6	55	F	18	50.5	s, ro, ap, q	560
7	70	M	12	49	m, ro, am, ama	515

*m, madopar; s, sinemet; ap, apomorphine; ca, cabergoline; am, amitriptiline; ama, amantadine; cl, clozapine; b, bromazepam; ro, ropinirol; v, venlafaxine; q, quetiapine; pr, pramipexol; d, diazepam; ci, citalopram; re, reboxetine; pe, pergolide.

Electrophysiological microrecording was performed starting from 10 mm above the anatomical target (Fig. 1) by means of microtargeting electrodes (Type BP, FHC, Bowdoinham, ME, USA) and a Neurotrek system (NeuroTrek, Alpha Omega, Nazareth, Israel). First, we encountered thalamic neurons in the ventral anterior thalamus (VA) and anterior ventrolateral thalamus (VLa) nuclei, which were identified because of a low background activity just below them, which corresponds to the zona incerta (Zi) (Fig. 1). Then, STN neurons were identified by a sustained and irregular firing pattern at a frequency ranging from about 25 to 45 Hz (Hutchison *et al.* 1998). Some STN neurons responded to contralateral proprioceptive stimuli and some neurons were related to tremor (4–6 Hz). Microstimulation (stimulus width: 60 μs; frequency: 130 Hz; intensity: 1–5 V) gave us further confirmation of good positioning of the electrode in the STN. In fact, microstimulation induced reduction of rigidity and/or disappearance of tremor in most of the cases. Only in some cases were side effects, such as dyskinesias, muscle contractions and tingling sensations, induced.

The microstimulation site with the best therapeutic effect represented the reference for the anatomical location of the different recorded units, and this was done by projecting the different recorded neurons on the atlas of Schaltenbrand & Wahren (1977). This procedure has been successfully adopted in our previous studies (e.g. Lanotte *et al.* 2005; Benedetti *et al.* 2009). A neuron was classified as thalamic only if it was located at a minimum of 2 mm above the superior border of STN. The typical firing pattern of STN neurons helped us to identify the superior border of STN (see above). We found a striking correlation between the electrophysiological criteria and the anatomical location.

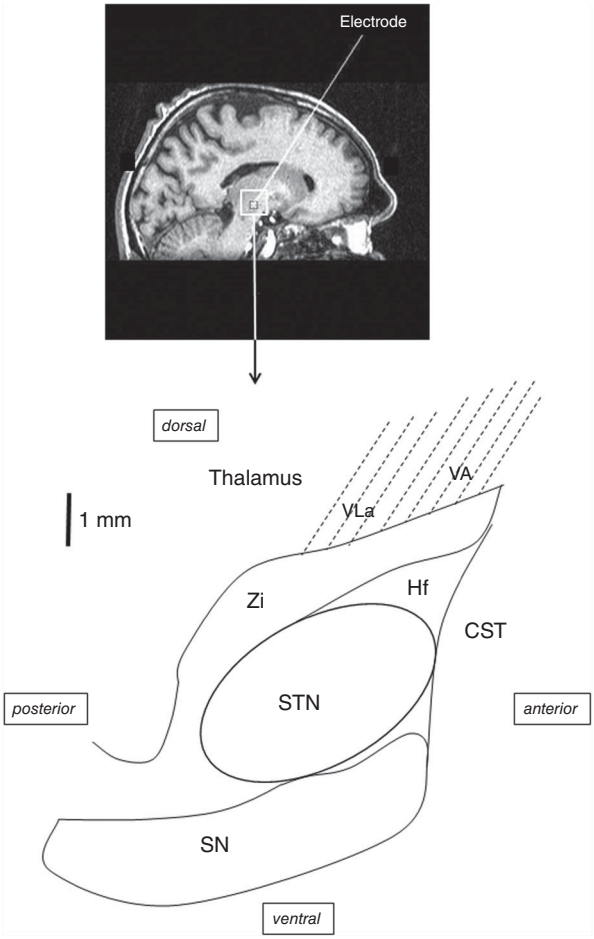

Figure 1. Recording from thalamic neurons
The broken lines show the electrode tracks and the recording area in the ventral anterior (VA) and anterior ventrolateral (VLa) thalamus. This recording region was easily identified electrophysiologically, because just above is a silent area corresponding to the zona incerta (Zi). STN = subthalamic nucleus, Hf = Forel's field, CST = corticospinal tract, SN = substantia nigra.

Procedure

The patients were randomly subdivided into six groups, of seven patients each (Fig. 2). The first group did not receive any treatment, and thus it represents the no-treatment (natural history) group, whereas the second group received a subcutaneous placebo treatment intraoperatively, along with suggestions of motor improvement: in this second group no previous pharmacological pre-conditioning was performed (Placebo 0 group). The other four groups received an intraoperative subcutaneous placebo after either one injection of the anti-Parkinson agent, apomorphine, 1 day before surgery (Placebo 1 group), two injections of apomorphine for 2 days before surgery (Placebo 2 group), three injections for 3 days before surgery (Placebo 3 group) or four injections for 4 days before surgery (Placebo 4 group) (Fig. 2). This was done by giving a 2 mg dose of apomorphine subcutaneously (the usual dose in our routine clinical practice) to the patients in the medication-off state, along with domperidone to prevent nausea and vomiting. A neurologist assessed symptom improvement by using the UPDRS scores, particularly muscle rigidity at the arm. Those patients who developed dyskinesias after apomorphine injection were omitted from the study to avoid possible dyskinetic effects intraoperatively after placebo. To allow the number of patients in the groups to be equal, when a patient was omitted because of dyskinesia, he/she was replaced with another patient. Overall, six patients showed dyskinetic responses to apomoprhine.

During surgery, when the first electrode was implanted, we recorded neuronal activity from VA and VLa, along with rigidity of both arms. Assessment was limited to arm rigidity because (1) tremor is subject to fluctuations during surgery and is not present in all patients, (2) bradykinesia shows a later onset compared with rigidity and requires a

more complex measurement and (3) complete assessment of all symptoms would prolong the discomfort of the patient. Therefore, only arm rigidity is shown in the figures. This is assessed on a scale ranging from 0 = no rigidity to 3 = severe rigidity.

After implantation of the first electrode, we implanted the second electrode. Left and right implantation was randomized across the patients, and the interval between first and second implantation was about 60 min in all patients. After recording from VA and VLa neurons for about 15 min and after contralateral arm rigidity assessment, a subcutaneous injection of saline solution (placebo) was administered along with the suggestion that it was the same drug of the previous days (groups

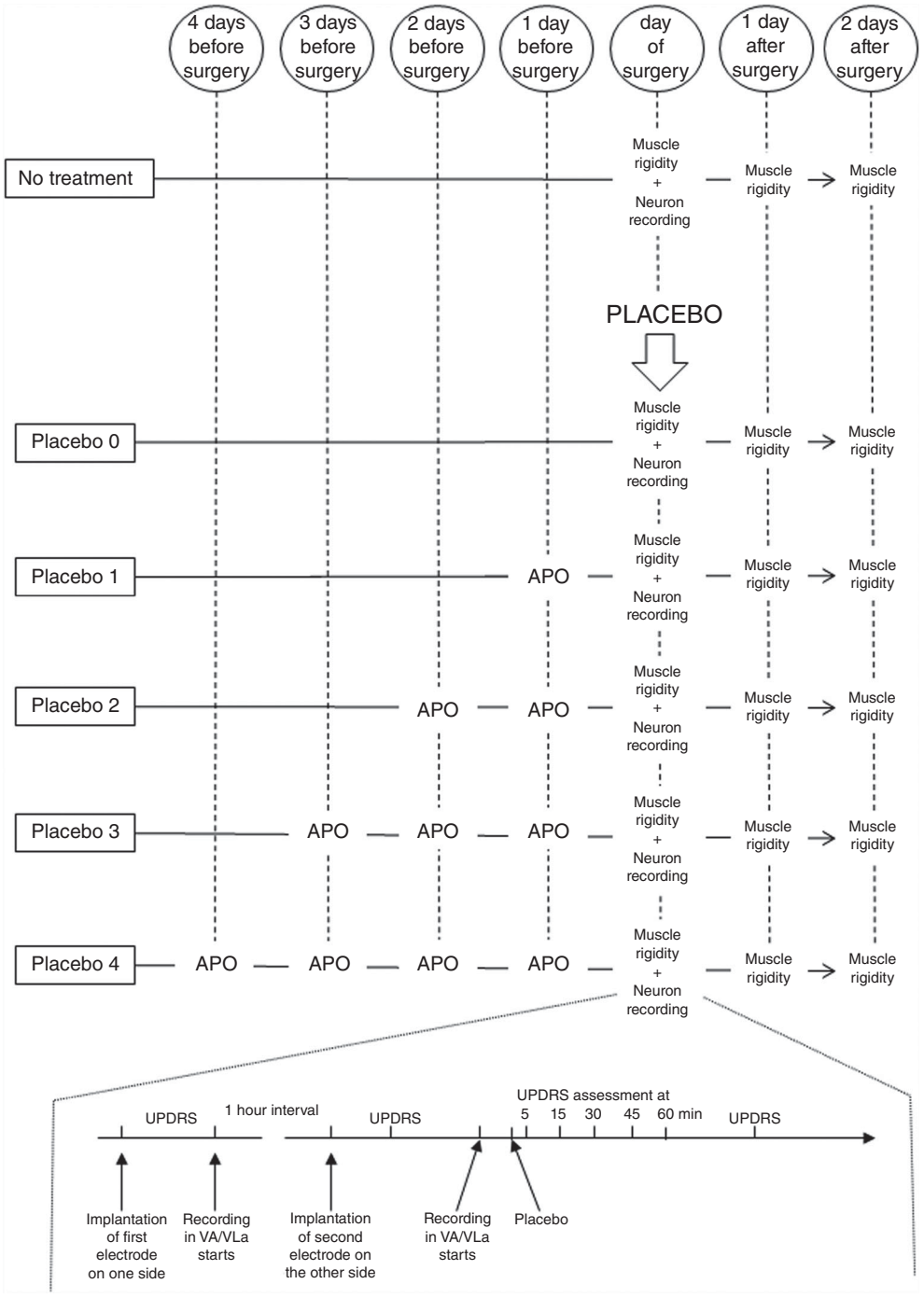

Figure 2. Experimental design
The treatment before, during and after surgery is shown for all six groups. The detailed experimental design on the day of surgery during the implantation of the electrodes is described at the bottom. APO = apomorphine.

Placebo 1, 2, 3 and 4). Therefore, the patients expected a motor improvement and a sensation of well-being. The patients were also told that an anti-nausea drug would be administered. Arm rigidity was assessed after 5, 15, 30, 45 and 60 min by a blinded neurologist, and recordings from VA and VLa were made with the patient at rest between assessments. It is important to point out that the blinded neurologist did not know the purpose of the study and that the arm rigidity assessment was done without knowing the subjective report of the patient. Indeed, the patients reported their sensations when the neurologist was out of the operating room, so that we could rule out any influence of the patients' reports on the blinded neurologist.

Each recording lasted 60–120 s, and this allowed us to record from as many neurons as possible. The electrode was moved within the limits of VA and VLa in search of different units, and the mean time of recording from each neuron after placebo was 88 s (range = 60–120 s). The investigator who recorded the neuron activity was blind, i.e. they did not know about the assessment of muscle rigidity by the neurologist.

Electrophysiological analysis

The analysis of neuron activity is described in detail in our previous studies (e.g. Benedetti *et al.* 2009). Briefly, we considered both single and multiunit recordings: when more than one unit was present, the single spikes were separated by using principal components analysis (AlphaSort, Alpha Omega Engineering, Nazareth, Israel). By considering two projections of the principal components, P1 and P2, we reconstructed an approximation of the original signal by means of:

$$S_A = Pj_1 * P_1 + Pj_2 * P_2$$

where S_A is the approximated signal, Pj_1 and Pj_2 are the projections of the signal on the first and second principal components, and P_1 and P_2 are the principal component vectors. The approximation error E_A was obtained following the formula:

$$E_A = \frac{S(S_A - S_O)^2}{|S_O|}$$

where S_A is the approximated signal, S_O is the original signal and $|S_O|$ is the absolute value (the magnitude) of the original signal. Thus, E_A is the sum over all vector elements of the squared differences between the original and approximated signals, normalized by the magnitude of the original signal. If a signal has E_A higher than the specified threshold level, it is considered to be an artifact. We adopted a stringent criterion, whereby only a pattern of $E_A < 0.1$ was considered for all spikes. After a signal was classified as a neuronal spike, we performed a cross interval histogram analysis to confirm the validity of the principal components analysis (Benedetti *et al.* 2009). Indeed, the cross interval histogram allowed us to assess whether two consecutive spikes of two different units were separated by intervals smaller than 3 ms, namely the refractory period of action potential. We found inter-spike intervals shorter than 3 ms, which indicates that the spikes belong to different units.

We calculated the mean firing rate for a single neuron by dividing the number of spikes by the duration of recording. We then calculated the mean across the neurons recorded at each time point for each patient. The difference in neuronal firing rate was derived by considering the difference in the mean rates within a single subject.

We also performed bursting analysis to see whether bursts of discharge were present in neuronal activity. The detailed methodology is described elsewhere by Kaneoke & Vitek (1996), Levy *et al.* (2001) and Benedetti *et al.* (2004, 2009). Briefly, we performed a discharge density histogram analysis, whereby the number of occurrences of no spikes, one spike, two spikes, etc., in each time interval was calculated. This histogram represents the probability distribution of the neuron's discharge density and is compared with a discharge density of a Poisson process with a mean of 1 by using a χ^2 test. The neurons were classified as non-bursting if either a random (Poisson) discharge pattern or a non-Poisson pattern with variance <1 were present, whereas they were classified as bursting if a non-Poisson pattern and variance >1 occurred.

Statistical analysis

Statistical analysis of the clinical (muscle rigidity scores) and neuronal (firing rate) placebo response was performed by using mixed ANOVA (amplitude, time, groups) followed by the *post-hoc* Bonferroni test and multiple comparisons correction. Before performing the ANOVA, we used Mauchly's sphericity test to verify that the variances of the differences between all possible pairs of groups were equal. In no case was sphericity violated. The values are expressed as the clinical improvement (reduction in muscle rigidity), as assessed by means of the UPDRS, at 5, 15, 30, 45 and 60 min after placebo. To do this, the differences between pre-treatment and post-treatment (either apomorphine or placebo) were calculated. Likewise, the differences between pre-treatment about 1 h before and pre-treatment just before apomorphine or placebo administration was calculated to assess possible natural changes before treatment. All post-treatment conditions were compared with the immediate pre-treatment measures. Neuronal discharge was analysed in the same way by comparing pre-treatment about 1 h before (during the first implantation) and pre-treatment just before placebo during the second implantation, which allowed us to assess possible natural changes of neuron activity before

treatment. Then, analysis of neuronal activity after placebo was performed. Again, all post-treatment conditions were compared with the immediate pre-treatment measures. The neuronal values are expressed as the percentage change in firing rate. The number of bursting and non-bursting neurons before and after placebo was compared by means of the chi-square (χ^2) test. In addition, linear regression analysis was performed to assess possible correlations between the number of apomorphine administrations and the magnitude (peak response) and duration of the placebo responses.

Results

The number of neurons recorded before placebo and at different time intervals after placebo is shown in Table 2. No differences were found in the number of neurons with bursting activity (shown in parentheses) before and after placebo. By contrast, significant differences were related to both clinical improvement (reduction in muscle rigidity) and firing rate of the thalamic neurons. The baseline firing rates (mean ± SD) were the same across the different groups (No treatment: 30.1 ± 14.7 Hz; Placebo 0: 27.5 ± 11.9 Hz; Placebo 1: 28.4 ± 18 Hz; Placebo 2: 25.8 ± 15.3 Hz; Placebo 3: 30.7 ± 12.8 Hz; Placebo 4: 27 ± 15.6 Hz).

Overall, by increasing the number of pre-operative administrations of apomorphine (Fig. 3), there was a significant increase in both the clinical (black circles) and the neuronal (grey columns) placebo responses ($F_{25,180} = 8.2824$, $P < 0.001$ and $F_{20,144} = 8.8491$, $P < 0.001$, respectively, by ANOVA). *Post-hoc* Bonferroni analysis showed that there were no significant differences in muscle rigidity (black circles), as assessed through the UPDRS, and in neuronal firing rate (grey columns) between the no-treatment group (Fig. 3*A*) and the Placebo 0 (Fig. 3*B*) and Placebo 1 (Fig. 3*C*) groups. Therefore, neither a placebo given for the first time (Placebo 0) nor a placebo after a single exposure to apomorphine (Placebo 1) produced any effect at the clinical and neuronal level.

When the placebo was given after two pre-operative administrations of apomorphine (Fig. 3*D*), the clinical response was significantly different from the no-treatment group at 5 min ($P < 0.001$ by Bonferroni *post-hoc*) and 15 min ($P < 0.001$) after placebo administration, and the neuronal response was significantly different from the no-treatment group at 15 min ($P < 0.005$). By increasing the pre-operative apomorphine exposures up to three (Fig. 3*E*), the clinical placebo response was significant at 5, 15, 30 and 45 min ($P < 0.001$ in all cases) and the neuronal placebo response at 15, 30 and 45 min ($P < 0.001$ in all cases). A further increase occurred by increasing the pre-operative apomorphine administrations up to four (Fig. 3*F*), which produced a clinical placebo response at 5, 15, 30, 45 and 60 min ($P < 0.001$ in all cases), and a neuronal placebo response at 15, 30, 45 and 60 min ($P < 0.001$ in all cases).

Therefore, the greater the number of pre-operative exposures to apomorphine, the larger the magnitude and the longer the duration of the clinical and neuronal placebo responses. In fact, a linear regression analysis showed a positive correlation between the number of pre-operative apomorphine administrations and the magnitude of both the clinical and the neuronal response ($r = 0.8243$, $R^2 = 0.6795$, $t_{33} = 8.364$, $P < 0.001$, and $r = 0.8002$, $R^2 = 0.6403$, $t_{33} = 7.666$, $P < 0.001$, respectively), as shown in Fig. 4*A*. Likewise, a positive correlation was found between the number of pre-operative apomorphine administrations and the duration of the clinical/neuronal response ($r = 0.9574$, $R^2 = 0.9167$, $t_3 = 5.744$, $P < 0.015$), as shown in Fig. 4*B*.

All these clinical placebo responses disappeared completely at 24 and 48 h, with the exception of the Placebo 4 group (Fig. 5). In fact, a decrease in muscle rigidity after placebo administration was still present at 24 h after surgery. The overall effect showed a significant difference across groups ($F_{25,180} = 3.1562$, $P < 0.001$ by ANOVA). This was attributable to a placebo response at 30 min after placebo administration in the Placebo 4 group ($P < 0.001$ by Bonferroni *post-hoc*). This effect disappeared completely after 48 h.

Discussion

The main findings of the present study can be summarized as follows. First, when a placebo was administered for the first time, neither clinical responses nor neuronal changes were observed, thus indicating that verbal suggestions of improvement are ineffective in inducing biological changes in the patient's brain. Second, as the number of prior exposures to apomorphine increased, both clinical and neuronal responses to placebo increased, thus showing the crucial role of learning in clinical/biological placebo responsiveness. Third, only four preoperative exposures to apomorphine induced long-lasting changes up to 24 h. In fact, the re-exposure to a placebo induced a placebo response 24 h after surgery in group Placebo 4.

Taken together, these data demonstrate that the neuronal changes induced by placebos in the patient's brain and previously described in other studies (Benedetti *et al.* 2004, 2009; Frisaldi *et al.* 2014) can be obtained only after pharmacological preconditioning. In particular, in the present study we found that placebos can mimic the clinical effect of apomorphine. In fact, it should be noted that placebo administration following four apomorphine preconditioning trials induced clinical responses that were as large as those to apomorphine (Fig. 3*F*). Unfortunately, we do not know whether placebos also mimicked the neuronal responses to apomorphine, as no apomorphine

Table 2. Number of total neurons (in parentheses those with bursting activity) recorded before placebo and after placebo in the first 15 min, between 15 and 30 min, between 30 and 45 min, and between 45 and 60 min

Patient	Before placebo	0–15 min after placebo	15–30 min after placebo	30–45 min after placebo	45–60 min after placebo
No treatment (no placebo was given but recordings were performed for 75 min)					
1	7 (1)	8 (1)	10 (0)	11 (0)	9 (1)
2	5 (1)	8 (1)	7 (0)	9 (0)	10 (1)
3	11 (3)	10 (1)	9 (2)	6 (0)	7 (0)
4	8 (0)	5 (0)	10 (1)	12 (1)	6 (0)
5	5 (0)	12 (1)	13 (1)	8 (0)	7 (0)
6	13 (0)	7 (0)	7 (0)	7 (0)	11 (1)
7	10 (2)	6 (0)	6 (0)	5 (1)	9 (0)
Placebo 0					
1	8 (1)	8 (0)	8 (1)	9 (0)	7 (1)
2	12 (2)	11 (0)	10 (2)	9 (1)	6 (0)
3	6 (0)	5 (0)	10 (0)	5 (0)	7 (0)
4	8 (2)	6 (1)	9 (0)	6 (0)	8 (1)
5	6 (1)	6 (0)	7 (0)	10 (1)	11 (1)
6	14 (3)	7 (0)	12 (2)	9 (0)	10 (1)
7	7 (1)	10 (0)	6 (1)	12 (0)	8 (1)
Placebo 1					
1	9 (1)	13 (1)	6 (0)	8 (0)	9 (0)
2	7 (0)	7 (0)	8 (0)	7 (0)	6 (1)
3	9 (1)	8 (0)	8 (0)	6 (1)	7 (1)
4	13 (3)	14 (1)	11 (2)	9 (0)	10 (1)
5	10 (1)	5 (0)	7 (0)	12 (1)	9 (0)
6	10 (2)	11 (0)	12 (1)	7 (2)	7 (0)
7	5 (0)	9 (0)	8 (0)	10 (1)	12 (0)
Placebo 2					
1	6 (1)	10 (1)	4 (0)	6 (1)	6 (0)
2	7 (0)	8 (0)	5 (0)	11 (2)	9 (1)
3	14 (4)	9 (0)	12 (1)	12 (1)	10 (0)
4	6 (0)	7 (0)	6 (1)	9 (0)	8 (0)
5	11 (2)	8 (0)	9 (0)	11 (2)	6 (0)
6	4 (0)	8 (1)	12 (1)	5 (0)	9 (0)
7	13 (2)	13 (0)	10 (1)	7 (0)	10 (1)
Placebo 3					
1	9 (1)	10 (1)	9 (0)	10 (0)	8 (0)
2	8 (1)	8 (1)	7 (1)	7 (0)	8 (0)
3	15 (3)	12 (0)	9 (1)	7 (0)	6 (1)
4	11 (0)	6 (0)	8 (0)	7 (2)	8 (0)
5	5 (0)	5 (0)	9 (1)	8 (0)	10 (1)
6	7 (1)	6 (0)	8 (1)	10 (2)	10 (0)
7	7 (2)	11 (1)	12 (0)	9 (0)	12 (1)
Placebo 4					
1	6 (1)	6 (0)	8 (1)	5 (0)	7 (0)
2	8 (1)	12 (1)	11 (1)	9 (0)	8 (0)
3	9 (2)	9 (1)	6 (0)	10 (0)	10 (0)
4	10 (2)	10 (0)	7 (0)	5 (0)	5 (0)
5	13 (1)	6 (0)	11 (2)	11 (1)	8 (0)
6	9 (0)	7 (0)	6 (0)	5 (0)	9 (0)
7	12 (1)	12 (1)	8 (0)	8 (1)	12 (0)

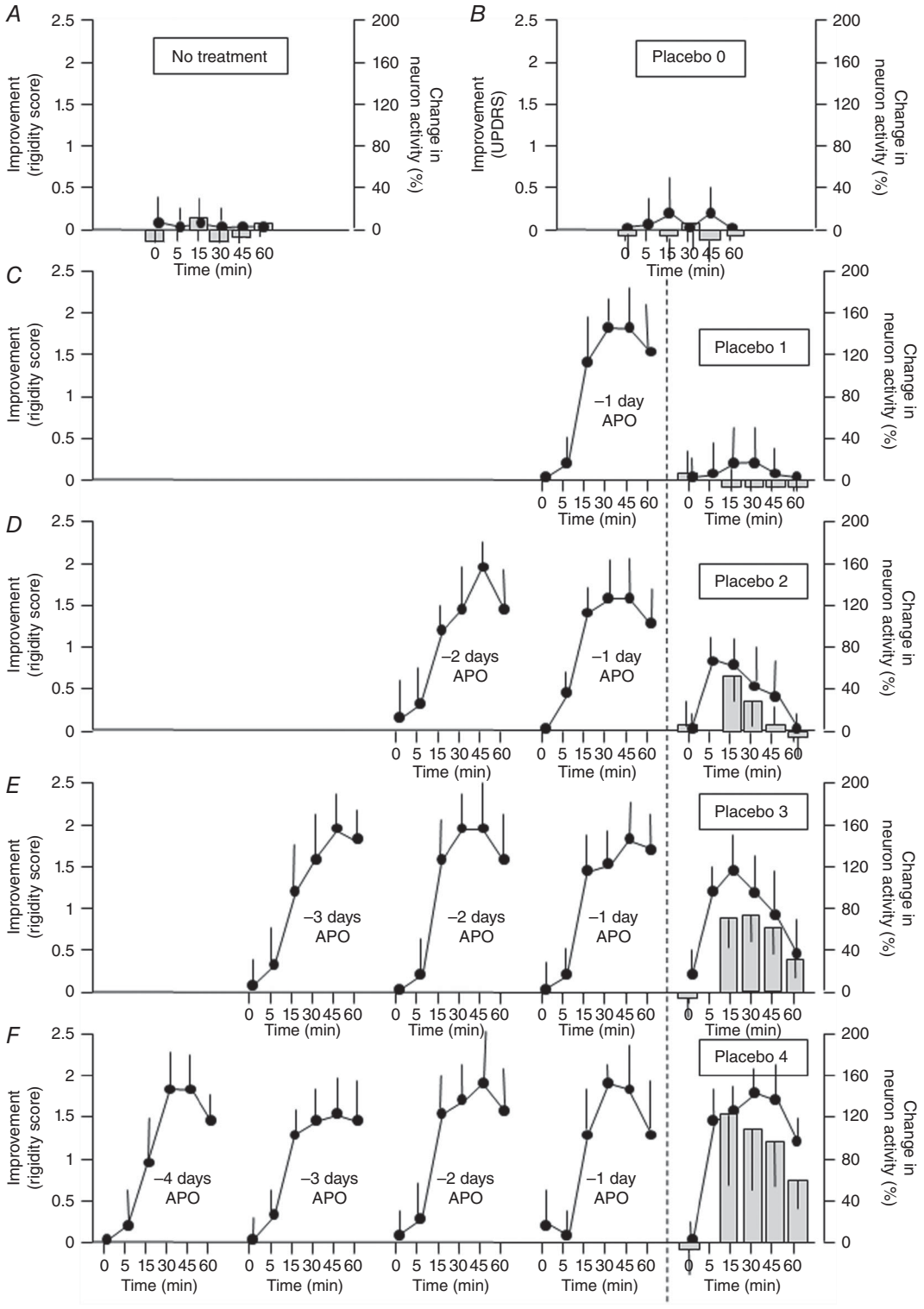

Figure 3. Comparisons across the six groups for both clinical and electrophysiological data
Arm rigidity (black circles) is expressed as clinical improvement of rigidity score (vertical bars represent standard deviations), on the basis of a rigidity scale ranging from 0 = no rigidity to 3 = severe rigidity. Thalamic neuron activity (grey columns) is expressed as the percentage increase in firing rate after placebo administration (vertical bars = standard deviations). Note that in group Placebo 4, after four preoperative exposures to apomorphine (APO), the clinical response to placebo (on arm rigidity) was as large as the response to apomorphine, along with substantial neuron activity changes (grey columns).

was given intra-operatively while recording from single neurons.

The mechanism underlying this enhancement of the placebo response following multiple exposures to apomorphine could be represented by classical conditioning. The ritual of the subcutaneous injection, acting as a conditioned stimulus (CS), associated several times with apomorphine delivery, acting as the unconditioned stimulus (US), leads to a conditioned response (CR), whereby the subcutaneous injection ritual alone is capable of inducing the same unconditioned response (UR) of apomorphine (i.e. muscle rigidity reduction), along with the increase in thalamic neuronal activity. Whether this is true Pavlovian conditioning or rather an enhancement of expectations, according to more recent cognitive interpretations of classical conditioning (Reiss 1980; Rescorla 1988; Kirsch *et al.* 2004), cannot be resolved by the present study. Nor can this study explain the mechanism of the disappearance of the placebo response after 48 h. For example, this could be due to either the repeated administration of placebo (e.g. at 24 h) or to an effect of time. This represents a crucial point that needs to be addressed in future research, to better understand whether the placebo response can be prolonged beyond 48 h.

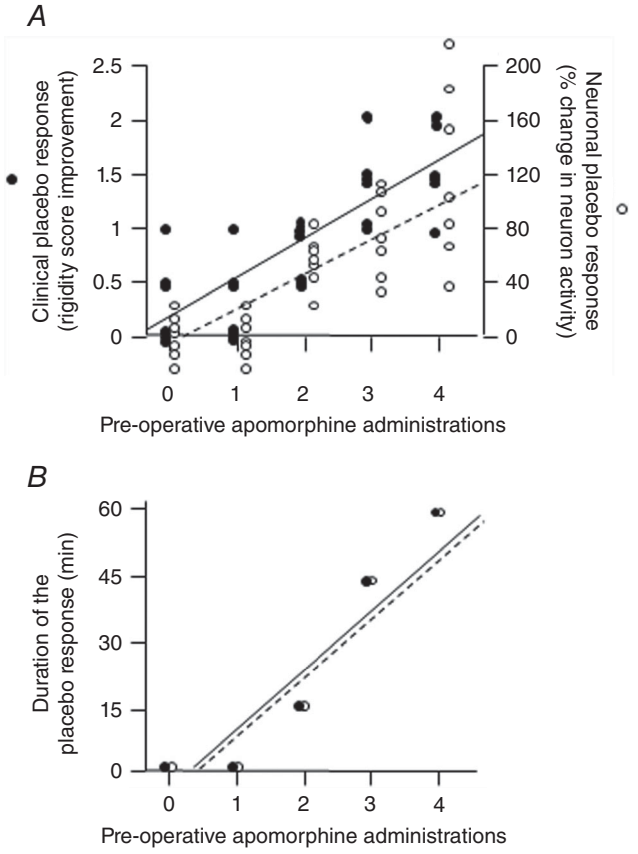

Figure 4. Linear regression analysis between the number of apomorphine exposures and placebo responses
A, positive correlation between the number of pre-operative apomorphine exposures and magnitude of clinical (black circles) and neuronal (white circles) placebo responses at 15 min after placebo. Each circle represents a single patient. *B*, positive correlation between the number of apomorphine administrations and the duration of clinical (black circles) and neuronal (white circles) placebo responses. Each circle represents the duration calculated by considering the significant difference relative to the no-treatment group (see Results for statistical analysis).

Apomorphine has been found to produce either no change in STN mean frequency discharge (Levy *et al.* 2001) or a pronounced decrease (Stefani *et al.* 2002). Although our study is on the thalamus and not on STN, it supports the association between the clinical improvement in Parkinsonian rigidity and the increase in neuronal activity in thalamic VA and VLa nuclei, which strengthens the pathophysiological model in which the hyperactivity of STN leads to inhibition of thalamic neurons via the substantia nigra pars reticulata (SNr) (Bergman *et al.* 1994; Blandini *et al.* 2000; Frisaldi *et al.* 2014). According to this model, an anti-Parkinson treatment would restore the normal activity in STN (Limousin *et al.* 1998; Benazzouz & Hallett, 2000), with the consequent decreased inhibition over the thalamus. The increased output from the motor thalamus would facilitate movement control by the motor cortex.

Besides the frequency of discharge of different neuronal populations, there is today compelling evidence that synchronized activity between different regions may be impaired in Parkinson's disease (Brown, 2003). For example, monkeys treated with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which represents an experimental model of parkinsonism, show oscillations below 30 Hz (Nini *et al.* 1995). Likewise, synchronization of single neurons in both STN and internal globus pallidus (GPi) at 11–30 Hz have been observed in Parkinson patients during intra-operative recording (Levy *et al.* 2000, 2001, 2002). In addition, Parkinson patients treated with levodopa show oscillations greater than 60 Hz between STN, GPi and cortex (Brown *et al.* 2001; Williams *et al.* 2002). All these data indicate that proper functioning of the basal ganglia circuitry is not mediated only by the neuronal frequency of discharge, but by oscillatory activitiy as well (Brown, 2003).

Which pattern, frequency of discharge or oscillations, is more important in the placebo response cannot be resolved by our study, and this certainly represents a challenge for future research. In fact, although we did not find any change in bursting activity in the thalamic neurons, it should be noted that we found only a few bursting neurons. Therefore, a definitive conclusion cannot be drawn. In addition, we do not know whether bursting activity in other regions, such as the STN (Benedetti *et al.* 2004), was affected by the different pre-operative exposures to apomorphine, and this needs to be further investigated in future research.

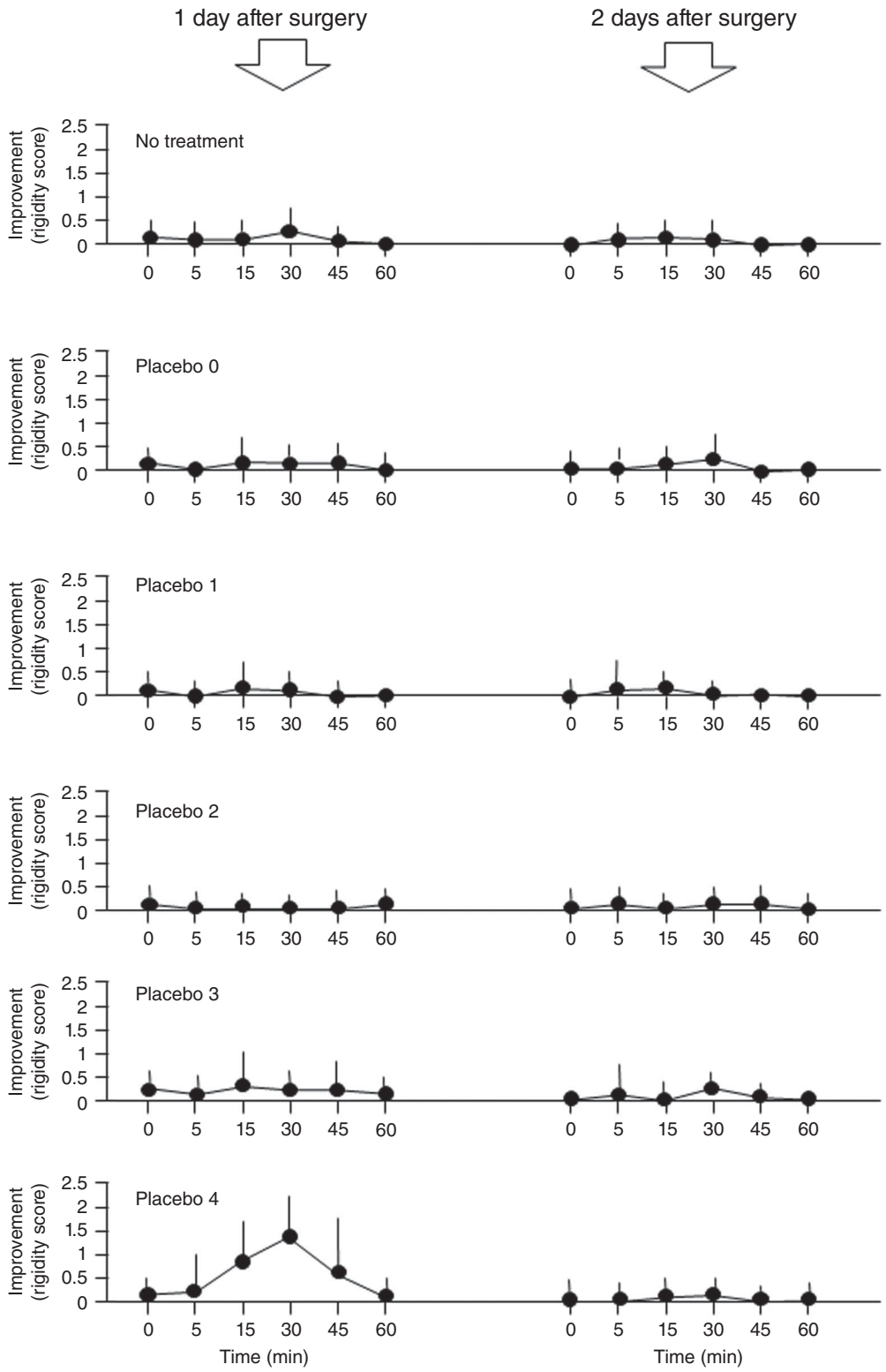

Figure 5. Placebo administration 1 and 2 days after surgery
No placebo response (on arm rigidity) was observed in all groups, with the exception of group Placebo 4 after 1 day. However, even in this group, the placebo response was completely absent after 2 days. Vertical bars = standard deviations.

Due to the obvious ethical limitations when recording intra-operatively, in the present study we recorded only from the VA and VLa nuclei of the thalamus. Therefore, we do not know whether the observed changes also took place in other regions of the basal ganglia that are 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). In other words, the observed changes in the thalamus could merely represent changes that actually took place in other regions. These regions have 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) and include STN, the major target for the surgical treatment of Parkinson's disease, the external globus pallidus (GPe), GPi and SNr. The main reason why our main target for single-neuron recording was only the thalamus depends on the time constraints during surgery. As the placebo-induced neuronal changes previously described (Benedetti *et al.* 2004, 2009; Frisaldi *et al.* 2014) 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), in the present study we decided to record from thalamic neurons only, thus overcoming the time limitations.

Another possible limitation of our study is related to the identification of the thalamic VA and VLa neurons. In fact, there is the possibility that some 'thalamic' neurons may be actually dorsal Zi neurons, because the border between these two regions is not always clear. However, as described in the Methods, we paid particular attention to recording from neurons at least 2 mm above the superior STN border, thus ruling out fibres and cells in the Zi. Finally, a further limitation is represented by the clinical assessment of arm rigidity only, due to time limitations during surgery. We decided to assess only wrist rigidity because it has been shown to be a reliable clinical response in our previous studies (Benedetti *et al.* 2004, 2009). If it becomes possible to overcome the intra-operative ethical constraints, future research should be aimed at assessing other symptoms as well, such as bradykinesia and tremor.

By taking all these limitations into account, our study emphasizes the importance of pharmacological preconditioning in placebo responsiveness, as described in several experimental models, such as pain, immune responses and hormone secretion (Benedetti *et al.* 2003; Colloca & Benedetti, 2006; Pacheco-Lopez *et al.* 2006). In particular, our study suggests that placebo non-responders can be turned into placebo responders when a learning procedure is carried out. As the number of pharmacological preconditioning exposures increases, the placebo responses become more robust and long-lasting. However, it should be acknowledged as a possible limitation that we did not use a within-subject design, whereby both a conditioning and a no-conditioning condition were tested in the same individual. Therefore, future studies should verify whether a Parkinson patient not responding to a placebo can become a placebo responder through a conditioning procedure. These findings may have profound implications for neurotherapeutics. In fact, the alternate replacement of drugs with placebos can be used in therapeutic protocols aimed at reducing drug intake.

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**, S13–S17.

Benedetti F (2013). Placebo and the new physiology of the doctor–patient relationship. *Physiol Rev* **93**, 1207–1246.

Benedetti F (2014). Placebo effects: from the neurobiological paradigm to translational implications. *Neuron* **84**, 623–637.

Benedetti F, Amanzio M, Rosato R & Blanchard C (2011). Non-opioid placebo analgesia is mediated by CB1 cannabinoid receptors. *Nat Med* **17**, 1228–1230.

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, Lanotte M, Colloca L, Ducati A, Zibetti M & Lopiano L (2009). Electrophysiological properties of thalamic, subthalamic and nigral neurons during the anti-parkinsonian placebo response. *J Physiol* **587**, 3869–3883.

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 Pakinson'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 (2006). How prior experience shapes placebo analgesia. *Pain* **124**, 126–133.

de la Fuente-Fernández 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-Fernández 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.

Diederich NJ & Goetz CG (2008). The placebo treatments in neurosciences: new insights from clinical and neuroimaging studies. *Neurology* **71**, 677–684.

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*, eds. Fahn S, Marsden CD & Calne DB, pp. 153–163. MacMillan, London.

Frisaldi E, Carlino E, Lopiano L, Lanotte M & Benedetti F (2014). Characterization of the thalamic–subthalamic circuit involved in the placebo response through single-neuron recording in Parkinson patients. *Cortex* **60**, 3–9.

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.

Goebel MU, Trebst AE, Steiner J, Xie YF, Exton MS, Frede S, Canbay AE, Michel MC, Heemann U & Schedlowski M (2002). Behavioral conditioning of immunosuppression is possible in humans. *FASEB J* **16**, 1869–1873.

Goetz CG, Laska E, Hicking C, Damier P, Müller T, Nutt J, Warren OC, Rascol O & Russ H (2008*b*). Placebo influences on dyskinesia in Parkinson's disease. *Mov Disord* **23**, 700–707.

Goetz CG, Leurgans S, Raman R & Stebbins GT (2000). Objective changes in motor function during placebo treatment in PD. *Neurology* **54**, 710–714.

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, Wuu J, McDermott MP, Adler CH, Fahn S, Freed CR, Hayser RA, Olanow WC, Shoulson I, Tandon PK & Leurgans S (2008*a*). Placebo response in Parkinson's disease: comparisons among 11 trials covering medical and surgical interventions. *Mov Disord* **23**, 690–699.

Guo JY, Wang JY & Luo F (2010). Dissection of placebo analgesia in mice: the conditions for activation of opioid and non-opioid systems. *J. Psychopharmacol* **24**, 1561–1567.

Hammond C, Bergman H & Brown P (2007). Pathological synchronization in Parkinson's disease: networks, models and treatments. *Trends Neurosci* **30**, 357–364.

Hutchison WD, Allan RJ, Opitz H, Levy R, Dostrovsky JO, Lang AE & Lozano AM (1998). Neurophysiological identification of the subthalamic nucleus in surgery for Parkinson's disease. *Ann Neurol* **44**, 622–628.

Kaneoke Y & Vitek JL (1996). Burst and oscillation as disparate neuronal properties. *J Neurosci Methods* **68**, 211–223.

Keitel A, Wojtecki L, Hirschmann J, Hartmann CJ, Ferrea S, Südmeyer M & Schnitzler A (2013). Motor and cognitive placebo-/nocebo-responses in Parkinson's disease patients with deep brain stimulation. *Behav Brain Res* **250**, 199–205.

Kirsch I, Lynn SJ, Vigorito M & Miller RR (2004). The role of cognition in classical and operant conditioning. *J Clin Psychol* **60**, 369–392.

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.

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 & Dostrowsky 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 & Dostrowsky JO (2002). Synchronised neuronal discharge in the basal ganglia of parkinsonian patients is limited to oscillatory activity. *J Neurosci* **22**, 2855–2861.

Lidstone SC, Schulzer M, Dinelle K, Mak E, Sossi V, Ruth TJ, de la Fuente-Fernández R, Phillips AG & Stoessl AJ (2010). Effects of expectation on placebo-induced dopamine release in Parkinson disease. *Arch Gen Psychiatry* **67**, 857–865.

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.

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.

McRae C, Cherin E, Yamazaki TG, Diem G, Vo AH, Russell D, Ellgring JH, Fahn S, Greene P, Dillon S, Winfield H, Bjugstad KB & Freed CR (2004). Effects of perceived treatment on quality of life and medical outcomes in a double-blind placebo surgery trial. *Arch Gen Psychiatry* **61**, 412–420.

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, Slovin 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.

Pollack AE (2001). Anatomy, physiology, and pharmacology of the basal ganglia. *Neurol Clin* **19**, 523–534.

Reiss S (1980). Pavlovian conditioning and human fear: an expectancy model. *Behav Ther* **11**, 380–396.

Rescorla RA (1988). Pavlovian conditioning: it's not what you think it is. *Am Psychol* **43**, 151–160.

Schaltenbrand G & Wahren W (1977). *Atlas for Stereotaxy of the Human Brain*. Thieme, New York.

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.

Stockhorst U, Steingruber HJ & Scherbaum WA (2000). Classically conditioned responses following repeated insulin and glucose administration in humans. *Behav Brain Res* **110**, 143–159.

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 bridles neuronal activity in the subthalamic nucleus and the substantia nigra reticulata. *FASEB J* **17**, 1820–1830.

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.

Additional information

Competing interests

The authors declare that they have no competing financial interests.

Author contributions

F.B. conceived and designed the research, performed the experiments, analysed the data and wrote the manuscript; E.F. and E.C. analysed the data and critically discussed the paper; L.G. and A.P. conceived and designed the research; M.Z., M.L. and L.L. designed the research, performed the experiments and critically discussed the paper. All authors have read and approved the final version of the manuscript. All experiments were carried out in the operating room of the Division of Neurosurgery of the Department of Neuroscience, University of Turin Medical School.

Funding

This study was supported by grants from Compagnia di San Paolo Foundation (F.B., M.L., L.L.), Giancarlo Quarta Foundation (F.B.) and the Carlo Molo Foundation (F.B.).