Memantine Inhibits ATP-Dependent K+ Conductances in Dopamine Neurons of the Rat Substantia Nigra *Pars Compacta*

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ABBREVIATIONS:

AP5, D-(-)-2-amino-5-phosphonoephanoic acid; $I_{HYPO}$, hypoxia-induced outward current; $I_{NMDA}$, NMDA-mediated inward current; $K_{ATP}$, adenosine triphosphate-sensitive potassium; MK-801, (5S,10R)-(+)−5-Methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate; NMDA, N-methyl-D-aspartate; SNc, substantia nigra pars compacta.

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ABSTRACT

Memantine is a non competitive NMDA receptor antagonist used in the clinical practice to treat neurodegenerative disorders that could be associated to excitotoxic cell death. Since memantine reduces the loss of dopamine neurons of the substantia nigra pars compacta (SNc) in animal models of Parkinson’s disease, we examined the effects of this drug on dopamine cells of the SNc. Beside inhibition of NMDA receptor-mediated currents, memantine (30 and 100 µM) increased the spontaneous firing rate of whole-cell recorded dopamine neurons in a midbrain slice preparation. Occasionally, a bursting activity was observed. These effects were independent from the block of NMDA receptors and were prevented in neurons dialyzed with a high concentration of ATP (10 mM). An increase in firing rate was also induced by the ATP-sensitive potassium (K\textsubscript{ATP}) channel antagonist tolbutamide (300 µM), and this increase occluded further effects of memantine. In addition, K\textsubscript{ATP} channel-mediated outward currents, induced by hypoxia, were inhibited by memantine (30 and 100 µM) in the presence of the NMDA receptor antagonist MK-801 (10 µM). An increase in the spontaneous firing rate by memantine was observed in dopamine neurons recorded with extracellular planar 8x8 mutielectrodes, in conditions of hypoglycemia. These results highlight K\textsubscript{ATP} channels as possible relevant targets of memantine effects in the brain. Moreover, in view of a proposed role of K\textsubscript{ATP} conductances in dopamine neurons degeneration, they suggest another mechanism of action underlying the protective role of memantine in Parkinson’s disease.
INTRODUCTION

Memantine (1-amino-3,5-dimethyl-adamantane) is a derivative of amantadine, used in the clinical practice to treat several neurological disorders associated to excitotoxic cell death, including Parkinson’s disease, amyotrophic lateral sclerosis, Alzheimer’s disease, epilepsy, stroke, spasticity, convulsions and vascular dementia (Fleischhacker et al., 1986; Ditzler, 1991; Sonkusare et al., 2005; Chen and Lipton, 2006; Volbracht et al., 2006). Its mechanism of action resides on the ability of memantine to bind NMDA receptors in a non competitive manner, acting as a low-affinity open-channel blocker (Lipton 2004; Lipton 2006). Because of its peculiar pharmacological properties, memantine is proposed to be beneficial, as it blocks excessive NMDA receptors activation, without interfering with their physiological activity. In so doing, it is a well tolerated NMDA receptor antagonist, able to reduce or prevent the excitotoxic damage, without producing undesired side effects, like hallucination, agitation, catatonia, centrally mediated increase in blood pressure and anesthesia, typical of other NMDA receptor antagonists.

Although NMDA receptors constitute the main target of memantine, other mechanisms of action have been reported, including reduced action potential firing in cultured neurons (Netzer and Bigalke, 1990), block of 5-HT3 receptors (Reiser at al., 1988; Rammes et al., 2001) and of nicotinic receptors (Maskell et al., 2003; Aracava et al., 2005). These results suggest the presence of additional mechanisms of action, whose relative importance may be dependent on the brain area under investigation.

An anti-parkinsonian activity has been described for memantine, in animal models of Parkinson’s disease and in parkinsonian patients (Danysz et al., 1997; Merello et al., 1999). In addition, memantine prevents cell death induced by 1-methyl-4-phenyl-1,2,3,6-
tetrahydropyridine (Lange et al., 1997; Kucheryanu and Kryzhanovskii, 2000). Therefore, we focused our attention onto the action of this drug on the dopamine neurons of the substantia nigra pars compacta (SNc), whose progressive degeneration is a hallmark of Parkinson’s disease. Indeed, several lines of evidence indicate an over-stimulation of glutamate receptors, especially of the NMDA subtype, as the main cause of the progressive loss of this neuronal population (Gardoni and Di Luca, 2006), thus, NMDA receptor antagonism by memantine in the SNc is expected to represent the main mechanism underlying its neuroprotective action. Accordingly, we now present evidence that memantine is effective in reducing NMDA-mediated currents of the dopamine neurons, however, we also demonstrate a novel mechanism of action of this drug, consisting on the ability to reduce ATP-mediated potassium (K\textsubscript{ATP}) conductances, opened in conditions of metabolic stress.
MATERIAL AND METHODS

Slice Preparation

Wistar rats (18-25 days old) were anaesthetized with halothane and killed by decapitation. All experiments have been carried out in accordance with the Declaration of Helsinki and follow international guidelines on the ethical use of animals from the European Communities Council Directive of 24 November 1986 (86/609/EEC). The brain was rapidly removed from the skull and horizontal midbrain slices (250 µm) were cut in cold (8-12°C) artificial cerebrospinal fluid (ACSF) and left to recover at 33.5°C for at least one hour. ACSF composition was the following (in mM): NaCl 126; KCl 2.5; MgCl2 1.2; CaCl2 2.4; NaH2PO4 1.2; NaHCO3 24; glucose 11; saturated with 95% O2, 5% CO2 (pH 7.4).

Patch-clamp Recordings

An individual slice was placed in a recording chamber, on the stage of an upright microscope (Axioscope FS, Carl Zeiss Spa, Arese, Italy) and submerged in a continuously flowing (2.5 ml/min) solution at 33°C (± 0.2°C). Neurons were visualized with infrared video microscopy (Hamamatsu Photonics Italia Srl, Milan, Italy). Borosilicate glass electrodes (3-4 MΩ) were filled with (in mM): K-Gluconate 135; KCl 10; MgCl2 2; CaCl2 0.045; EGTA 0.1; HEPES 10; ATP 2; GTP 0.3 (pH 7.3, with KOH). In experiments with high calcium buffer, EGTA concentration was raised to 10 mM and CaCl2 to 4 mM, while K-Gluconate was reduced to 115 mM. In experiments with high ATP, the filling solution was the same as the control solution, but with 10 mM ATP. Whole-cell voltage clamp (–60 mV holding potential) or current clamp experiments were carried out with a MultiClamp 700A amplifier (Molecular Devices Co, Sunnyvale, USA).
filtered at 1 kHz and digitized at 10 kHz. Dopamine neurons were identified electrophysiologically on the basis of a prominent Ih in response to hyperpolarizing voltage steps and a voltage sag when negative current steps were applied in current-clamp mode (Grillner and Mercuri, 2002). Data are expressed as mean ± s.e.m and compared using the Student’s t-test, with $p < 0.05$ as minimum level for significance.

Pressure applied N-methyl-D-aspartate (NMDA; 10 psi, 0.5-1.0 s) was used to obtain NMDA-mediated inward currents ($I_{NMDA}$), through a patch electrode filled with NMDA (100 µM) dissolved in ACSF, connected to a Pneumatic Pico-pump PV 800 (WPI, Berlin, Germany). The puff electrode was positioned above the slice, in close proximity of the recorded neuron.

Hypoxic insults were obtained by exposing the slices for 2-3 min to ACSF saturated with 95% N₂, 5% CO₂.

**Multielectrode Recordings**

Individual slices were placed over an 8 x 8 array of planar microelectrodes, each 20 x 20 µm in size, with an interpolar distance of 100 µm (MED-P2105; Matsushita Electric Industrial Co., Ltd. Kadoma, Japan). Slices were submerged under a nylon mesh and positioned over the multielectrode array under visual control, through an upright microscope (Leica DM-LFS, Leica Microsystems, Wetzlar, Germany), in such a way that the area close to the medial terminal nucleus of the accessory optic tract covered most of the electrodes (Geracitano et al., 2005). Extracellular signals were acquired using the Panasonic MED64 System (Alpha MED Sciences, Kadoma, Japan). Signals were low-cut filtered at 100 Hz and digitized at 20 kHz with a 6071E Data Acquisition Card (National Instruments, Austin, USA) using the MED64 Conductor Software (Alpha MED
Sciences, Japan). The frequency of the fast transients corresponding to spontaneous action potentials was calculated off line with Spike2 software (Cambridge Electronic Design Ltd, Cambridge, UK), using an amplitude threshold adjusted by visual inspection in each individual active channel. Spikes recorded by a single channel could differ in shape and amplitude, reflecting spontaneous action potentials arising from more than one neuron, therefore, spike sorting discrimination of multi-unit responses was achieved by generating spike templates with Spike2 (Cambridge Electronic Design Ltd, Cambridge, UK), sorted with a Normal Mixtures algorithm on independent clusters obtained from principal component data.

On average, we detected spikes from 34.6 ± 1.5 active channels in each midbrain slice (n = 14), however, following the spike sorting procedure, activity arising from a total of 82.7 ± 3.6 cells per slice could be obtained. Since dopamine neurons of the SNc are selectively hyperpolarized by dopamine (Grillner and Mercuri, 2002), we used sensitivity to dopamine (30 µM) as a criterion to discriminate spikes originating from this neuronal population. 43.4 ± 2.0 cells per slice, out of the total number of cells, met this pharmacological criterion, and were considered for further analysis. Hypoglycemic conditions were obtained perfusing the slices in ACSF containing 1 mM glucose, for 25 to 30 min, before being challenged with memantine or tolbutamide (Figs. 6 and 7). This procedure caused an overall reduction of firing frequency in the dopamine cells population, and in some of them a complete loss of activity occurred. Only the dopamine cells whose firing could still be detected during the experimental session in hypoglycemia were considered to evaluate the effects of memantine or tolbutamide.
Drugs

All drugs, with the exception of NMDA, that was pressure applied, were applied in the bath. They included, 3,4-Dihydroxyphenethylamine (dopamine), D-(-)-2-amino-5-phosphonoctanoic acid (AP5), 5S,10R-(+)-5-Methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate (MK-801), 1-Buthyl-3-(4-methylbenzenesulfonyl)urea (tolbutamide), from Sigma-Aldrich, Milan, Italy; 1,2,3,4,10,14b-Hexahydro-2-methylpyrazino[2,1-a]pyrido[2,3-c][2]benzazepine (mirtazapine), [1α,4(S),6β,14α,16β]-20-Ethyl-1,6,14,16-tetramethoxy-4-[[2-(3-methyl-2,5-dioxo-1-pyrrolidinyl) benzoyloxy]methyl]aconitane-7,8-diol citrate (methyllycaconitine), from Tocris Cookson Ltd, Bristol, UK; 1-amino-3,5-dimethyladamantane (memantine), from Lundbeck Italia SpA, Milan, Italy.
RESULTS

Memantine Inhibits NMDA-Mediated Currents

Dopamine neurons of the SNc were identified on the basis of electrophysiological criteria (see Methods) and recorded in voltage-clamp mode, at -60 mV holding potential. We investigated the effects of memantine on NMDA-mediated inward currents ($I_{\text{NMDA}}$) evoked by pressure application of NMDA (100 µM; 10 psi, 0.5-1.0 s) at 3 min interval, through a glass pipette positioned in close proximity of the recorded neuron. $I_{\text{NMDA}}$ (190.5 ± 18.4 pA, n = 26) remained constant upon repeated application of NMDA and was sensitive to the NMDA receptor antagonist MK-801 (10 µM; n = 4; Fig. 1). Perfusion with memantine (30 µM) reversibly reduced $I_{\text{NMDA}}$ amplitude to 48.8 ± 2.9 % of control (n = 15). Rising memantine concentration to 100 µM produced further inhibition of $I_{\text{NMDA}}$ amplitude (26.7 ± 3.0 % of control, n = 15; Fig. 1B). These results confirmed the ability of memantine to inhibit NMDA receptor-mediated responses in dopamine neurons of the SNc.

NMDA receptor-independent Increase of Dopamine Neurons Firing Rate by Memantine

We then investigated the effects of memantine on dopamine neurons firing properties. Neurons were recorded in current-clamp mode, in order to detect their typical spontaneous tonic action potentials discharge (Grillner and Mercuri, 2002). We found that memantine, on its own, was capable of increasing the basal firing frequency of the recorded neurons. At 30 µM, memantine reversibly increased the firing frequency from 1.1 ± 0.2 to 2.0 ± 0.3 Hz ($p < 0.002$ paired t-test, n = 11; Fig. 2A). At higher concentration (100 µM), memantine increased the firing rate from 1.4 ± 0.1 to 3.2 ± 0.3
Hz (\( p < 0.001 \) paired t-test, \( n = 21 \); Fig. 2A,D) and in 12 cells it transformed their typical tonic firing into rhythmic bursts of action potentials (Fig. 2B).

In order to examine whether this effect of memantine could be due to reduction of tonic NMDA receptors stimulation within the neuronal circuitry, we repeated the same experiments in the presence of MK-801 (10 \( \mu \text{M} \)) or AP5 (50 \( \mu \text{M} \)), however, both these NMDA receptor antagonists did not mimic nor prevent the effects of memantine (100 \( \mu \text{M} \)) on dopamine neurons firing (Fig. 2C,D).

Memantine has also been reported to act as an antagonist of 5-HT3 and \( \alpha \)7-containing nicotinic receptors (Reiser at al., 1988; Rammes et al., 2001; Maskell et al., 2003; Aracava et al., 2005), however, in the presence of the 5-HT3 receptor antagonist mirtazapine (100 \( \mu \text{M} \)) and of the \( \alpha \)7-containing nicotinic receptor antagonist methyllycaconitine (10 nM), memantine (100 \( \mu \text{M} \)) still produced a significant increase in dopamine neurons firing rate (Fig. 2D).

**No effect of Memantine in Multielectrode Recordings of Dopamine Neurons Firing Rate**

We then tried to corroborate these results using a less invasive technique of spontaneous firing recording, in the same midbrain slice preparation, consisting of single units’ detection from an 8x8 array of planar electrodes (Geracitano et al., 2005). Since dopamine neurons of the SNc are selectively hyperpolarized by dopamine (Grillner and Mercuri, 2002), spikes originating from dopamine neurons were identified on the basis of their sensitivity to brief (30-60 s) exposure to dopamine 30 \( \mu \text{M} \). Using this pharmacological criterion, we recorded the spontaneous firing arising from a total of 302 presumed dopamine neurons, from 6 midbrain slices. Their basal firing rate was 2.08 \( \pm \)
0.10 Hz and no significant alteration in spontaneous firing we observed following 100 µM memantine perfusion (2.05 ± 0.01 Hz in memantine, \( p > 0.27 \) paired \( t \)-test; Fig. 3).

**High Intracellular ATP Prevents the Effects of Memantine on Dopamine Neurons Firing**

In order to elucidate why memantine increased dopamine neurons firing rate only when they were recorded with patch-clamp electrodes, we reasoned that intracellular dialysis associated with this technique might be responsible for some form of alteration in the cellular physiology, unmasking a novel property the drug, independent of its ability to antagonize NMDA receptor-mediated responses.

We first increased the calcium buffering properties of the patch-clamp electrodes filling solution, using high (10 mM) EGTA, however, memantine 100 µM still increased the neuronal firing (\( n = 3 \); data not shown). In contrast, when the dopamine neurons were recorded with high (10 mM) intracellular ATP, no significant change in their firing rate was observed in 100 µM memantine (from 1.6 ± 0.2 to 1.7 ± 0.2 Hz in memantine; \( n = 8 \), \( p > 0.2 \) paired \( t \)-test; Fig. 4A,C).

**The Block of K\textsubscript{ATP} Channels Occludes the Effects of Memantine on Dopamine Neurons Firing**

DA neurons are highly enriched in ATP-sensitive potassium (K\textsubscript{ATP}) channels, opened when intracellular ATP levels are reduced under conditions of metabolic stress (Mercuri et al., 1994; Guatteo et al., 1998; Marinelli et al., 2000; Liss et al., 2005). We thus hypothesized that memantine depolarized the dopamine neurons by blocking K\textsubscript{ATP}...
channels, partially opened in neurons recorded using a standard 2 mM ATP filling solution.

In agreement with this hypothesis, we found that perfusion with the $K_{\text{ATP}}$ channel antagonist tolbutamide (300 µM) did not change the firing rate of dopamine neurons recorded with 10 mM intracellular ATP (from 1.7 ± 0.1 to 1.6 ± 0.1 Hz in tolbutamide; $n = 9$, $p > 0.3$ paired $t$-test), while it increased in the firing rate of neurons recorded using 2 mM ATP (from 1.3 ± 0.2 to 2.5 ± 0.3 Hz in tolbutamide, $p < 0.01$ paired $t$-test, $n = 6$; Fig. 4C). Moreover, in the same neurons, no further increase in the frequency of action potential discharge was observed when 100 µM memantine was added, in the continuous presence of tolbutamide (2.5 ± 0.3 Hz in tolbutamide and memantine; $p > 0.54$ paired $t$-test, $n = 6$; Fig. 4B,C).

**Memantine Reduces $K_{\text{ATP}}$-mediated Outward Currents Induced by Hypoxia**

The previous experiments strongly suggest that memantine reduces the opening of tolbutamide-sensitive $K_{\text{ATP}}$ channels. To further confirm this hypothesis, we directly tested the involvement $K_{\text{ATP}}$ conductances by briefly exposing the slice (2-3 min) to a hypoxic medium. These experiments were performed in the continuous presence of MK-801 (10 µM), in order to rule out indirect actions through NMDA receptors. As previously demonstrated (Guatteo et al., 1998), this experimental protocol induces the development of a $K_{\text{ATP}}$-dependent outward current, in dopamine neurons recorded in voltage-clamp mode. As shown in Fig. 5, bath perfusion of memantine 30 µM reversibly reduced hypoxia-induced outward current ($I_{\text{HYPO}}$) to 73.6 ± 3.8 % of control ($n = 12$). Rising memantine concentration to 100 µM produced further inhibition of $I_{\text{HYPO}}$ to 47.9 ±
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3.6 % of control (n = 11). Moreover, \( I_{\text{HYPO}} \) was abolished by tolbutamide (300 µM, n = 4), thus confirming an effect of memantine on \( K_{\text{ATP}} \) conductances.

**Multielectrode Recordings of Firing Rate Increase in Hypoglycemic Conditions**

Exposure of the dopamine neurons to a hypoglycemic medium inhibits SNc dopamine neurons through activation of \( K_{\text{ATP}} \) channels (Marinelli et al., 2000). Therefore, we tested whether the spontaneous firing rate of dopamine neurons recorded with the multielectrode system could be increased by memantine in hypoglycemic conditions. In order to avoid an excessive metabolic stress, we perfused the slices in 1 mM glucose, a threshold dose for dopamine neurons hyperpolarization (Marinelli et al., 2000). As shown in Fig 6, when the recordings were obtained in ACSF containing 1 mM glucose, a low overall firing rate was detected (compare with Fig 3). In these experimental conditions, memantine 100 µM reversibly increased the firing rate of presumed dopamine neurons. When glucose was raised to 2 mM in the same slice, the overall firing rate increased, and memantine (100 µM) became ineffective, in accordance with what was previously observed in normoglycemic conditions (Fig. 3). Similar results were obtained from a total of 47 presumed dopamine neurons, from 4 midbrain slices perfused in 1 mM glucose. Their basal firing rate increased from 0.37 ± 0.09 to 1.51 ± 0.28 Hz (\( p < 0.0001 \) paired t-test) in memantine 100 µM.

In accordance with the hypothesis of an involvement of \( K_{\text{ATP}} \) channels, we found that tolbutamide (300 µM) increased the spontaneous firing rate in hypoglycemic conditions and no further increase was induced by memantine (100 µM; Fig.7). When glucose was raised to 10 mM in the same slice, the overall firing rate increased, and tolbutamide (300 µM) became ineffective (Fig. 7). In 74 presumed dopamine neurons recorded from a
total of 4 midbrain slices perfused in 1 mM glucose, tolbutamide (300 µM) increased their firing rate from 0.51 ± 0.05 to 1.72 ± 0.12 Hz ($p < 0.0001$ paired $t$-test). Exposure to memantine (100 µM) in hypoglycemia and tolbutamide, did not produce further increase in firing rate (1.56 ± 0.11 Hz).
DISCUSSION

Our results provide a direct electrophysiological demonstration of a novel mechanism of action of the amantadine derivative memantine. We have shown that memantine, beside its expected property of NMDA receptor antagonist, reduces neuronal hyperpolarization mediated by the opening of $K_{\text{ATP}}$ channels, in dopamine neurons of the SNc, in a dose-dependent manner.

Three lines of evidence point at this conclusion. First, we have shown that action potential discharge of the dopamine neurons increased in frequency upon memantine perfusion, in dopamine neurons recorded using the patch-clamp whole-cell technique.

This effect was evident when the internal patch-clamp electrode solution contained 2 mM ATP, while, under high (10 mM) internal ATP, memantine was ineffective. During whole-cell recordings, ATP internal concentration is largely imposed by the filling solution composition, thus, the concentration of ATP in the patch-clamp electrode greatly affects the degree of $K_{\text{ATP}}$ channels opening in response to metabolic stress. For instance, previous experiments from our laboratory have shown that dopamine neurons exposed to a hypoxic medium develop a $K_{\text{ATP}}$-dependent current only when they are recorded with 2 mM ATP in the patch-clamp electrode solution, while no $K_{\text{ATP}}$ outward current is induced by hypoxia if internal ATP is set to 10 mM (Guatteo et al., 1998). On this base, we hypothesized that memantine increased the firing rate of dopamine neurons recorded in whole-cell conditions, by closing $K_{\text{ATP}}$ channels partially open under 2 mM internal ATP. This hypothesis was confirmed by the observation that the $K_{\text{ATP}}$ channel antagonist tolbutamide produced an analogous increase in the firing rate of dopamine neurons recorded with 2 mM internal ATP, while the same antagonist was
ineffective when intracellular ATP was set to 10 mM. Moreover, the frequency increase induced by tolbutamide in 2 mM ATP occluded any additional increase by memantine. The second evidence supporting our hypothesis derives from experiments conducted using the non-invasive, extracellular 8x8 planar multielectrode device. Presumed dopamine neurons, identified on the basis of their sensitivity to exogenously applied dopamine, were insensitive to memantine in control experimental conditions. However, memantine markedly increased dopamine neurons firing rate, in slices perfused in 1 mM glucose ACSF, instead of 10 mM. Previous experiments from our laboratory demonstrated that 1 mM glucose is a threshold hypoglycemic condition for the development of a K\textsubscript{ATP}-dependent hyperpolarization in dopamine neurons of the SNc (Marinelli et al., 2000). Conceivably, the reduced basal firing rate in 1 mM glucose, compared to controls, was due to a more hyperpolarized state of the dopamine neuronal population, because of the opening of K\textsubscript{ATP} conductances in 1 mM glucose. Thus, memantine increased dopamine neurons firing rate by closing these constitutively open K\textsubscript{ATP} channels. This hypothesis was confirmed by the observation that the K\textsubscript{ATP} channel antagonist tolbutamide mimicked the effects of memantine, and occluded additional increase in the firing rate by memantine in hypoglycemia. Both memantine and tolbutamide were ineffective under higher glucose concentration, because no tonic K\textsubscript{ATP}-dependent current was present in these conditions. Indeed, these experiments also suggest that the tonic activation K\textsubscript{ATP} conductances observed in our patch-clamp whole-cell recordings, using 2 mM internal ATP, is a non-physiological phenomenon, as no such a tonic K\textsubscript{ATP} current seems to be evident using the less invasive multielectrode extracellular technique.
Finally, the third, more direct, piece of evidence of memantine effects on $K_{\text{ATP}}$ conductances derives from our experiments using brief exposures of the dopamine neurons to hypoxia. Dopamine neurons respond to oxygen deprivation with an early hyperpolarization, largely due to opening of $K_{\text{ATP}}$ channels (Mercuri et al., 1994; Guatteo et al., 1998; Geracitano et al., 2005). Memantine reversibly reduced this tolbutamide-sensitive outward current, even in the presence the open-channel NMDA receptor antagonist MK-801, thus, ruling out any possible indirect effect through inhibition of NMDA receptors.

As shown in Fig. 2, memantine not only increased the firing rate of the dopamine neurons, but also changed occasionally their firing mode, from tonic to bursting behavior. This change in the firing pattern may have important functional implications, as burst firing of the dopamine neurons has been associated to increased release of dopamine in the areas of nigral projection (Gonon and Buda, 1985; Suaud-Chagny et al., 1992; Floresco et al., 2003; Phillips et al., 2003). However, such a change was observed using high doses of memantine and, more importantly, we never observed burst firing in neurons recorded using the extracellular multielectrode technique. Probably, burst generation by memantine is linked to some unspecific effect, amplified by a more invasive technique, like whole-cell patch-clamp recording. However, we cannot exclude the possibility that this burst-inducing effect of memantine emerges under more complex pathological alterations of the intracellular composition, somehow mimicked in our patch-clamp recording conditions.

**Clinical relevance**
A question arising from our experimental observation is whether the above effects, obtained from dopamine neurons recorded in an *in vitro* slice preparation, also occur in patients under memantine treatment. According to *in vivo* measurements, treatment with a standard 20 mg daily dose of memantine should result in a steady-state brain level of this drug approaching the low micromolar range (Hesselink et al., 1999; Danysz et al., 2000); therefore, it is suggested that results obtained using higher concentrations may reflect unspecific and non clinically relevant effects (Chen and Lipton, 2006). However, attention should be put into the limiting factor offered in a slice preparation by the need of the drug to diffuse within the brain tissue, during a relatively short time of bath perfusion. Consequently, the effective concentration of the drug reaching the neuronal membranes is not the same as that of the extracellular medium. Indeed, an almost complete block of NMDA receptor-mediated responses can be achieved in isolated cell cultures at concentrations of memantine close to 10 μM (Chen et al., 1992), while, in our brain slice preparation, inhibition of NMDA receptor mediated responses hardly exceeded 50% at 30 μM of memantine, or 75% at a concentration of 100 μM (Fig. 1B). This difference supports the clinical relevance of our present results, as they should be compared to those obtained on isolated neurons, using lower concentrations of memantine. In addition, we have also shown that the effects of 100 μM memantine were completely prevented in patch-clamp recordings obtained with a 10 mM ATP filling solution, and the same concentration of memantine did not modify the firing rate of the dopamine neurons using the multielectrode device, in normoglycemic conditions. Both these observations rule out unspecific effects simply due to a high dose of memantine.

**Memantine and Parkinson’s disease**
According to our observations, memantine does not affect the basal firing activity of the dopamine neurons in physiological conditions, while, in conditions of metabolic stress, a significant effect of memantine emerges, resulting in recovery of firing activity of previously silenced dopamine neurons. This property may be particularly relevant in terms of firing dependent dopamine release and in relation to prevention of neuronal loss in Parkinson’s disease. The activity of complex I of the mitochondrial respiratory chain is reduced in dopamine neurons of parkinsonian patients (Schapira, 2001), and drugs acting as inhibitors of complex I, like rotenone or 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine induce dopamine neurons degeneration (Betarbet et al., 2000; Przedborski and Vila, 2003). Inhibition of mitochondrial complex I may lead to reduction of ATP and increased production of reactive oxygen species (Hoglinger et al., 2003; Testa et al., 2005), causing the opening of K\textsubscript{ATP} channels and dopamine neurons silencing (Avshalumov et al., 2005; Geracitano et al., 2005; Berretta et al., 2005). A recent report by Liss and colleagues (2005) proposed that the selective vulnerability of the SNc dopamine neurons in Parkinson’s disease is casually correlated with the opening of K\textsubscript{ATP} conductances in these neurons, thus, the presence of functional K\textsubscript{ATP} channels promotes the selective loss of SNc dopamine neurons in both a genetic model of Parkinson’s disease and in response to mitochondrial complex I inhibition. At present, the mechanism through which K\textsubscript{ATP} channel opening contributes to dopamine neurons degeneration is still unclear. There is evidence that increasing neuronal excitability protects dopamine neurons from degeneration (Salthun-Lassalle et al., 2004), for this reason Liss and colleagues (2005) proposed that drugs acting at K\textsubscript{ATP} channels of SNc dopamine neurons, should cause a recovery from their functional silencing, thus providing a clinical benefit in the treatment of Parkinson’s disease. Indeed, memantine
has been shown to prevent cell death associated to Parkinson’s disease (Danysz et al., 1997; Lange et al., 1997; Merello et al., 1999; Kucheryanu and Kryzhanovskii, 2000), although prevention of excitotoxic neuronal damage through an uncompetitive inhibition of NMDA receptors has been proposed as its underlying mechanism of action. Our results show that memantine does inhibit NMDA responses in the SNc, however, memantine may also result beneficial in Parkinson’s disease patients because it reduces dopamine neurons silencing through closure of $K_{ATP}$ conductances.
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REFERENCES


FOOTNOTES

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LEGENDS FOR FIGURES

Fig. 1. Dose-dependent inhibition of the NMDA receptor mediated inward current ($I_{\text{NMDA}}$) evoked on dopamine neurons by pressure application of NMDA (100 µM). (A) Bottom, plot of $I_{\text{NMDA}}$ amplitude against time, in response to perfusion with memantine (30 µM) and MK-801 (10 µM). On top, raw traces of $I_{\text{NMDA}}$, acquired at the times indicated by the corresponding letters in the plot. (B) Histogram of $I_{\text{NMDA}}$ amplitude expressed as % of control (mean ± s.e.m.), following exposure to 30 and 100 µM memantine, and 10 µM MK-801.

Fig. 2. NMDA receptor-independent effects of memantine on dopamine neurons spontaneous firing rate. (A) Plot of the instantaneous firing frequency against time from a single dopamine neuron, showing the reversible increase in action potential discharge induced by perfusion of 30 and 100 µM memantine. (B) Trace records from a dopamine neuron exposed to 100 µM memantine, showing a reversible change in the firing mode of action potential discharge, from tonic to bursting activity. (C) Trace records from a dopamine neuron exposed to 100 µM memantine in the continuous presence of MK-801 (10 µM). (D) Histogram of the increase in firing rate (mean ± s.e.m.) induced by 100 µM memantine, in control conditions, in the presence of MK-801 (10 µM), AP5 (50 µM) and mirtazapine (10 µM) + methyllycaconitine (10 nM). * $p < 0.05$, *** $p < 0.001$; paired $t$-test.

Fig. 3. Lack of effect of memantine on dopamine neurons firing rate using the extracellular multielectrode array technique. Plot of the average spontaneous firing frequency (mean ± s.e.m) against time (10 s bin size) obtained from the raster plots.
shown above. On top, raster plots are shown of single unit action potentials, obtained from a total of 44 neurons sensitive to dopamine (30 µM, left) in a single midbrain slice. The same neurons were insensitive to memantine (100 µM, right).

Fig. 4. Involvement of K$_{\text{ATP}}$ conductances in the effects of memantine on dopamine neurons firing rate. (A) Trace records from a dopamine neuron recorded with a patch pipette filling solution containing 10 mM ATP. Exposure to 100 µM memantine did not result in significant alteration of the spontaneous firing rate. (B) Trace records from a dopamine neuron recorded with a patch pipette filling solution containing 2 mM ATP. Perfusion with the K$_{\text{ATP}}$ channel antagonist tolbutamide (300 µM) produced an increase in the firing rate and no further increase resulted from perfusion of memantine (100 µM) in the continuous presence of tolbutamide. (C) Histogram of dopamine neurons firing rate (mean ± s.e.m.) recorded using 10 mM ATP (left) or 2 mM ATP (right) in the patch pipette filling solution. The spontaneous firing rate did not change significantly in neurons recorded in 10 mM ATP, exposed to memantine (100 µM) or tolbutamide (300 µM). Conversely, a significant increase was induced by tolbutamide (300 µM) in neurons recorded with 2 mM ATP, but no additional increase in the firing rate was observed in the same neurons, when memantine (100 µM) was added in the continuous presence of 300 µM tolbutamide. ** $p < 0.01$; paired t-test.

Fig. 5. Inhibition by memantine of K$_{\text{ATP}}$-dependent hypoxia-induced outward current (I$_{\text{HYPO}}$). All experiments were conducted in the continuous presence of MK-801 (10 µM). (A) Trace record obtained from a single dopamine neuron, showing I$_{\text{HYPO}}$ generated in response to repeated 2 min exposures (vertical arrows) to ACSF saturated with 95% N$_2$, ...
5% CO₂. Memantine 30 and 100 µM reversibly inhibited I_{HYPO}, in a dose-dependent manner. Perfusion with the Kₐtcp channel blocker tolbutamide (300 µM) completely abolished I_{HYPO}, unmasking a small inward current followed by an electrogenic post-hypoxic outward response (Mercuri et al., 1994; Guatteo et al. 1998). (B) Histogram of I_{HYPO} amplitude expressed as % of control (mean ± s.e.m.), following exposure to 30 and 100 µM memantine, and 300 µM tolbutamide.

Fig. 6. Increase of dopamine neurons firing rate by memantine using the extracellular multielectrode array technique, in hypoglycemic conditions. Plot of the average spontaneous firing frequency (mean ± s.e.m.) against time (10 s bin size) obtained from the raster plots shown above. On top, raster plots are shown of single unit action potentials, obtained from a total of 22 neurons sensitive to dopamine (30 µM, not shown) in a single midbrain slice. Perfusion of memantine (100 µM) in ACSF containing 1 mM glucose caused a marked, reversible increase in the firing rate (left). Raising the glucose concentration to 2 mM caused an overall recovery of the firing frequency and no effect by memantine was observed in this experimental condition (right).

Fig. 7. Increase of dopamine neurons firing rate by tolbutamide using the extracellular multielectrode array technique, in hypoglycemic conditions. Plot of the average spontaneous firing frequency (mean ± s.e.m.) against time (10 s bin size) obtained from the raster plots shown above. On top, raster plots are shown of single unit action potentials, obtained from a total of 26 neurons sensitive to a 1 min exposure to dopamine (30 µM, not shown) in a single midbrain slice. Perfusion of tolbutamide (300 µM) in ACSF containing 1 mM glucose caused a marked increase in the firing rate. In
this conditions, memantine (100 µM) did not produce further increase in action potential firing (left). Restoring normoglycemic conditions caused an overall recovery of the firing frequency and no effect by tolbutamide was observed in this experimental condition (right).
Figure 1

A

(b) (c) (d)

memantine 30 µM
MK-801 10 µM

INMDA amplitude (pA)

(n=4)

BA

relative INMDA amplitude (% of control)

50 pA

5 s

B

n=15

n=15

n=4

memantine 100 µM
dashed line represents control
Figure 2

A

memantine 30 μM

memantine 100 μM

instantaneous frequency (Hz)

time (min)

B

control

memantine 100 μM

wash

memantine 100 μM

C

in MK-801 10 μM

control

memantine 100 μM

wash

D

spike rate (Hz)

control

MK-801

AP5

mirtazapine + methyllycaconitine

*** p < 0.001

* p < 0.05
Figure 3

DA 30 µM

memantine 100 µM

Spike rate (Hz)

time (min)
Figure 4

A 10 mM intracellular ATP

control

memantine 100 µM

wash

B 2 mM intracellular ATP

control

tolbutamide 300 µM

memantine 100 µM in tolbutamide

wash

C

spike rate (Hz)

10 mM intracellular ATP

2 mM intracellular ATP

control

memantine 100 µM

tolbutamide 300 µM

memantine + tolbutamide

n=6

n=8

n=9

**
Figure 5

(A) In MK-801 10 µM

- Memantine 30 µM
- Memantine 100 µM
- Tolbutamide 300 µM

Hypoxia

10 min

30 pA

(B) Relative IHYPO amplitude (% of control)

- Memantine
- Tolbutamide 300 µM

n=4, n=12, n=11

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Figure 6
Figure 7

Comparison of glucose levels (1 mM and 10 mM) and the effects of tolbutamide (300 µM) and memantine (100 µM) on spike rate (Hz) over time (min).