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TRANSLATIONAL NEUROSCIENCES - ORIGINAL ARTICLE

The dopaminergic stabilizer pridopidine increases neuronal activity of pyramidal neurons in the prefrontal cortex

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Abstract The dopaminergic stabilizer pridopidine demonstrates state-dependent effects on locomotor activity, counteracting both hypo- and hyperactivity in rats. Pridopidine has been shown to display both functional dopamine D2 receptor antagonist properties and increase in biomarkers associated with NMDA-mediated glutamate transmission in the frontal cortex. To further characterise the effects of pridopidine on prefrontal cortex (PFC) neurons, a series of in vivo electrophysiological studies were performed in urethane-anaesthetised rats. Pridopidine, administered at doses from 10 to 60 mg/kg (i.v.), dose dependently increased pyramidal cell firing in the majority of the neurons tested. Pridopidine induced a significant increase of 162 % in mean firing activity of PFC neurons, versus initial basal firing activity as the cumulative dose of 30 mg/kg, i.v., was administered. This enhancement of activity was due to increased firing frequency of already spontaneously active neurons, rather than an increase in population activity. The increase was partially reversed or prevented by a sub-threshold dose of the dopamine D1 receptor antagonist SCH23390 (0.5 mg/kg, i.v.). Microiontophoretic application of pridopidine had only moderate activating effects. The selective dopamine D1 receptor agonist A-68930 also had limited effects when administered by microiontophoretic application, but exerted a dose dependent (0.2–3 mg/kg, i.v.) activation of firing in the majority of neurons tested (10/16). However, inhibition of firing by systemic administration of A-68930 was also observed in a subgroup of neurons (6/16). Both activation and inhibition of firing induced by systemic administration of A-68930 were reversed by the systemic administration of SCH23390. The present data suggests that pridopidine enhances pyramidal cell firing via an indirect dopamine D1 receptor-mediated mechanism. These effects of pridopidine may serve to strengthen the cortico-striatal communication and to improve motor control in Huntington's disease for which pridopidine is currently in development.

Keywords Prefrontal cortex · In vivo electrophysiology · Dopamine stabilizer · Dopamine D1 receptor · Huntington's disease

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Introduction

Pridopidine [4-(3-(methylsulfonyl)phenyl)-1-propylpiperidine; ACR16] (Sonesson et al. 2000) belongs to a class of compounds often referred to as dopaminergic stabilizers. Dopaminergic stabilizers demonstrate state-dependent behavioural effects. They are able to both decrease hyperactivity induced by psychostimulants and stimulate activity in hypoactive animals habituated to their environment, whilst having only subtle effects on spontaneous locomotor activity (Natesan et al. 2006; Pettersson et al. 2010; Ponten et al. 2010). The dopaminergic stabilizer pridopidine induces subcortical neurochemical effects resembling those induced by dopamine D2 receptor antagonists, e.g. a dose-dependent increase in striatal 3,4-dihydroxyphenylacetic acid (DOPAC) (Pettersson et al. 2010; Ponten et al. 2010). Furthermore, similar to dopamine D2 antagonists, pridopidine lacks intrinsic activity and displaces dopamine from striatal dopamine D2 receptors in vivo, in a dose range

where the agent is active in models assessing antipsychotic-like efficacy (Natesan et al. 2006). In vitro, pridopidine has shown fast-off dopamine D2 receptor antagonism in the micromolar range (Dyhring et al. 2010), and also affinity in similar concentration to several other monoaminergic receptors (Ponten et al. 2013) as well as nanomolar affinities to the sigma-1 receptor (Sahlholm et al. 2013).

The state-dependent behavioural effects of pridopidine have been hypothesized to be mediated by a dual mechanism of action, consisting of both antagonism of subcortical dopamine transmission as well as an increase of extracellular dopamine in the prefrontal cortex (PFC) (Ponten et al. 2010). Furthermore, pridopidine induces frontal cortex expression of the immediate early gene encoding activity-regulated cytoskeletal protein (Arc) mRNA (Waters et al. 2009), which is associated with synaptic activity (Steward and Worley 2001; Kawashima et al. 2009). Dopamine in the PFC is known to play a key role in cognition as well as in a broad spectrum of other brain activities (Alexander et al. 1986; Sullivan and Brake 2003). Most prefrontal dopamine innervations, originating from the midbrain, terminate onto pyramidal glutamate projecting neurons and modulate various cortical excitatory descending pathways. This includes the massive prefrontal projections to the basal ganglia, which are known to regulate, as part of a series of complex feedback loops, executive, motor and affective functions. As a result, dopamine manipulation in the PFC is known to affect locomotor activity, as well as dopamine neurotransmission in striatal regions (Cools 2011; Pycock et al. 1980; Rezvani et al. 2008; Robinson and Stitt 1981; Sullivan and Brake 2003).

Pridopidine has been tested clinically in Huntington's Disease (HD) (de Yebenes et al. 2011; Kieburtz 2011; Landwehrmeyer 2011), a movement disorder associated with cognitive decline and abnormal frontostriatal connectivity. Pridopidine was found to be well tolerated and to improve overall motor function as measured by the Unified Huntington's Disease Rating Scale Total Motor Score (de Yebenes et al. 2011). Electrophysiological studies in animal models of HD have pointed out abnormal synaptic plasticity in the PFC, which can be rescued by dopamine D1 receptor agonists (Dallerac et al. 2011; Tang et al. 2007). Hence, inadequate prefrontal dopamine modulation may be present in HD, suggesting a potential therapeutic benefit by activating cortical dopamine D1 receptors.

The present study was undertaken to explore the effects of pridopidine on neuronal activity in the PFC. By using the in vivo electrophysiological paradigm, we investigated the effects of pridopidine on the electrophysiological characteristics of pyramidal PFC neurons. We also investigated possible involvement of the dopamine D1 receptors in the effects of pridopidine. To this end, we examined if the selective dopamine D1 receptor agonist A-68930 could affect PFC neuronal activity and if the dopamine D1 receptor antagonist SCH23390 could counteract the effects of pridopidine.

Methods

Subjects

Experiments were performed on adult (260–350 g) male Sprague–Dawley rats (Charles River, UK) housed (2–6/group) at constant temperature and humidity under a 24 h light/dark cycle (lights on from 6 a.m. to 6 p.m.) with free access to food and water. All experiments were carried out in accordance with the UK Animals Scientific Procedures Act (1986) and Home Office guidelines and approved by internal ethical committee at De Montfort University.

Drugs

4-(3-(Methylsulfonyl)phenyl)-1-propylpiperidine, hydrochloric salt (pridopidine) was provided by Neurosearch, Sweden; haloperidol hydrochloride and R(+)-7-Chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine hydrochloride (SCH23390) were purchased from Tocris (UK); *N*-methyl-D-Aspartate (NMDA) and *cis*-(±)-1-(Aminomethyl)-3,4-dihydro-3-phenyl-1H-2-benzopyran-5,6-diol hydrochloride (A-68930) were purchased from Sigma (UK). All other chemicals were of laboratory grade and obtained from standard commercial sources. Doses of test compounds are provided as mg/kg of hydrochloric salt.

Electrophysiological experiments

Animals were deeply anaesthetized throughout the experiment with urethane (1.2–1.7 g/kg administered by intraperitoneal (i.p.) injection, with additional doses administered if necessary), secured to a stereotaxic frame and maintained at 36–37 °C. An incision was made across the top of the head and a hole was drilled through the bone at the coordinates of the PFC. Single or multibarrel glass electrodes were pulled on a PP-830 electrode puller (Narishige, Japan). The single glass electrodes were filled by hand with an electrolyte solution (in mM: NaCl 147, KCL 4, adjusted to pH 6 with sodium hydroxide). In case of multibarrel electrodes, the central barrel, used for recording, was filled with the same solution, and the four-side barrels were filled with at least one of the following solutions: NMDA (30 mM in 200 mM of NaCl, pH 7), pridopidine (30–100 mM in 200 mM NaCl, pH 4), A-68930 or dopamine (in some experiments only, 30 mM in 200 mM NaCl, pH 4) and 2 M of NaCl solution for automatic current balancing. The tips of the electrodes were broken down of 3–5 μm in diameter. Impedance of the

recording barrel was typically between 3 and 6 MΩ and impedances of the lateral channel ranged between 40 and 80 MΩ. The microelectrodes were lowered into the PFC at the following coordinates using a hydraulic micromanipulator (Narishige): anteroposterior 2.5–3.7 mm from bregma, lateral 0.3–1.1 mm, dorsoventral 1.5–4.5 mm below cortical surface. In some experiments the recording site was marked by the ejection of Pontamine Sky Blue (−500 nA, 25 min) to verify histological location. NMDA was typically applied using an ejection current of −10 nA for short period (10–60 s) in order to identify the presence of quiescent neurons which can become active only in the presence of ejected excitatory agents. Between each application of NMDA a small positive retention current (+3 to 5 nA) was applied to prevent any leakage of the drug from the pipette. Pridopidine, A-68930 and dopamine were applied during longer periods, 100–600 s, at currents ranging from +10 to 20 nA.

Outputs from the electrode were sent to a Neurolog AC pre-amplifier and amplifier (Digitimer, UK). Signals were filtered and sent to an audio amplifier, a Tektronix 2201 digital storage oscilloscope, and a 1401 interface connected to a computer running Spike 2 (CED, Cambridge, UK) for data capture and analysis. Pyramidal neurons were identified according to previous electrophysiological criteria established from previous studies carried on formally identified pyramidal neurons (Hajos et al. 2003; Kargieman et al. 2008; Puig et al. 2005; Tseng and O'Donnell 2007; Wang et al. 2011): a broad-action potential (>1 ms), with a biphasic or triphasic, large wave form (>1 mV), starting with a positive inflection, a relatively slow firing rate typically between 1 and 50 spikes/10 s and an irregular firing pattern, often with burst activity (Fig. 1). Drugs were administered intravenously (i.v.) 7–15 min after stable recording.

Data treatment and analysis:

Values are expressed as the mean ± standard error of the mean (SEM). Mean basal firing activity was evaluated after the neuron had attained a stable firing rate, generally after at least 10 min of recording. Predrug values of firing rate were obtained by averaging the firing rate over a period of at least 4 min immediately prior to the i.v. administration, post drug values were obtained by averaging firing over a period of 5 min, immediately after the third minute following the administration. In general, subsequent i.v. injections were separated by 5–10 min intervals. For evaluating population activity, spontaneously active neurons were recorded during at least 2 min using a single glass electrode and each electrode descents were distant from each other by at least 200 µm. No more than eight electrode descents were performed per animal.

Changes in firing rate or population activity were assessed by using ANOVA followed by Neuman–Keuls post hoc test, or Student's t test when appropriate. Neuronal burst activity was evaluated as the percentage of spikes occurring in bursts versus the total number of spikes. Burst was defined, according to established criteria on PFC neurons (Labonte et al. 2009) as a train of at least two spikes with an initial interspike interval of 45 ms or less, and a termination interval greater than or equal to 90 ms. Burst parameters were calculated over periods of 200–500 consecutive interspike time intervals.

Results

A total of 307 neurons from 157 rats were included in the present study. All the neurons tested exhibited the electrophysiological characteristics of pyramidal neurons corresponding to the criteria presented in methods. For representative firing rate histograms see Fig. 1. The basal firing activity of the neurons recorded was variable, from 0.5 to 60 spikes/10 s, but typically below 35 spikes/10 s (95 % of the neurons tested in basal condition). All neurons included in the present data were recorded in the dorsal part of the medial PFC in subregions, which include the cingulated and the prelimbic cortex (lateral 0.5–0.7 mm, ventral 1.5–4.5 mm and anterior to bregma 2.7–3.7 mm) and the medial part of frontal cortex (lateral (0.7–1.1, ventral 1.5–3 mm). All recording sites that were marked by ejection of pontamine blue ($n = 12$), were confirmed to be within the PFC at similar location from what was determined from stereotaxic measurement.

Dose-dependent activating effects of pridopidine (Fig. 2a)

In a group of 13 neurons, pridopidine was cumulatively administered up to a dose of 60 mg/kg, by injecting subsequent doses of 10, 20, and 30 mg/kg, each with 5–10 min interval between i.v. injections. Pridopidine induced a significant increase of 162 % in mean firing activity of PFC neurons, versus initial basal firing activity at the cumulative dose of 30 mg/kg, i.v. (Fig. 2a). A higher dose of pridopidine (60 mg/kg i.v., cumulative dose) induced an increase of 214 % versus the basal values. However, as only 5 out of the 13 neurons tested had their firing further enhanced by the last dose, and the remaining neurons showed no change between the 30 and the 60 mg/kg doses, there was no statistical difference between 30 and 60 mg/kg cumulative doses in mean firing. For an example of a neuron, displaying clear activating effects of the 60 mg/kg dose see Fig. 1a. At the lower dose (10 mg/kg, i.v.), pridopidine did not have any significant effects on the mean basal firing activity.

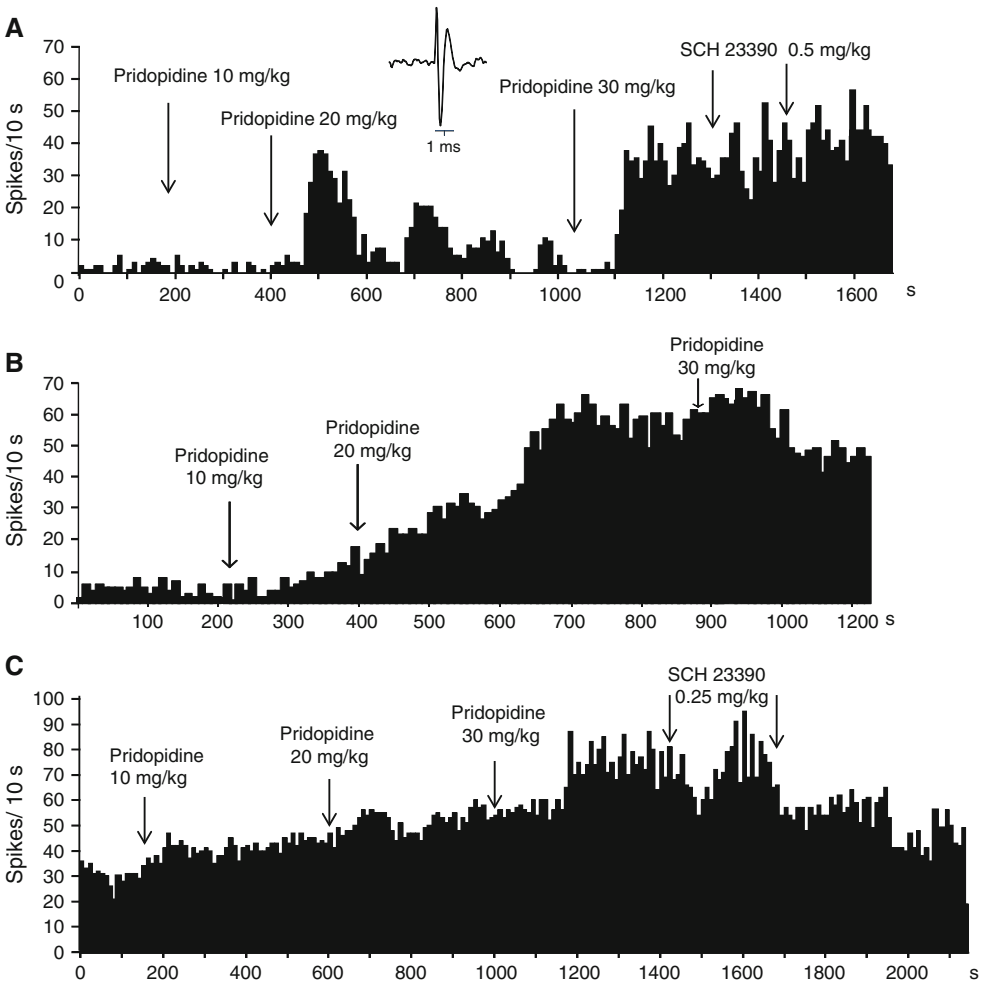

Fig. 1 Representative firing rate histograms of prefrontal cortex neurons before and following the i.v. administration of subsequent doses of pridopidine (cumulative dose 60 mg/kg). **a** Neuron exhibited a strong but temporary activation at the dose of 30 mg/kg, and a more consistent activation at the dose of 60 mg/kg, which was insensitive to the subsequent administration of the dopamine D1 receptor antagonist SCH23390. **b** Example of a neuron that displayed a progressive and strong increase, which was more significant at the dose of 30 mg/kg. **c** Neuron exhibited a dose-dependent increase in firing, which was partially reversed following two doses of SCH23390

As a comparison, we have also tested the effects of the typical antipsychotic and non selective dopamine receptor antagonist haloperidol (Creese et al. 1996) on the firing activity of PFC neurons. Fig. 2b shows that PFC neurons did not display any significant changes in firing activity by acute administration of haloperidol at doses within effective therapeutic ranges (Creese et al. 1996). In addition, neurons treated with saline ($n = 10$), administered in the same conditions as pridopidine, did not exhibit any significant change in firing (Fig. 2c), a result which is in agreement with our previous published observation (Gronier 2011).

Pridopidine preferentially stimulated PFC neurons already spontaneously active (Fig. 3)

The average effects of pridopidine administered at the dose of 30 mg/kg (i.v.) were examined on a larger population of neurons ($n = 34$, which included the latter group of 13 neurons tested with cumulative doses and a group of 21 neurons tested directly with the dose of 30 mg/kg). These neurons were either spontaneously active neurons ($n = 27$) or silent neurons ($n = 7$). The presence of silent neurons was detected by recording neuronal activity using an iontophoretic electrode and applying intermittently, every 100 s, a small current of NMDA which induced a reversible neuronal activation (duration of application: 30 s). Silent neurons and active neurons did not differ in their action potential characteristics. Pridopidine significantly increased the firing activity of the PFC neurons overall ($p < 0.0001$, $n = 34$, Paired Student's t test, Fig. 3a). However, there were some variations between neurons in their response to pridopidine: progressive increase in firing in 38 % of the neurons tested ($n = 13$), immediate and potent increase in 18 % of the neurons tested ($n = 6$), none or only minor activation in 41 % of the neurons ($n = 14$, see examples of individual firing activity in Fig. 1).

Interestingly, when the neurons were separated according to their response to pridopidine into responders ($n = 20$, neurons with firing activity increasing by more than 5 spikes/10 s over baseline) and non-responders ($n = 14$, change by <5 spikes/10 s), we found that the baseline firing activity was significantly higher in the group of neurons that responded to pridopidine (Fig. 3b). This

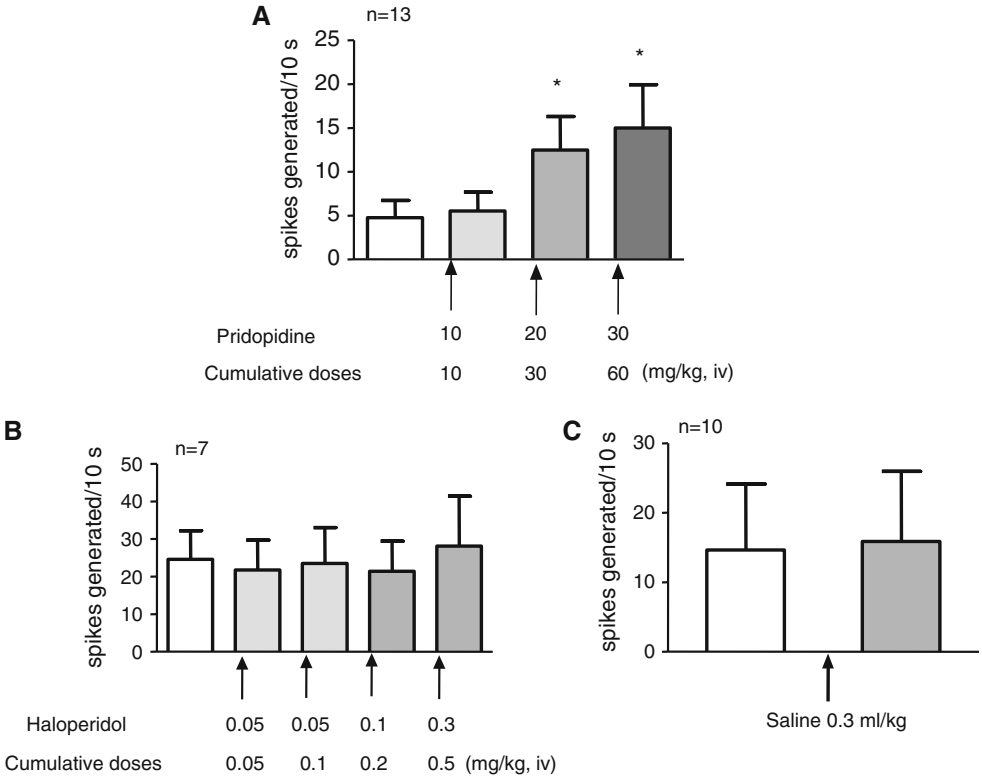

Fig. 2 **a** Mean firing activity of 13 medial prefrontal cortex neurons before and after three subsequent i.v. injections of pridopidine (cumulative dose 60 mg/kg). The same neurons were tested during the complete sequence. The figure shows that pridopidine significantly stimulates the firing activity of PFC neurons at the dose of 30 and 60 mg/kg. There was no significant difference between the dose of 30 and 60 mg/kg. $^{*}p < 0.05$, compared to baseline, Neuman–Keuls test after significant repeated measure ANOVA. **b** Mean firing activity of seven prefrontal cortex neurons before and after three subsequent i.v. administration of the typical antipsychotic haloperidol (cumulative dose 0.5 mg/kg). **c** Mean firing activity of PFC neurons before and after a saline administration (0.3 ml/kg, i.v.). The same neurons were studied during the complete sequence

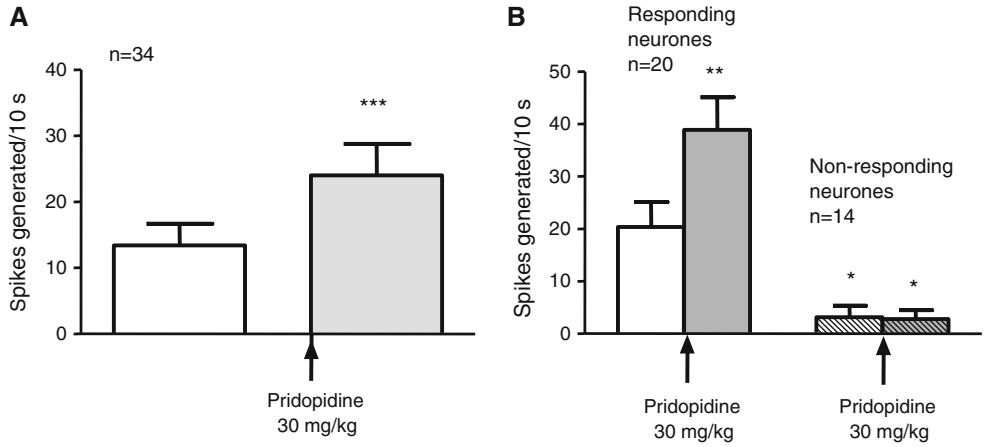

Fig. 3 **a** Mean firing activity of medial prefrontal cortex neurons before and after the administration of the dopaminergic stabiliser pridopidine (30 mg/kg, i.v.). The same neurons were tested in the complete sequence. The Figure shows that the average baseline activity increased by 84 % following the administration of pridopidine. $^{***}p < 0.0001$ compared to baseline, paired Student's *t* test. **b** Mean firing activity of the same prefrontal cortex neurons as in the *left Figure*, but separated in two groups: group responding to pridopidine by an increase in firing by more than five spikes/10 s (*left*), group non responding (*right*). The *Figure* shows that the group responding to pridopidine had a higher baseline activity (before pridopidine administration). $^{*}p < 0.05$, $^{**}p < 0.01$, compared to the values obtained in the responding neurons in baseline conditions, Neuman–Keul's test after significant ANOVA

may be explained by the fact that pridopidine affects preferentially the neurons that are spontaneously active rather than silent neurons. Indeed, none of the neurons that were in a silent state ($n = 7$) turned into an active firing-mode following pridopidine administration. On the other hand, neurons with a low baseline activity, provided they were active in baseline conditions (1–9 spikes/10 s, $n = 15$), and neurons with a higher baseline firing activity (>9 spikes/10 s) were equally activated by pridopidine. Active neurons with a low firing rate had their firing increasing from 3.7 ± 0.7 to 14.5 ± 4.3 spikes/10 s ($p < 0.02$, $n = 15$, paired Student's *t* test); neurons with a higher firing rate had their firing increased from 35.8 ± 4 to 54.4 ± 8 spikes/10 s ($p < 0.002$, $n = 19$, paired

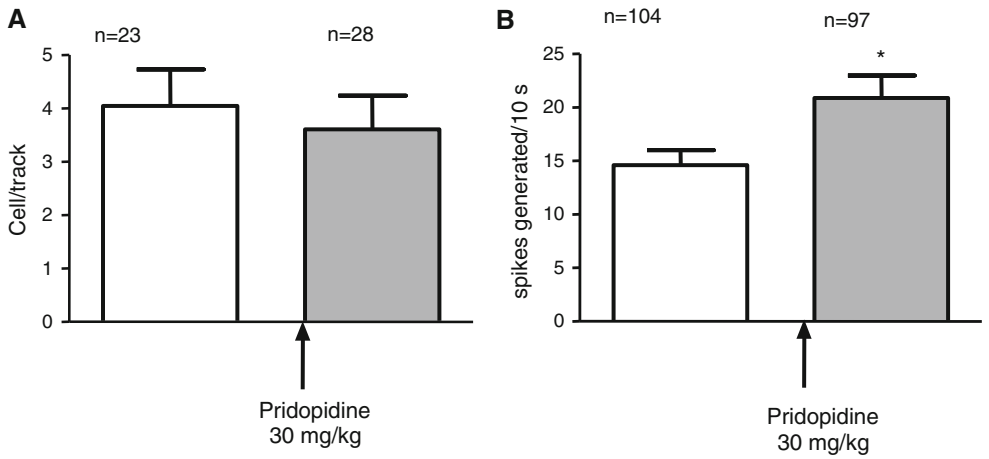

Fig. 4 **a** Population activity (expressed as the average number of cells encountered per electrode descent) of PFC neurons in basal condition or after the i.v. administration of pridopidine (30 mg/kg). **b** Mean firing rate of the same PFC neurons in basal condition or after the i.v. administration of pridopidine (30 mg/kg, i.v.). Neurons were recorded in saline-treated animals or 10 min to 2 h after the i.v. administration of pridopidine (9 animals in total, as indicated in "Results"). *$p < 0.03$, compared to respective controls, unpaired Student's t test

Student's t test, Figure not shown). To verify the observation that pridopidine preferentially activates already spontaneously active neurons (and does not turn on quiescent neurons to an active state), we examined the effects of a 30 mg/kg (i.v.) dose of pridopidine on the population activity of PFC neurons.

Pridopidine did not increase population activity of PFC neurons (Fig. 4)

The average number of active PFC neurons per electrode vertical pass, i.e. population activity, was examined in six animals, treated either with saline (0.3 ml/kg, i.v., $n = 3$) or pridopidine (30 mg/kg, i.v., $n = 3$) and in three animals treated first with saline (during 3 electrode tracks) and then with pridopidine (30 mg/kg, i.v.). Figure 4 shows that there was no significant difference in population activity in pridopidine-treated and saline-treated conditions. This was observed both when data from animals separately treated with saline (0.3 ml/kg, i.v.) and pridopidine (30 mg/kg, i.v.) were compared, and when comparison included animals treated subsequently with saline and pridopidine (Fig. 4a).

Still, the average firing of the PFC neurons, recorded during these experiments, was found to be significantly higher in animals treated with pridopidine (30 mg/kg, i.v., Fig. 4b), as also observed in the acute experiments (Figs. 1a, 2a).

Activating effects of pridopidine were reduced by the dopamine D1 receptor antagonist SCH23390 (Fig. 5; Table 1)

As pridopidine has been found to elicit release of dopamine in the PFC and as dopamine D1 receptors are the more abundant postsynaptic dopamine receptors in this region, the possible involvement of the dopamine D1 receptor in pridopidine-induced electrophysiological effects was investigated. First, we examined whether the activation induced by pridopidine (30 mg/kg, i.v.) could be reduced by the administration of the dopamine D1 receptor antagonist SCH23390. Though the firing obtained following the administration of SCH23390 (0.5 mg/kg, i.v.) in pridopidine already treated neurons (30 mg/kg, i.v.) did not decrease to a level that reached statistical significance, it was also no longer significantly increased versus corresponding baseline value (Fig. 5a).

The ability of SCH23390 to reduce the effect of pridopidine varied from one neuron tested to one another. A marked reduction of the effects of pridopidine (assessed as a reduction in firing rate by more than 5 spikes/s) following the administration of SCH23390, was only observed in five neurons out of 12 (Table 1).

In another series of experiments, we examined whether a pre-administration of SCH23390 could prevent the excitatory effect of pridopidine. Figure 5b shows that the activating effects of pridopidine were reduced by the pre-administration of SCH23390. The effects of pridopidine (30 mg/kg, i.v.) in neurons pretreated with SCH23390 turned out to be no longer significant versus either baseline or SCH23390 treatment alone (Fig. 5b). Consequently, the proportion of neurons that respond to pridopidine is significantly lower in the group of neurons pretreated with the D1 receptor antagonist than in the control (non-pretreated) group (33 vs. 70 %, Fisher exact test, $p < 0.03$). Only 4 neurons (33 %) pretreated with SCH23390 out of the tested 12 neurons, demonstrated excitatory effect (>5 spikes/10 s) following pridopidine. On the other hand, as

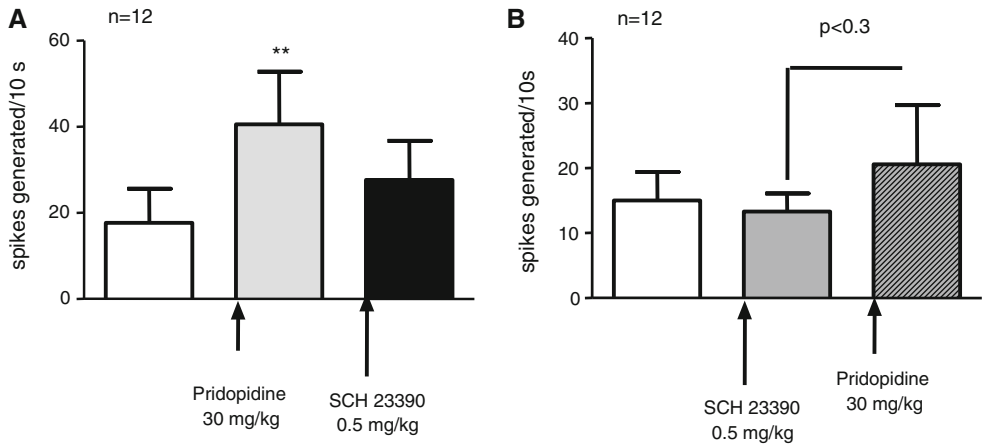

Fig. 5 Mean firing activity of prefrontal cortex neurons measured: **a** Before and after the administration of pridopidine (30 mg/kg, i.v.), followed by the administration of the dopamine D1 receptor antagonist SCH23390 (0.5 mg/kg, i.v.). **b** Before and after the administration of SCH23390 (0.5 mg/kg, i.v.), followed by the administration of pridopidine (30 mg/kg, i.v.). The same neurons were recorded during the complete sequence. Both Figures show that the excitatory effect of pridopidine is partially prevented or partially reversed by antagonism of dopamine D1 receptors. $^{**}p < 0.01$; compared to baseline, Neuman–Keuls test after significant repeated measures ANOVA

Table 1 Proportion of neurons responding by a significant change in firing rate to pridopidine and SCH23390 administered in different orders

Doses Response	Pridopidine 30 mg/kg (%)	Pridopidine 60 mg/kg (%)	Pridopidine after SCH23390 (%)	SCH23390 after pridopidine (%)
Increased				
All cells	19/34 (56)	9/17 (53)	4/12 (33)	1/12 (8)
Active cells	19/27 (70)	9/15 (60)	4/12 (33)	1/10 (10)
No change				
All cells	14/34 (41)	7/17 (41)	3/12 (25)	6/12 (50)
Active cells	7/27 (27)	5/15 (33)	3/12 (25)	4/10 (40)
Decreased				
All cells	1/34 (3)	1/17 (6)	5/12 (42)	5/12 (42)
Active cells	1/27 (3)	1/15 (7)	5/12 (42)	5/10 (50)

All neurons and only spontaneously active neurons were separately considered. A change (defined as the difference between pre-drug and post-drug values) of more than five spikes/10 s was considered a significant response for an individual neuron

indicated in Table 1, the proportion of non-pretreated cells significantly activated by pridopidine was 55 %, or 70 % if only active neurons are considered. All neurons pre-treated with SCH23390 before pridopidine were spontaneously active. Figure 5b also shows that SCH23390 has no significant effect on its own on baseline activity. Similar lack of effect for SCH23390 (0.5 mg/kg, i.v.) has previously been observed in another recent electrophysiological study (Gronier 2011).

Dopamine D1 receptor agonist A-68930 activated PFC neurons (Figs. 6, 7)

The effects of the selective and brain-penetrating dopamine D1 receptor agonist A-68930 were also examined. A-68930, tested on 18 neurons, at cumulative doses up to 1.5 mg/kg, i.v., exerted a significant excitatory effect at the dose 0.5 mg/kg, i.v. (Fig. 6a). Higher doses produced variable responses from one neuron to one another (Fig. 6b, c). Ten out of the 16 neurons that were tested with higher doses (up to 3 mg/kg, i.v.) responded by additional activation of their firing activity. Such activation was irregular in some neurons tested, with period of large activation followed by periods of silence (Fig. 7b). This activation was nevertheless significantly reversed by the administration of the dopamine D1 receptor selective antagonist SCH23390 (0.5 mg/kg i.v., Fig. 6b). Another group of six neurons had their firing gradually decreased following the subsequent administration of the different doses of A-68930. This inhibition of firing activity was also reversed by the administration of SCH23390 (Figs. 6c, 7c), but only in three out of the five neurons tested. When

A-68930 was tested in silent neurons ($n = 3$) that were identified as indicated before by alternated iontophoretic NMDA applications, no effect of A-68930 could be obtained (not shown).

Local application of pridopidine and A-68930 on PFC neurons increased mean firing activity (Figs. 8, 9)

In these experiments pridopidine was applied iontophoretically on PFC neurons during 10–15 min at currents varying from 10 to 20 nA. We found that the application of pridopidine produced a moderate increase in firing activity which was statistically significant, but only seen in 6 out of the 10 neurons tested (Fig. 8). It was observed that most of the neurons tested did not return to their baseline level of activation after the end of the application of pridopidine. Ejection of positive currents from a concentrated saline solution did, however, not induce any change in neuronal activity of PFC neurons (not shown).

We also tested the effect of the iontophoretic application of A-68930 (+5–15 nA) onto 20 PFC neurons. Variability among neurons in the nature of the response to the application of A-68930 was observed: slight to moderate activation (from 4 to 10 spikes/10 s over baseline) in 10 neurons (Fig. 9a, c), moderate decrease in activity in 5 neurons (Fig. 9b), and no effect in 5 neurons (not shown). Most of these effects were reversible as they disappeared progressively after cessation of the drug application, but were not always consistent from one application to one another, which prevented us from attempting to block this response with antagonist. As a comparison, the iontophoretic application of dopamine (10–20 nA) was producing moderate to large inhibition of firing activity in the majority of the neurons tested (not shown), in agreement with what has been already observed in the literature (Gronier 2011).

Pridopidine and A-68930 increased burst activity of PFC neurons:

Administration of pridopidine (30 mg/kg, i.v.) was found to significantly increase the percentage of spikes firing in

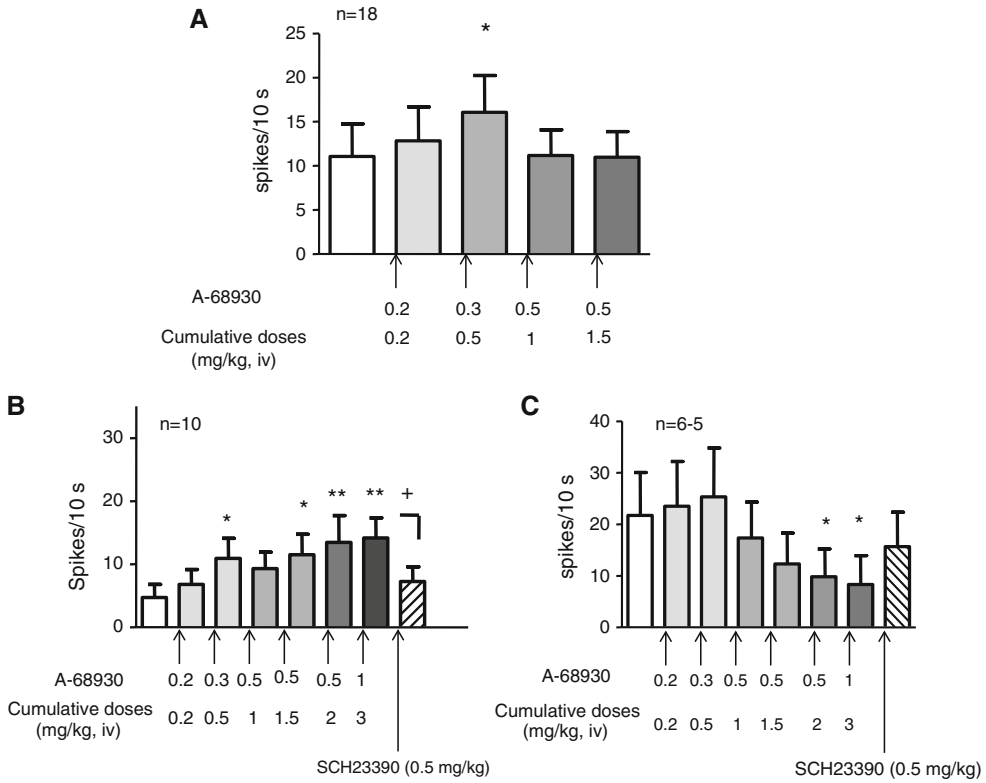

Fig. 6 **a** Mean firing activity of 18 prefrontal cortex neurons before and after several subsequent i.v. injections of the dopamine D1 receptor agonist A-68930 (cumulative dose 1.5 mg/kg). A-68930 has a slight but significant activating effect at the dose of 0.5 mg/kg i.v., but not at higher doses. Sixteen of the neurons represented above have been tested at higher doses and separated according to their response to the A-68930. **b** Ten neurons tested have responded to the higher doses of A-68930 by an activation of their firing activity which is significantly reversed by the dopamine D1 receptor antagonist SCH23390. **c** Six neurons tested had their firing gradually decreased. This was also reversed by the dopamine D1 receptor antagonist SCH23390 for 3/5 of the neurons tested. The same neurons were tested during the complete sequence. $^{**}p < 0.01$, $^{*}p < 0.05$, compared to respective baseline, Neuman–Keuls test after significant repeated measures ANOVA. $^{+}p < 0.05$, compared to corresponding values obtained immediately before the administration of SCH23390

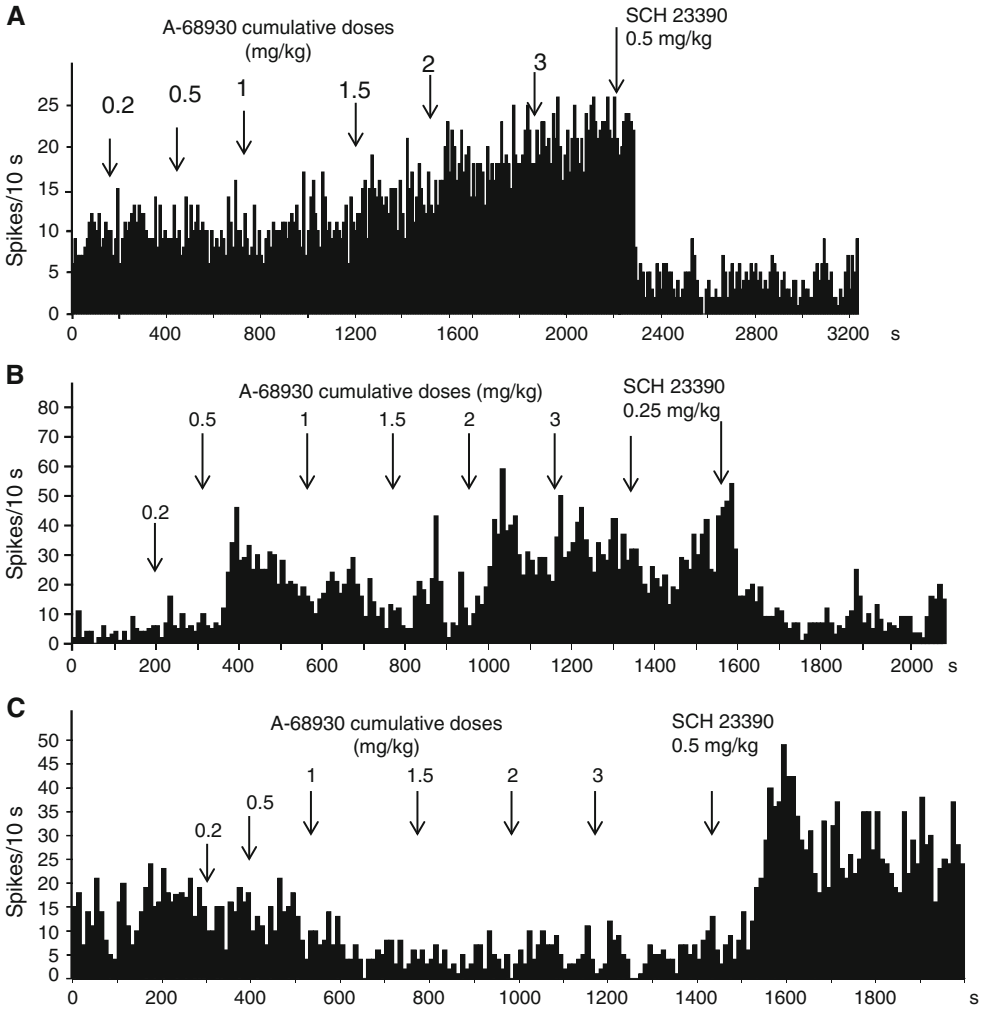

Fig. 7 Representative firing rate histograms of three prefrontal cortex neurons before and after the i.v. administration of subsequent doses of the dopamine D1 receptor agonist A-68930 (cumulative dose 3 mg/kg) followed by the administration of the dopamine D1 receptor antagonist SCH23390 (0.5 mg/kg, i.v.). **a** This neuron displayed a progressive increase in firing which was maximum within the doses of 1.5–3 mg/kg and was completely suppressed by SCH23390 (0.5 mg/kg). **b** This neuron displayed irregular increases in firing. Maximum of activation was obtained after the dose of 2 mg/kg and was completely suppressed following two injections of SCH23390 (cumulative dose 0.5 mg/kg). **c** This neuron displayed a decrease in firing activity which was completely reversed by SCH23390 (0.5 mg/kg, i.v.)

burst in the spontaneously active cells (from 19 ± 5 to 30 ± 5 %, $n = 27$, $p < 0.001$ paired Student's t test). Changes in burst activity were not significantly correlated with changes in firing activity as some neurons showed dramatic changes in burst activity with only moderate or no change in firing (Spearman rank correlation = 0.22, NS).

In the 10 neurons that were activated by a 3 mg/kg, i.v., dose of A-68930, a significant increase in burst activity was also observed (from 17 ± 7 to 32 ± 7 %, $n = 27$, $p < 0.002$ paired Student t test). Such changes in burst activity were not significantly correlated with changes in firing (Spearman rank correlation = 0.46, NS).

Discussion

Our data demonstrates that pridopidine affects the firing and burst activity of prefrontal cortical neurons. More than half of the neurons tested in our paradigm responded to the administration of pridopidine by a moderate to large activation of firing, overall almost doubling their initial basal firing (Fig. 3). It is anticipated that most of the neurons recorded in the present study are pyramidal glutamatergic neurons, which are by far the more represented type of neurons in the PFC (Povysheva et al. 2008) and our recording characteristics match with those of previous electrophysiological studies on identified PFC pyramidal neurons (Hajos et al. 2003; Kargieman et al. 2008; Puig et al. 2005; Tseng and O'Donnell 2007; Wang et al. 2011). GABA interneurons display different electrophysiological properties (Gui et al. 2010; Tierney et al. 2008; Tseng and O'Donnell 2007), still, it cannot be excluded that a small group of GABA interneurons that could not be distinguished electrophysiologically from glutamate pyramidal neurons have been included in the present study.

Our data extends the previous observation that pridopidine, at similar dose-range as in this study, can stimulate the expression of the gene encoding activity-regulated cytoskeleton-associated protein (Arc) in the PFC (Waters 2009). Arc is a key regulator of neuronal plasticity

(Bramham et al. 2010) and a marker of neuronal activity with expression triggered by synaptic NMDA-mediated glutamate signalling (Kawashima et al. 2009; Steward and Worley 2001).

There were, however, interindividual variations regarding the responses of PFC neurons to pridopidine. A significant proportion of neurons remained non responsive and there were also large differences in the amplitude of the excitatory response between neurons. Interestingly, we found that half of the neurons that were insensitive to pridopidine were silent neurons which were only activated by the local microiontophoretic application of an excitatory substance like NMDA. Accordingly, all silent neurons tested in the present study were insensitive to pridopidine, administered either systemically or iontophoretically. In agreement with these data, we did not find that pridopidine could alter PFC population activity, as assessed by the number of spontaneously active neurons per electrode track (Fig. 4). The notion that pridopidine did not seem to be able to turn on PFC neurons from a quiescent state to an active state, indicates that pridopidine may function by strengthening specific neurotransmission system(s) that already tonically modulate the basal activity of PFC pyramidal neurons, rather than generating directly neuronal excitability. It is noteworthy that when the silent neurons were excluded from the statistics, it raised the proportion of neurons responding to pridopidine to 70 % (Table 1).

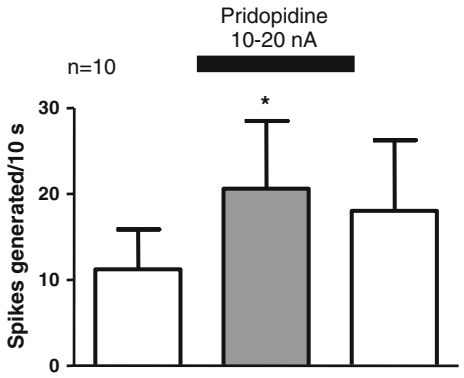

Fig. 8 Mean firing activity in basal condition before and during the iontophoretic application of pridopidine. The figure shows that the baseline activity is significantly increased during pridopidine application. Most neurons did not return to baseline after the end of application of pridopidine. *$p < 0.05$, compared to respective control, Student's paired t test

Whether the effects of pridopidine observed in the PFC are mainly caused by an action on neuronal circuits present in the PFC, or by stimulation of excitatory inputs from region(s) outside the PFC has not been thoroughly investigated in the present study. However, the observation that local application of pridopidine induced a moderate long-lasting activating effect on some neurons argues in favour of the presence of local effects occurring nearby PFC neurons, but probably not directly onto the PFC neurons.

The increase in single-cell firing induced by pridopidine was partially prevented or antagonized by the selective dopamine D1 receptor antagonist SCH23390 (Fig. 5). This suggests that the activating effect of pridopidine is, at least in part, dependent on stimulated dopamine D1 receptors. To the best of our knowledge, pridopidine has not shown

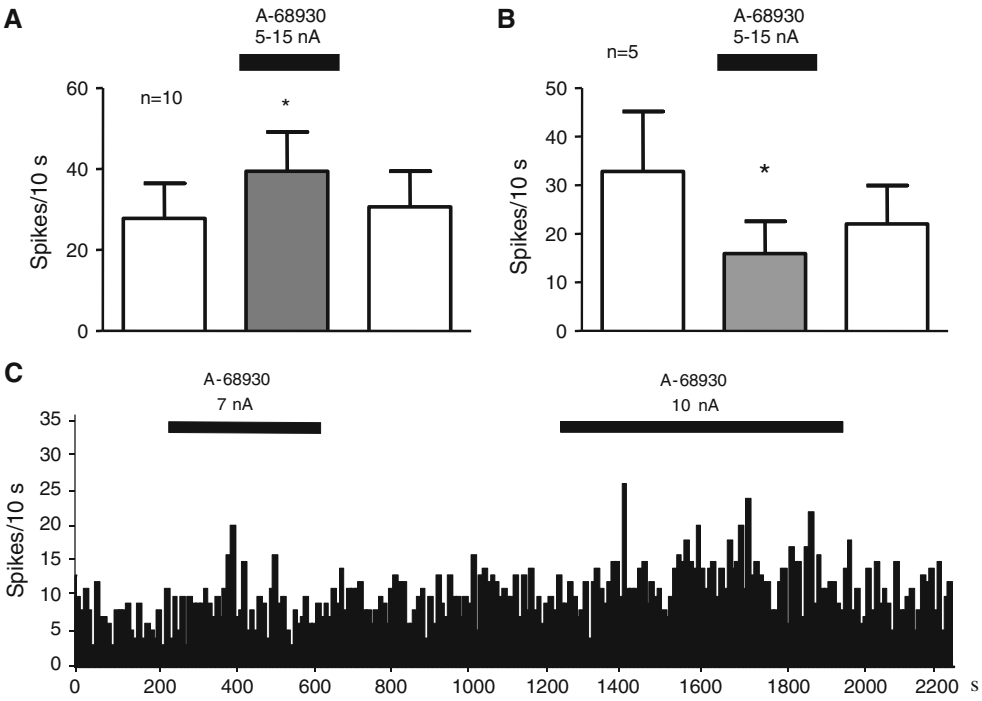

Fig. 9 **a**, **b** Mean firing activity before, during and after the micro-iontophoretic application of the dopamine D1 receptor selective agonist A-68930. Two types of response could be identified: reversible and mild activation of firing (**a**) and reversible inhibition of firing (**b**). *$p < 0.05$, compared to respective baseline and post application values, Newman-Keuls test after significant repeated measure ANOVA. **c** Individual firing rate histograms a representative PFC neurons showing mild excitatory effects of the microiontophoretic application of the selective dopamine D1 receptor agonist A-68930

any pronounced affinity to dopamine D1 receptors. The stimulation of dopamine D1 receptors by pridopidine is probably indirect and may be associated with the action of pridopidine on dopamine turnover and release. In vivo microdialysis experiments have shown that extracellular levels of dopamine can increase up to 200 % of baseline in the rat medial PFC after a systemic administration of pridopidine (Ponten et al. 2010). To note, the administration of the potent dopamine/noradrenaline uptake blocker and psychostimulant methylphenidate (Easton et al. 2007) has been shown to stimulate electrical activity in PFC neurons, in parallel with an activation of expression of Arc protein (Gronier et al. 2009), via (partially at least) a mechanism associated to an increase in dopamine levels and stimulation of local dopamine D1 receptor (Gronier 2011).

The mechanism by which pridopidine can enhance extracellular levels of dopamine in the PFC is currently not fully understood. It cannot be solely explained by the antagonism of pridopidine on dopamine D2 inhibitory autoreceptors in dopamine terminals, as dopamine terminals are scarce in the PFC. Also, it is believed that most of the dopamine present in the PFC is stored and released by noradrenaline terminals, which lack dopamine D2 receptors (Devoto and Flore 2006). Other drugs with greater potency over dopamine D2 receptors such as sulpiride (Ichikawa and Meltzer 1999), raclopride (Andersson et al. 1995) as well as haloperidol, a non-selective but potent dopamine D2 antagonist, (Eltayb et al. 2005; Li et al. 2007; Yamamura et al. 2009) have been shown to produce none or only very moderate increase in dopamine release in the PFC. In the present study, we have observed that haloperidol, tested within a large dose range, was devoid of significant effects on the firing activity of PFC neurons. Pridopidine, as well as compounds with atypical antipsychotic profile, enhance noradrenaline levels in PFC (Ponten et al. 2010). Increase of noradrenaline levels have also been suggested to affect dopamine levels as dopamine can be co-released with noradrenaline from noradrenaline terminals in the PFC (Devoto and Flore 2006). Pridopidine has also been found to interact with sigma receptors at lower concentration than that reported for dopamine D2 receptors (Sahlholm et al. 2013). Sigma receptors agonists, such as pentazocine, have been reported to enhance dopamine release in the PFC by a mechanism that is independent of monoamine transporters (Gudelsky 1995) and to stimulate the firing activity of VTA dopamine neurons (Gronier and Debonnel 1999).

Our results suggest that dopamine D1 receptors partially mediate the activating effect of pridopidine, indicating that dopamine D1 receptors should have excitatory effects on PFC pyramidal neurons. This assumption is supported by our observation that the selective dopamine D1 receptor agonist A-68930 promotes the electrical activation of PFC neurons.

To the best of our knowledge, this is the first study to demonstrate that systemic administration of the dopamine D1 receptor agonist A-68930 produces a moderate activation of PFC neurons at a low dose (0.5 mg/kg, i.v.). More complex effects were observed when A-68930 was administered at higher doses, or by micro-iontophoresis. Indeed, we observed that higher doses (1–3 mg/kg, i.v.) of A-68930 either further activated the firing activity (62 % of the neurons tested) or produced a gradual inhibition of firing, leading sometimes to a complete inactivation of the neurons. Both inhibitory and excitatory effects induced by A-68930 could be reversed by the selective dopamine D1 receptor antagonist SCH23390. As A-68930 could also exert moderate effects (either activating or inhibiting) when applied iontophoretically, its site of action is probably within the PFC, still, other indirect mechanisms involving excitatory or inhibitory inputs to the PFC, cannot be ruled out. In line with our present results, systemic administration of dopamine D1 receptor agonists, including A-68930, have been shown to turn on the expression of immediate early genes such as c-fos and arc mRNA (Isacson et al. 2004; Moro et al. 2007).

Local application of the partial dopamine D1 receptor agonist SKF38393 has shown moderate inhibitory effects or no effect in previous studies (Parfitt et al. 1990; Sesack and Bunney 1989). Potential sources of discrepancy between the present study and these previous studies may be found in, e.g. the selection of dopamine D1 receptor agonist, criteria of characterisation of pyramidal neurons and the methods used for local application. Nevertheless, as in previous studies we have identified a group of neurons which did respond by an inhibition of firing following the application of the dopamine D1 receptor agonist.

Our observation that A-68930, at higher doses, can produce either activating or inhibitory effects, depending of the neurones tested, indicates that it may act on opposite neuronal processes regulating the firing activity of pyramidal neurons. Receptors of the D1 family are found on both pyramidal neurons—particularly on spines and axon terminals—and GABAergic interneurons (Seamans and Yang 2004), predominantly on parvalbumin (PV) interneurons which are the strongest source of inhibition to pyramidal cells (Glausier et al. 2009; Povysheva et al. 2008). It is therefore possible that the inhibition of firing produced by the higher doses of A-68930 (or by its local application) in some neurons was caused by a strong activation of adjacent PV neurons that overcomes the excitatory effects provided by the D1 receptors located on pyramidal neurons.

Surprisingly, direct application of dopamine by microiontophoresis techniques has never been found to activate the firing activity of PFC neurons (Sesack and Bunney 1989; Seamans and Yang 2004), but rather to exert

moderate to large inhibition, as in our study. Electrophysiological studies on iontophoretic dopamine have shown large difference in effects, depending on the injected current of applied dopamine and the brain region investigated (Seamans and Yang 2004). Interestingly, in the striatum, iontophoretic dopamine potentiated glutamate-evoked firing when applied at low ejection currents but dramatically depressed firing at high level of ejection (Hu and Wang 1988). Interestingly, Pirot et al. (1992) found that the inhibitory effects of iontophoretic dopamine on pyramidal PFC neurons are partly caused by activation of GABA interneurons. This indicates that with the iontophoresis technique, ejected drugs could reach distal site of action such as adjacent inhibitory interneurons. In contrast, we found in a recent electrophysiological study that moderately raising dopamine concentration nearby PFC neurons, simply by passive diffusion through the recording electrode, in combination with dopamine uptake blockade, can powerfully stimulate their electrical activity in a dopamine D1 receptor-dependent manner (Gronier 2011). Spontaneous leak of dopamine from the recording electrode should cause a more local increase in dopamine level, which when further amplified by a dopamine uptake blocker, provides enough dopamine in the synaptic cleft to stimulate postsynaptic D1 receptors and to activate firing of PFC neurons. Overall, these results further support the hypothesis that increasing the level of dopamine in the PFC near the site of recording could stimulate neuronal activity, through activation of cortical D1 receptors. Therefore, we could hypothesise that pridopidine elicits neuronal activation in PFC partly through evoked-dopamine release and D1 receptor stimulation. However, this would not be the only mechanism involved in the pridopidine-induced neuronal activation. First, pridopidine and the dopamine agonist do not display exactly the same profile of activation of PFC neurons (as the D1 agonist can also promote inhibition in some neurones). Secondly, the dopamine D1 receptor antagonist SCH23390 only partially reversed or prevented the effect of pridopidine, which confirms that the drug can also activate PFC neurons via interaction with other receptors. As a matter of fact, pridopidine has non-negligible affinity for the adrenergic alpha-2 receptor, 5-HT1A receptor and sigma receptor (Ponten et al. 2013; Sahlholm et al. 2013), which all have potential excitatory effects on PFC neurons. Future studies would need to investigate the putative role of these different receptors.

To note, A-68930, like pridopidine, activated spontaneously active neurons but not silent neurones, contrarily to classical excitatory substances. The spontaneous activity of PFC neurons has been shown to be mediated by mutual excitation between glutamatergic pyramidal neurons and regulated by inhibitory GABAergic interneurons. Therefore, stimulation of dopamine D1 receptors, the role of which is to "modulate and amplify" synaptic signals (Kritzer and Goldman-Rakic 1995), may function by strengthening the tonic glutamate regulation of firing activity of PFC neurons, rather than by directly activating electrical activity. Dopamine D1 receptors and some NMDA receptors have been shown to be co-localised and to interact synergically in the PFC (Kruse et al. 2009; Wang and O'Donnell 2001). Interestingly, our present study has demonstrated an important propensity of pridopidine and A-68930 to elicit burst activity in PFC neurons, in a way that seemed, at least in part, independent of the increase in firing activity. Spontaneous burst activity in the PFC, which is thought to promote synaptic potentiation by providing more efficient neurotransmitter release and dendritic depolarisation in terminals, is in part regulated by the synergic tonic activation of metabotropic glutamate receptor 5 and of NMDA glutamate receptors subtypes (Homayoun and Moghaddam 2006; Sidiropoulou et al. 2009). Therefore, it is possible that enhancement of the function of these receptors, through dopamine D1 receptors, is indirectly involved in the burst-promoting effects of both pridopidine and A-68930.

The interaction between dopamine D1 and NMDA receptors leading to an increase in excitability and burst activity of PFC neurons may be essential in regulating glutamatergic corticostriatal transmission, critically controlling the induction of long-term potentiation and long-term depression at corticostriatal synapses. This may impact on cortical as well as on striatal synaptic plasticity and possibly, via this way, help improve motor control in Huntington's disease (HD). As a matter of fact, there is evidence of dopamine-dependent loss of synaptic plasticity in PFC and abnormal prefrontostriatal processing in animal models of HD (Dallerac et al. 2011; Hohn et al. 2011).

In conclusion, our study demonstrates that pridopidine can stimulate neuronal activity in the PFC, by a mechanism involving in part dopamine D1 receptors. In agreement with this result, our study also showed that activation of central dopamine D1 receptors can stimulate the electrical activity of PFC neurons. We hypothesise that these actions may improve corticostriatal connectivity and play a role in the therapeutic benefit of pridopidine in patients with Huntington's disease.

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