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Safinamide Differentially Modulates In Vivo Glutamate and GABA Release in the Rat Hippocampus and Basal Ganglia

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ABSTRACT

Safinamide has been recently approved as an add-on to levodopa therapy for Parkinson disease. In addition to inhibiting monoamine oxidase type B, it blocks sodium channels and modulates glutamate (Glu) release in vitro. Since this property might contribute to the therapeutic action of the drug, we undertook the present study to investigate whether safinamide inhibits Glu release also in vivo and whether this effect is consistent across different brain areas and is selective for glutamatergic neurons. To this aim, in vivo microdialysis was used to monitor the spontaneous and veratridine-induced Glu and GABA release in the hippocampus and basal ganglia of naive, awake rats. Brain levels of safinamide were measured as well. To shed light on the mechanisms underlying the effect of safinamide, sodium currents were measured by patch-clamp recording in rat cortical neurons. Safinamide maximally inhibited the veratridine-induced Glu and GABA release in hippocampus at 15 mg/kg, which reached free brain concentrations of 1.89–1.37 μM. This dose attenuated veratridine-stimulated Glu (but not GABA) release in subthalamic nucleus, globus pallidus, and substantia nigra reticulata, but not in striatum. Safinamide was ineffective on spontaneous neurotransmitter release. In vitro, safinamide inhibited sodium channels, showing a greater affinity at depolarized (IC_{50} = 8 μM) than at resting (IC_{50} = 262 μM) potentials. We conclude that safinamide inhibits in vivo Glu release from stimulated nerve terminals, likely via blockade of sodium channels at subpopulations of neurons with specific firing patterns. These data are consistent with the anticonvulsant and antiparkinsonian actions of safinamide and provide support for the nondopaminergic mechanism of its action.

Introduction

Safinamide ((S)-(+)-2-[4-(3-fluorobenzyl) oxybenzyl] aminopropanamide methanesulfonate; XADAGO) is a drug originally identified as an anticonvulsant (Fariello et al., 1998; Fariello, 2007) that has recently been approved in European Union and United States as an add-on to a stable dose of levodopa (L-dopa), alone or in combination with other PD medicinal products, for the treatment of patients with mid- to late-stage fluctuating idiopathic Parkinson disease (PD) (Borgohain et al., 2014a,b).

Safinamide is a small-molecule drug (Pevarello et al., 1999) that is orally bioavailable (80%–92%) and highly brain-penetrant in rodents and nonhuman primates (Onofrj et al., 2008). In addition to reversible and highly selective monoamine oxidase-type B (MAO-B) inhibition, safinamide is endowed with nondopaminergic properties, such as blockade of voltage-gated sodium and calcium channels and inhibition of glutamate (Glu) release in vitro (Salvati et al., 1999; Caccia et al., 2006). Safinamide's ability to reduce Glu release might provide additional therapeutic effects to MAO-B inhibition. In fact, the progressive loss of dopamine (DA) neurons in the substantia nigra pars compacta (i.e., the neuropathological hallmark of PD) leads to changes in the firing rates and patterns of different subpopulations of neurons, resulting in a (hyper)synchrony and oscillatory activity within the basal ganglia and the cortex (Lopez-Azcarate et al., 2010; Wichmann et al., 2011).

Hyperactivity of Glu-releasing neurons of motor cortex and subthalamic nucleus (STN) plays a causative role in this process. In fact, both corticostriatal and corticosubthalamic inputs drive STN hyperactivity, which is a consistent finding across animal models of PD (Bergman et al., 1994; Hassani et al., 1996; Meissner et al., 2005) and PD patients (Magnin et al., 2000; Brown et al., 2001). STN overactivity sustains motor symptoms since it causes overstimulation of nigrothalamic GABA neurons, resulting in thalamic inhibition and impairment of motor planning and execution (Albin et al., 1989; Wichmann et al., 2011). Therefore, in addition to reinstating nigrostriatal DA transmission, normalizing overactive Glu transmission may prove useful in relieving PD

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ABBREVIATIONS: AP, antero-posterior; DA, dopamine; DLS, dorsolateral striatum; DV, dorso-ventral; Glu, glutamate, GP; globus pallidus; HPLC, high-performance liquid chromatography; L-dopa, levodopa; MAO-B, monoamine oxidase-type B; ML, medio-lateral; PD, Parkinson's disease; SNr, substantia nigra reticulata; STN, subthalamic nucleus.

symptoms. This approach may extend its efficacy beyond motor deficits, since overactive glutamatergic transmission is believed to contribute also to the neurodegeneration associated with PD (Rodriguez et al., 1998; Duty, 2012; Ambrosi et al., 2014) and to motor fluctuations and dyskinesia that develop along the chronic therapy with L-dopa (Chase et al., 2000; Sgambato-Faure and Cenci, 2012). Compared with classic Glu release inhibitors (e.g., riluzole), safinamide might present some advantages and a more favorable clinical profile since the use-dependent nature of the safinamide block of sodium channels might orient its action toward overactive glutamatergic neurons, leaving physiologic transmission unaffected. Nonetheless, evidence that safinamide inhibits Glu release in vivo is still lacking since safinamide has been shown to attenuate the veratridine-induced Glu release only in rat hippocampal slices (Salvati et al., 1999) and synaptosomes (Caccia et al., 2006) in vitro. Therefore, the main aim of the present study was to investigate the ability of safinamide to modulate spontaneous and veratridine-stimulated Glu release by using in vivo microdialyis in awake, freely moving rats. The effect of safinamide was first investigated in the hippocampus to confirm previous in vitro studies, as well as to draw a dose response, and then in four nuclei of the basal ganglia complex— namely, dorsolateral striatum (DLS), STN, globus pallidus (GP), and substantia nigra reticulata (SNr) to ascertain whether the effect of the drug was consistent across different brain areas. Spontaneous and veratridine-induced GABA release was also monitored to investigate whether the effect of safinamide was selective for glutamatergic neurons. Brain levels of safinamide were measured to determine the drug concentration at the tested doses and to provide a clinically relevant pharmacokinetic and pharmacodynamic support for the nondopaminergic properties of safinamide. Finally, to shed light on the mechanism of action of safinamide, sodium currents in rat cortical neurons were monitored by whole-cell patch-clamp recording.

Materials and Methods

Animal Subjects. Experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals and were approved by the Italian Ministry of Health (see license numbers to follow). Experimenters were blinded to treatments. Male Sprague-Dawley rats (275–300 g; Charles River, Calco, Italy) were used in the microdialysis and pharmacokinetic studies. Rats were housed in standard facilities with a temperature- and humidity-controlled environment (20–22°C and 45%–65%, respectively) and free access to food (4RF21 standard diet; Mucedola, Settimo Milanese, Milan, Italy) and water under regular lighting conditions (12-hour dark/light cycle). Animals were housed in groups of five in a 55 × 33 × 20-cm polycarbonate cage (Tecniplast, Buguggiate, Varese, Italy) with Scobis Uno bedding (Mucedola) and environmental enrichments. Certified timed pregnant Wistar rat dams (Harlan, San Pietro al Natisone, Italy) on gestational days 17–19 were used to prepare neuronal cultures for patch-clamp experiments. Adequate measures were taken to minimize animal pain and discomfort. At the end of the experiments, the rats were sacrificed with an overdose of isoflurane.

Experimental Protocols and Design. Ninety-five rats were used for the microdialysis experiments, 84 for the study of veratridine-stimulated neurotransmitter release and 11 for the study of spontaneous release. The experimental protocols were approved by the Italian Ministry of Health (licenses 170/2013B and 714/2016-PR-B). As for the design of the experiments (Fig. 1, C and D and Fig. 2), each rat was randomized to saline/veratridine or safinamide/veratridine (0.5, 5, or 15 mg/kg, Fig. 1, C and D; 5 or 15 mg/kg, Fig. 2) in the first and second microdialysis sessions, ensuring that no rat received the same treatment in the two sessions. Rats underwent two microdialysis sessions (i.e., at 24 and 48 hours after probe implantation), after which they were sacrificed with an isoflurane overdose, and placement of the probes was verified histologically. For study of veratridine-stimulated release (Fig. 1, A and B, 3, 4, and 5), each animal implanted with a single microdialysis probe was randomized to saline/veratridine or safinamide/veratridine (30 mg/kg, Fig. 1, A and B; 15 mg/kg, Figs. 3–5) in the first microdialysis session, and treatments crossed in the second session. For the study on spontaneous Glu and GABA release, rats implanted with one probe in STN and another in the contralateral SNr were randomized to saline or veratridine 15 mg/kg in the first microdialysis session, and treatments crossed in the second session. Overall, seven animals were discarded for probe misplacement or probe clogs during microdialysis.

In Vivo Microdialysis. Intracerebral microdialysis was performed as previously described (Morari et al., 1996; Paolone et al., 2015). One probe of concentric design was stereotaxically implanted under isoflurane anesthesia in five different brain regions according to the following coordinates (in millimeters) from the bregma and the dural surface (Paxinos and Watson, 1986): hippocampus (1-mm dialyzing membrane, antero-posterior (AP) −3.14, medio-lateral (ML) ± 1.8, dorso-ventral (DV) −4.2.), STN (1-mm dialyzing membrane, AP −3.7, ML ± 2.5, DV −8.6), SNr (1-mm dialyzing membrane, AP −5.5, ML ± 2.2, DV −8.3), DLS (3-mm dialyzing membrane, AP +1.0, ML ± 3.5, DV −6.0) and GP (2-mm dialyzing membrane, AP −1.3, ML ± 3.3, DV −6.5). When veratridine-stimulated neurotransmitter release was studied, each animal was implanted with one probe at the time. Conversely, when spontaneous neurotransmitter release was studied, each animal was implanted with two probes at the same time, one in the STN and another in the contralateral SNr. Probes were secured to the skull with dental cement. The wound was infiltrated with local anesthetic (lidocaine 2%) before surgery completion. Twenty-four hours after surgery, the probes were perfused with a modified Ringer's solution (1.2 mM $CaCl_2$, 2.7 mM KCl, 148 mM NaCl, and 0.85 mM $MgCl_2$) at a flow rate of 3.0 μl/min, and sample collection (every 20 minutes) began after 6 hours of rinsing. At least four baseline samples were collected before systemic (i.p.) administration of saline or safinamide. Thirty minutes later, veratridine (10 μM) was perfused for 30 minutes through the probe by reverse dialysis; at the end of veratridine perfusion, sample collection was continued for 80 minutes.

Endogenous Glu and GABA Analysis. Glu and GABA were measured by high-performance liquid chromatography (HPLC) coupled with fluorometric detection as previously described (Paolone et al., 2015). Thirty microliters of *o*-phthaldialdehyde/mercaptoethanol reagent was added to 30-μl aliquots of sample, and 50 μl of the mixture was automatically injected (Triathlon autosampler; Spark Holland, Emmen, Netherlands) onto a 5-C18 Hypersil ODS analytical column (3-mm inner diameter, 10-cm length; Thermo Fisher Scientific, Waltham, MA) perfused at a flow rate of 0.48 ml/min (Jasco PU-2089 Plus quaternary pump; Jasco, Tokyo, Japan) with a mobile phase containing 0.1 M sodium acetate, 10% methanol and 2.2% tetrahydrofuran (pH 6.5). Glu and GABA were detected by means of a fluorescence spectrophotometer FP-2020 plus (Jasco) with the excitation and the emission wavelengths set at 370 and 450 nm, respectively. Under these conditions, the limits of detection for Glu and GABA were ~1 nM (i.e., ~147 pg/ml) and ~0.5 nM (i.e., 51 pg/ml), and the retention times were ~3.5 and ~18.0 minute, respectively.

Brain Pharmacokinetic Analysis. Twenty-seven rats were used for pharmacokinetic analysis. The experimental protocols were approved by the Italian Ministry of Health (license no. 38200). Safinamide was administered at three dose levels (5, 15, and 30 mg/kg, i.p.), and brains were removed 40, 60, and 80 minutes later to match the veratridine perfusion time in the microdialysis study. Brain samples were homogenized by sonication (Covaris, Woburn, MA); after protein precipitation, the total safinamide concentration was measured by HPLC-tandem mass spectrometry on a Sciex API4000

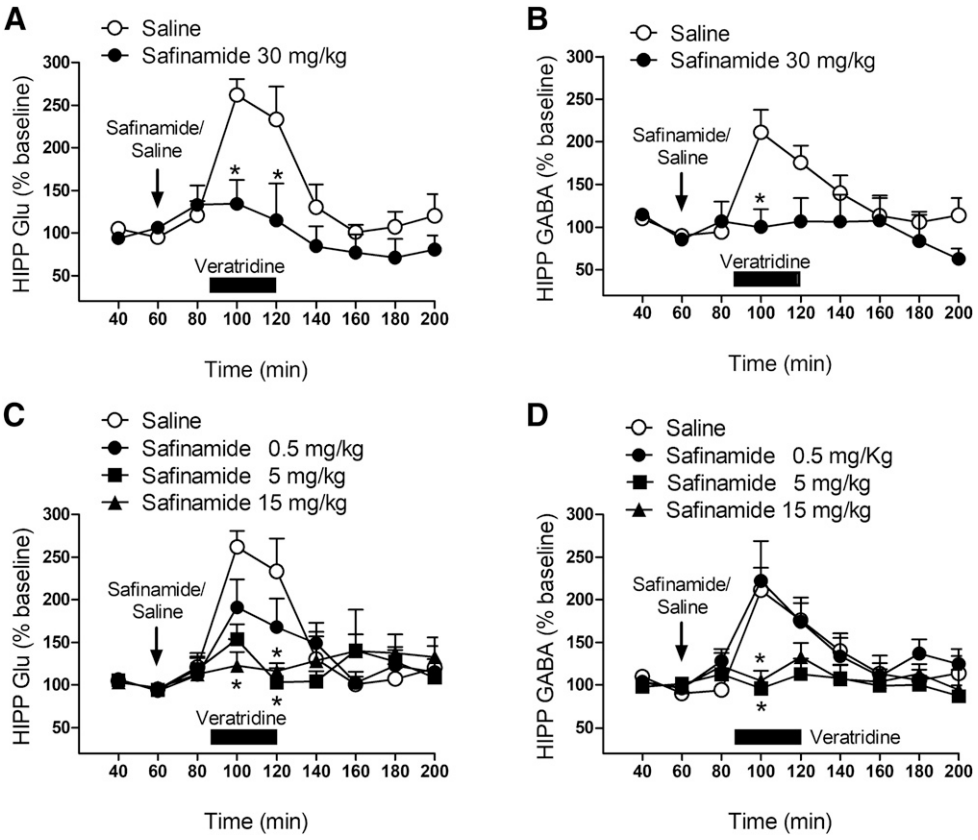

Fig. 1. Glu and GABA dialysate levels after systemic administration of saline or safinamide (0.5–30 mg/kg, i.p., arrow) in combination with reverse dialysis of veratridine (10 μM, 30 minutes, black bar) in the hippocampus (HIPP) of awake, freely moving rats. Data are expressed as percentage of basal pretreatment levels (calculated as the mean of the two samples preceding treatment) and are means ± S.E.M. of $n = 5$ (safinamide 30 mg/kg); (A and B), $n = 6$ (safinamide 0.5 mg/kg) or $n = 8$ (safinamide 5 and 15 mg/kg); (C and D) rats per group. Basal Glu and GABA levels were 41.6 ± 1.1 and 37.6 ± 1.3 nM, respectively. $^*P < 0.05$ vs. saline (two-way repeated measures ANOVA followed by the Bonferroni test for multiple comparisons).

mass spectrometer (AB Sciex, Framingham, MA). Samples (5 μl) were injected using a CTC analytics HTS Pal autosampler (Zwingen, Switzerland) onto a Synergi MAX-RP 30 ×2.0 mm, 4-μm column (Phenomenex, Macclesfield, UK) at an eluent flow rate of 1.5 ml/min. Analytes were eluted using a high-pressure linear gradient program by an HP1100 binary HPLC system (Agilent Technologies, Waldbronn, Germany). To calculate the free brain concentration, the fraction of unbound safinamide in the brain ($f_{u,b}$) was determined by

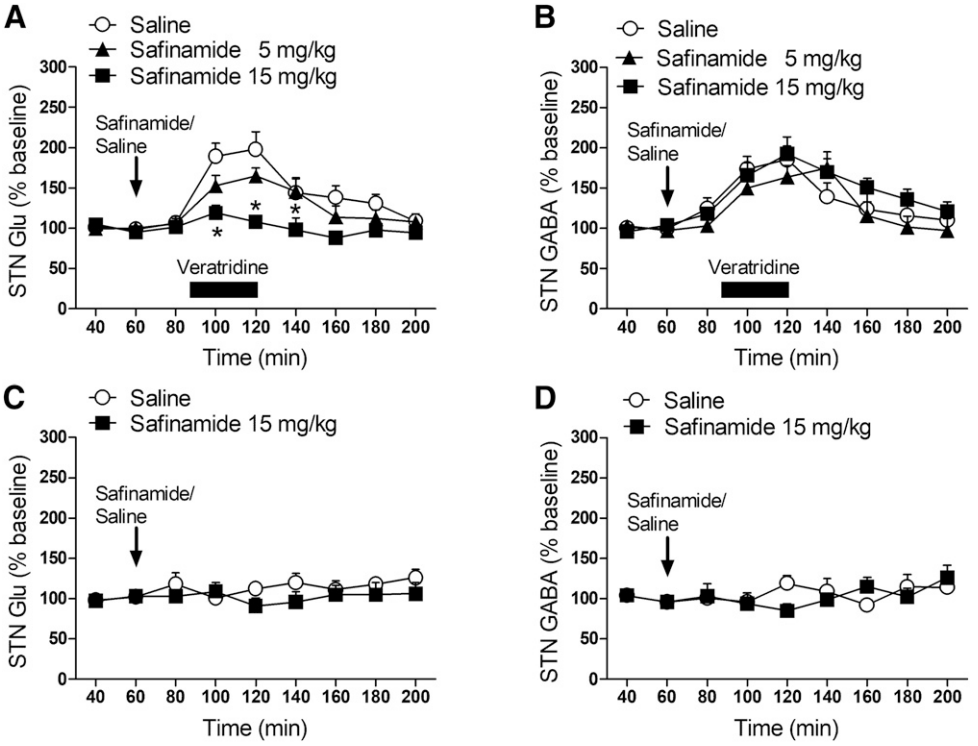

Fig. 2. Glu and GABA dialysate levels after systemic administration of saline and safinamide (5 and 15 mg/kg, i.p., arrow) in combination with reverse dialysis of veratridine (10 μM, 30 minutes, black bar) in the subthalamic nucleus (STN) of awake freely moving rats (A and B). The effect of saline and safinamide on spontaneous Glu and GABA release is also shown (C and D). Data are expressed as percentage of basal pretreatment levels (calculated as the mean of the two samples preceding treatment) and are mean ± S.E.M. of $n = 9$ rats per group. Basal Glu and GABA levels were 22.5 ± 2.7 and 11.4 ± 1.2 nM, respectively. $^*P < 0.05$ vs. saline (two-way repeated measures ANOVA followed by the Bonferroni test for multiple comparisons).

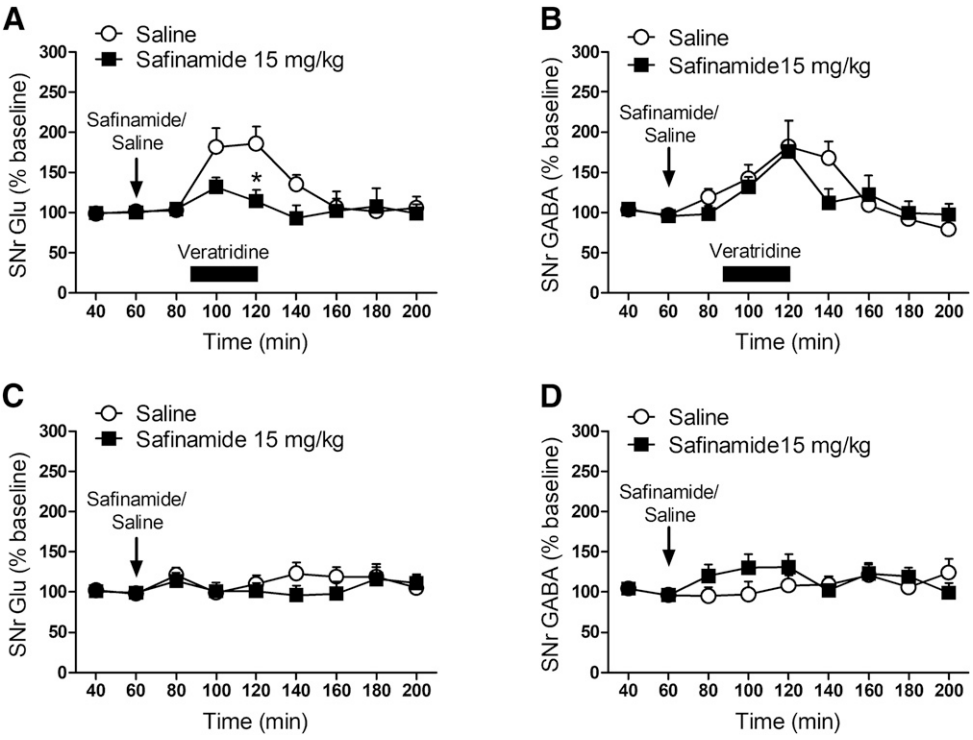

Fig. 3. Glu and GABA dialysate levels after systemic administration of saline or safinamide (15 mg/kg, i.p., arrow) in combination with reverse dialysis of veratridine (10 μM, 30 minutes, black bar) in the substantia nigra reticulata (SNr) of awake, freely moving rats (A and B). The effect of saline and safinamide on spontaneous Glu and GABA release is also shown (C and D). Data are expressed as percentage of basal pretreatment levels (calculated as the mean of the two samples preceding treatment) and are mean ± S.E.M. of n = 8 rats per group. Basal Glu and GABA levels in SNr were 17.5 ± 2.3 and 10.0 ± 1.4 nM, respectively. *$P < 0.05$ vs. saline (two-way repeated measures ANOVA followed by the Bonferroni test for multiple comparisons).

in vitro equilibrium dialysis (Summerfield et al., 2007). The $f_{u,b}$ percent was 3.27.

Cortical Neuron Preparation. Primary cultures of cortical neurons were prepared from 17- to 18-day-old Wistar rat fetuses obtained from two dams (experimental protocol approved by the Italian Ministry of Health, license no. 84/2001 B), as previously described (Brewer, 1995). The brain cortex was dissected out and repeatedly rinsed in ice-cold Hanks' balanced salt solution, and the meninges were peeled off. After mechanical dissociation, 5 ml of complete Dulbecco's modified Eagle's medium + 10% fetal bovine serum + 2 mM glutamine + Pen-Strep 100 U–100 μg/ml were added. The cell suspension was centrifuged at 1000 rpm for 3 minutes, and the pellet was resuspended in 5 ml of a serum-free growth medium composed by neurobasal medium, supplemented with 2% B27, 2 mM Glutamine, Pen-Strep (100 U–100 μg/ml). Cells were counted, diluted in neurobasal medium, and plated at a density of 400,000 cells onto poly-D-lysine (5 μg/ml)-treated 35-mm Petri dish. Neurobasal medium was changed once a week, and cells were used from day 6 until day 11 after plating.

Whole-Cell Patch-Clamp Recording. The experiments were carried out according to standard whole-cell patch-clamp recording techniques (Hamill et al., 1981) at room temperature (25°C). Neuronal cells were continuously superfused (RSC-200 solution changer; Bio-Logic Science Instruments, Seyssinet-Pariset, France) with an extracellular solution containing (in millimolars) NaCl (60), choline chloride (60), $CaCl_2$ (1.3), $MgCl_2$ (2), $CdCl_2$ (0.4), $NiCl_2$ (0.3), TEACl (20), glucose (10), and HEPES (10). Patch pipettes (Harward borosilicate glass tubes Sutter Instrument Co, Novato, CA) were pulled using a Sutter P-87 electrode puller and filled with an internal solution consisting of (in millimolars): CsF (65), CsCl (65), NaCl (10), $CaCl_2$ (1.3), $MgCl_2$ (2), EGTA (10), HEPES (10), and MgATP (1). Patch electrodes had a tip resistance of 2–3 MΩ. Membrane currents were recorded and filtered at 5 kHz using an Axopatch 200B amplifier, and data were digitized using an Axon Digidata 1322A (Axon Instruments, Sunnyvale, CA). Voltage command protocols and data acquisitions were controlled using Axon pClamp8 software. Measuring and reference electrodes were AgCl-Ag electrodes. Access resistance ranged from 5 to 10 MΩ; linear leakage and capacitative currents were eliminated using a P/4 leak subtraction protocol. Safinamide (20 mM stock solution in distilled water) was diluted in external solution and applied for 2 minutes to reach an equilibrium response.

Voltage Protocol and Data Analysis. To obtain the steady-state inactivation curves of sodium currents, currents were activated by applying 2-second conditioning prepulses from −110 to 0 mV from a holding potential (V_h) of −110 mV and then stepping the cell to +10 mV for 30 milliseconds (test pulse). The peak

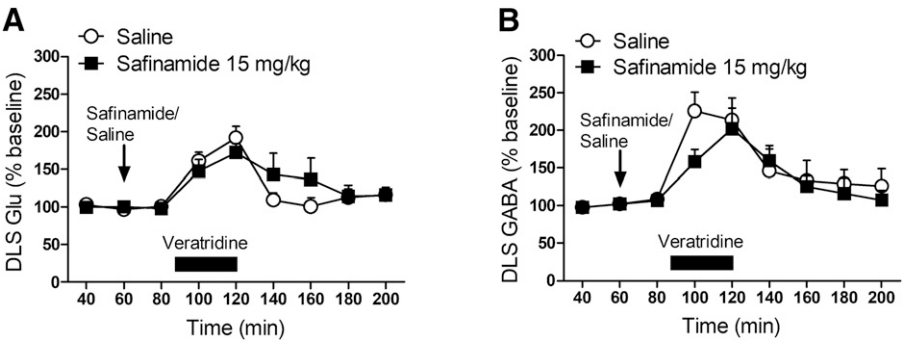

Fig. 4. Glu (A) and GABA (B) dialysate levels after systemic administration of saline or safinamide (15 mg/kg, i.p., arrow) in combination with reverse dialysis of veratridine (10 μM, 30 minutes, black bar) in the DLS of awake, freely moving rats. Data are expressed as percentage of basal pretreatment levels (calculated as the mean of the two samples preceding treatment) and are mean ± S.E.M. of n = 8 rats per group. Basal Glu and GABA levels were 15.8 ± 2.2 and 9.1 ± 1.1 nM, respectively. *$P < 0.05$ vs. saline (two-way repeated measures ANOVA followed by the Bonferroni test for multiple comparisons).

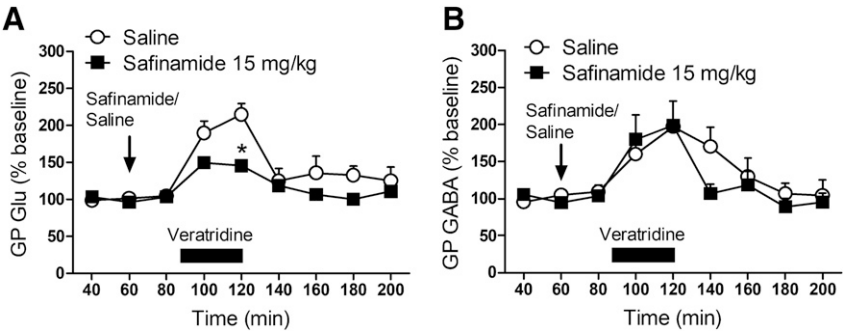

Fig. 5. Glu (A) and GABA (B) dialysate levels after systemic administration of saline or safinamide (15 mg/kg, i.p., arrow) in combination with reverse dialysis of veratridine (10 μM, 30 minutes, black bar) in the globus pallidus (GP) of awake, freely moving rats. Data are expressed as percentage of basal pretreatment levels (calculated as the mean of the two samples preceding the treatment) and are mean ± S.E.M. of n = 7 rats per group. Basal Glu and GABA levels were 16.4 ± 2.0 and 9.7 ± 1.1 nM, respectively. *$P < 0.05$ vs. saline (two-way repeated measures ANOVA followed by the Bonferroni test for multiple comparisons).

currents (I) were normalized with respect to the maximal peak current at −110 mV (I_{max}), and plotted against the respective preconditioning potentials. The steady-state inactivation data were fitted with the Boltzmann function according to the following equation: $I/I_{max} = 1/\{1+\exp[(V_{pre} - V_{half})/k_h]\}$, where V_{pre} is the preconditioning potential, V_{half} the potential at which half-maximal current inactivation occurs, and k_h the corresponding slope factor.

To test the effect of safinamide on sodium currents, neuronal cells were clamped at −90 mV, and then a two-step protocol was used to determine the voltage dependence of the block (Kuo and Lu, 1997). Sodium currents were activated by a 30-millisecond step pulse to +10 mV (test pulse) from 2-second preconditioning potential (at resting and half-maximal current inactivation).

Inhibition curves were obtained by plotting the tonic block in the resting and depolarized conditions versus drug concentration. Concentration-response data were fitted according to the following logistic equation (eq. 1) using Origin 6.0 software (Microcal Software, Northampton, MA):

$$y = A2 + (A1\text{-}A2)/[1 + (x/IC_{50})\ p] \quad (1)$$

A1 and *A2* are fixed values of 0 and 1 corresponding to 0% and 100% current inhibition, x is the drug concentration, IC_{50} is the drug concentration resulting in 50% current inhibition and p is the corresponding slope factor.

The apparent affinity of the drug for the inactivated state of the sodium channel (K_i) was determined according to eq. 2:

$$1/K_{dep} = h/K_r + (1\text{-}h)/K_i \quad (2)$$

where K_r is the affinity for the resting/closed state, K_{dep} is the IC_{50} in the depolarized condition, and h and (1 hour) are the fractions of channels present at the resting and depolarized potentials, respectively (Bean et al., 1983). The K_i value represents an estimation of the inactivated-state block, without the resting-state block component. Use-dependent inhibition of sodium currents by safinamide was also determined from analysis of the effect on a train of 15 test pulses (each pulse from −70 to +10 mV, 10-millisecond duration), applied at 1 and 10 Hz. The ratio of the amplitudes of the last to the first pulse was determined in the presence and in the absence of the drug.

Data Presentation and Statistical Analysis. In microdialysis studies, Glu and GABA levels were expressed as percentage ± S.E.M. of basal values (calculated as mean of the two samples preceding the treatment). This normalization was adopted in this and previous studies (see, for instance, Morari et al., 1996; Paolone et al., 2015) to account for variability in baseline levels across rats and experimental sessions. Absolute basal values were given in figure legends. Statistical analysis (GraphPad Prism; GraphPad Software, San Diego, CA) was performed by two-way repeated measure analysis of variance (ANOVA), followed by the Bonferroni post hoc test for multiple comparisons. Values were considered statistically significant if $P < 0.05$. In pharmacokinetic experiments, safinamide brain concentrations were expressed (in micromolars) as mean ± SD.

Materials

Veratridine was purchased from Tocris (Bristol, UK) and dissolved in dimethylsulfoxide to provide stock solution of 10 mM. All subsequent dilutions were made in Ringer. Safinamide methansulphonate was provided by Zambon SpA (Bresso, Italy), dissolved in saline, and administered i.p. as free base (volume of 1.0 ml/Kg body weight). Neurobasal medium (21103-049), B27 (17504-044), glutamine (25030-024), Pen-Strep 100× (15140-122), and Hanks' balanced salt solution (14170-088) were purchased from Life Technologies (Monza, Italy). Dulbecco's modified Eagle's medium and fetal bovine serum were purchased from GIBCO, Italy, and GE Healthcare (Little Chalfont, UK) HyClone, respectively.

Results

Glu and GABA Release In Hippocampus. The hippocampus was the first area investigated because previous studies indicated that safinamide inhibited veratridine-induced Glu release from hippocampal slices (Salvati et al., 1999) and synaptosomes (Caccia et al., 2006). Reverse dialysis of veratridine (10 μM; 30 minutes) in the hippocampus of naive rats evoked a prompt and transient +150% elevation of Glu (Fig. 1A) and GABA (Fig. 1B) levels. We first explored the effect of 30 mg/kg of safinamide in a small group of rats (n = 5) and found that this dose prevented the effect of veratridine both on Glu (treatment $F_{1,8} = 1.31$, $P = 0.28$; time $F_{8,64} = 8.25$, $P < 0.0001$; time × treatment interaction $F_{8,64} = 2.47$, $P = 0.0211$; Fig. 1A) and GABA (treatment $F_{1,8} = 4.04$, $P = 0.0791$; time $F_{8,64} = 3.76$, $P = 0.0012$, time × treatment interaction $F_{8,64} = 2.83$, $P = 0.0093$; Fig. 1B) release. Then we tested lower doses of safinamide (Fig. 1, C and D). Safinamide caused a slight, albeit not significant, reduction of veratridine-stimulated Glu release at 0.5 mg/kg and full inhibition at 5 and 15 mg/kg (treatment $F_{3,8} = 0.77$ $P = 0.52$, time $F_{8,208} = 9.70$ $P < 0.0001$, time × treatment $F_{24,208} = 3.31$ $P < 0.0001$; Fig. 1C). Likewise, safinamide dose dependently inhibited the veratridine-induced GABA release in the same dose-range (treatment $F_{3,8} = 4.27$, $P = 0.0145$; time $F_{8,200} = 6.59$, $P < 0.0001$; time × treatment $F_{24,200} = 1.96$, $P = 0.0066$; Fig. 1D). Based on this dose-finding study in hippocampus, we selected 15 mg/kg safinamide as a starting dose for further microdialysis studies in the basal ganglia.

Glu and GABA Release in STN. Reverse dialysis of veratridine (10 μM for 30 minutes) in the STN of naive rats evoked local Glu (Fig. 2A) and GABA (Fig. 2B) levels. The time course and magnitude of the veratridine-induced Glu and GABA release in STN were similar to those in the

hippocampus. Safinamide caused a dose-dependent inhibition of veratridine-evoked subthalamic Glu release (treatment $F_{2,8} = 6.74$, $P = 0.0047$; time $F_{8,192} = 16.61$, $P < 0.0001$; time × treatment interaction $F_{16,192} = 3.19$, $P < 0.0001$; Fig. 2A). In particular, safinamide prevented the rise in Glu levels at 15 mg/kg, and it caused a nonsignificant inhibition at 5 mg/kg. Safinamide did not significantly affect the veratridine-stimulated subthalamic GABA release (Fig. 2B), although a tendency toward a delayed normalization (i.e., longer stimulation) was observed. No significant effect of safinamide on spontaneous Glu and GABA levels in STN was observed after administration of safinamide 15 mg/kg (Fig. 2, C and D).

Glu and GABA Release in SNr. The time course and extent of the response of nigral Glu and GABA to veratridine (Fig. 3) were substantially similar to those observed in STN. Safinamide (15 mg/kg i.p.) attenuated the veratridine-evoked Glu response (Fig. 3A) (treatment $F_{1,8} = 2.31$, $P = 0.15$; time $F_{8,112} = 6.91$, $P < 0.0001$; time × treatment interaction $F_{8,112} = 2.85$, $P = 0.0064$) but did not significantly affect the veratridine-evoked GABA release (Fig. 3B). Moreover, safinamide did not affect the spontaneous Glu and GABA release in SNr (Fig. 3, C and D).

Glu and GABA Release in DLS. Reverse dialysis of veratridine in the DLS of naive rats transiently evoked local Glu (Fig. 4A) and GABA (Fig. 4B) levels; however, safinamide (15 mg/kg, i.p.) did not significantly affect the veratridine-stimulated neurotransmitter release in this brain area.

Glu and GABA Release in GP. The Glu and GABA response to reverse dialysis of veratridine in GP (Fig. 5, A and B) was superimposable to those observed in the other nuclei. Safinamide (15 mg/kg i.p.) significantly inhibited the veratridine-evoked Glu release (treatment $F_{1,8} = 5.11$, $P < 0.0431$; time $F_{8,96} = 20.53$, $P < 0.0001$; time × treatment interaction $F_{8,96} = 3.35$, $P = 0.0020$; Fig. 5A) without affecting the veratridine-stimulated GABA response (Fig. 5B).

Safinamide Brain Levels. In a separate group of rats, the brain levels of safinamide were measured 40, 60, and 80 minutes after the administration of 5, 15, or 30 mg/kg safinamide. Free brain concentrations, derived by taking into account the brain-binding tissue of safinamide, correlated with doses, being highest for the 30 mg/kg dose and lowest for the 5 mg/kg dose at any time points examined (Table 1). In addition, for all doses, a gradual and linear decline was observed from the first through the last time point examined. During veratridine perfusion (100–120 minutes), safinamide-free brain levels for the 5, 15, and 30 mg/kg doses were in the 0.70–0.44, 1.89–1.70, and 4.77–3.04 μM concentration ranges, respectively.

TABLE 1

Free brain concentrations of safinamide in rats

Safinamide was administered at 5, 15, and 30 mg/kg (i.p.), and brains were removed 40, 60, and 80 minutes later to match with the veratridine perfusion in microdialysis studies. Safinamide content was analyzed by HPLC-tandem mass spectrometry. Data are mean ± S.D. of three rats per group.

Time after safinamide (*min*)	Safinamide free brain concentrations (μM)		
	5	15	30
min	*mg/kg*	*mg/kg*	*mg/kg*
40	0.70 ± 0.01	1.89 ± 0.76	4.77 ± 2.52
60	0.44 ± 0.06	1.70 ± 0.73	3.04 ± 0.86
80	0.31 ± 0.05	1.37 ± 1.14	2.71 ± 0.37

Sodium Channel Inhibition in Rat Cortical Neurons. Voltage pulses to +10 mV evoked fast inward sodium currents from cortical neurons, whose amplitude was dependent on the voltage of the conditioning pulse (see *Materials and Methods*). The conditioning voltage at which maximal (resting state, V_{rest}) and 50% maximal sodium current (half maximal inactivation state, V_{half}) could be evoked were −110 and −53 mV, respectively (Fig. 6A). According to the observed steady-state inactivation curve, the effects of safinamide on sodium currents and voltage/state dependence of the block were tested at preconditioning potentials of −110 mV (V_{rest}) and −53 mV (V_{half}). As shown in Fig. 6B, safinamide (1–300 μM) reduced the amplitude of the peak sodium currents (tonic block) in a concentration-dependent manner. When currents were stimulated to a V_{test} of +10 mV from a V_h of −110 mV, the IC_{50} value was 262 μM. The inhibitory effect of safinamide was voltage-dependent since a significantly lower IC_{50} value (8 μM) was obtained when the holding potential was depolarized to −53 mV. Washout resulted in complete reversal of the inhibition. The affinity constant for the inactivated state of the sodium channel (K_i) was 4.1 μM.

To test whether the sodium currents showed a use-dependent (phasic) block in the presence of safinamide, trains of repeated voltage steps to +10 mV from a V_h of −70 mV were applied at two different frequencies (i.e., 1 and 10 Hz) (Fig. 6, C and D). At 1 Hz, in the absence of the drug, a small decay occurred in the peak amplitude of evoked currents, and the ratio of the current amplitudes at the 15th and 1st pulse was 0.97 ± 0.006. In the presence of safinamide (12.5 μM), no significant use-dependent drug action was observed at 1 Hz (0.90 ± 0.01). In contrast, when repetitive impulses were applied at the frequency of 10 Hz, safinamide produced a reduction in the evoked currents owing to development of the use-dependent block (phasic block). In fact, safinamide significantly reduced the ratio from 0.89 ± 0.03 (control conditions) to 0.67 ± 0.05.

Discussion

The present study provides the first evidence that safinamide inhibits in vivo Glu release in rat brain regions involved in cognition and movement. Since veratridine is a voltage-dependent sodium-channel opener, which in previous microdialysis studies has been shown to stimulate striatal Glu and GABA release in a tetrodotoxin-sensitive fashion (Young et al., 1990; Waldmeier et al., 1996), it appears that safinamide inhibits neuronal Glu and GABA release from overactive Glu and GABA terminals.

Safinamide is a small molecule that was originally identified as an anticonvulsant (PNU-151774E) (Salvati et al., 1999) and recently approved as add-on to levodopa in PD therapy (Borgohain et al., 2014a,b). Previous studies (Salvati et al., 1999; Caccia et al., 2006) indicated that safinamide inhibited the veratridine-induced Glu release from hippocampal slices ($IC_{50} = 56$ μM) and synaptosomes ($IC_{50} = 9$ μM), possibly via inhibition of sodium and calcium channels. The present study indicates that this inhibitory control is relevant also in vivo but likely relies on inhibition of voltage-operated sodium channels. In fact, inhibition of Glu release occurred at safinamide doses generating free brain concentrations in the range of the affinity values for sodium channels. Whole-cell patch-clamp recording in cortical neurons showed that

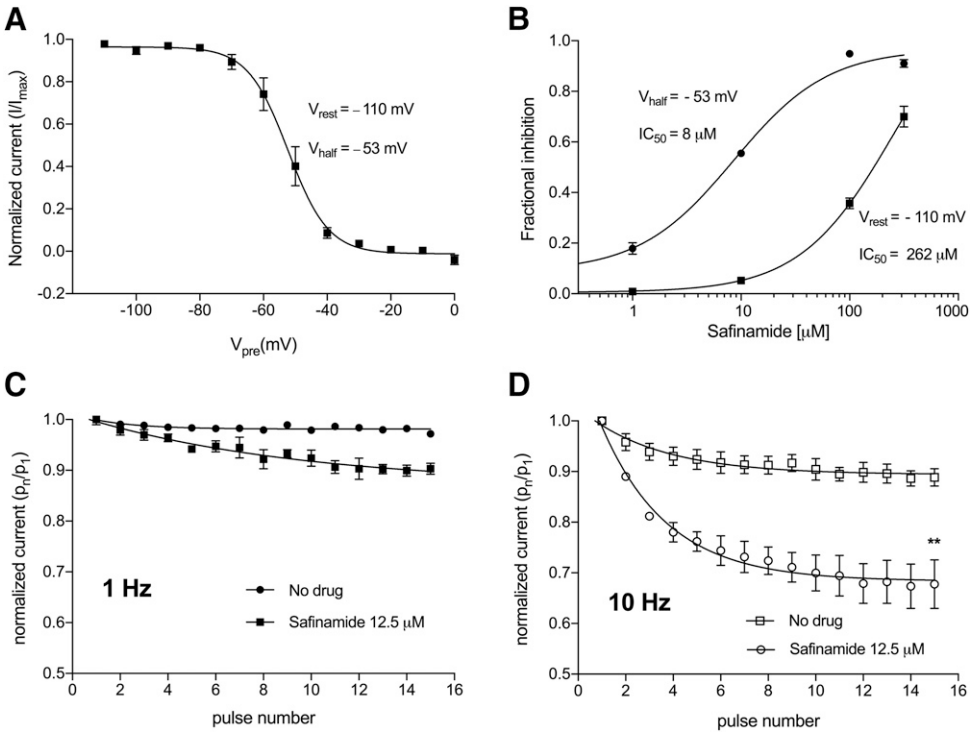

Fig. 6. Effects of safinamide on sodium currents in rat cortical neurons. (A) Steady-state inactivation curves of sodium currents. Inactivation curves were obtained by applying a 2- second conditioning prepulse from −110 to 0 mV from a holding potential of −110 mV and then stepping the cell to +10 mV for 30 millisecond (test pulse). Each current was normalized to the maximal current, and then the average and the S.E. were plotted versus the preconditioning potentials and fitted according to the Boltzmann equation. (B) Voltage- dependence block of sodium currents by safinamide. Neuronal cells were clamped at −90 mV, and then a two-step protocol was used: sodium currents were activated by a 30-millisecond step pulse to +10 mV (test pulse) from 2-second preconditioning potential of −110 mV (V_{rest}) and −53 mV (V_{half}). Drug concentration-inhibition curves were obtained by plotting the tonic block in the resting and depolarized conditions versus drug concentration. Each point represents the mean ± S.E.M. of eight to nine cells. (C and D) Use- and frequency-dependence block of sodium currents by safinamide. A pulse protocol with repetitive impulses to +10 mV (10-millisecond duration) was used at different stimulating frequencies (1 and 10 Hz). Amplitudes of the currents were normalized to the current amplitude of the first impulse in the absence (square symbols) and in the presence of 12.5 μM safinamide (circle symbols). The tonic component of the block by safinamide was cleared by normalization, and only the use-dependent component was reported. $^{**}P < 0.01$ significantly different from no drug condition (Student's *t* test, two-tailed for unpaired data).

safinamide inhibited the fast sodium currents in a concentration- and state-dependent manner. At a depolarized potential of −53 mV, when half of the available sodium channels was in the inactivated state, as during neuronal overexcitation, safinamide more potently inhibited sodium currents (IC_{50} = 8 μM) than at resting potential (IC_{50} = 262 μM). The preferential interaction of safinamide with the inactivated state of the channel (K_i = 4.1 μM) allows it to reduce channel availability for reactivation and thus to inhibit neuronal excitability. Moreover, the tonic inhibition of sodium currents in depolarized conditions was enhanced by the use- and frequency-dependent action of safinamide, resulting in further significant inhibition during sustained repetitive firing and ineffectiveness at normal firing rate (Salvati et al., 1999). Indeed, we did not observe any significant effects of safinamide on spontaneous Glu and GABA release in vivo.

The finding that maximal inhibition of Glu release was observed at free brain concentrations (<1.89 μM) below the observed K_i for sodium currents (4.1 μM) is line with the view that free brain concentrations <50% of in vitro K_i are sufficient to deliver significant in vivo functional sodium channels inhibition (Large et al., 2009). Consistently, safinamide conferred a significant protection (50%–60%) from convulsions in rats when free brain concentrations were in the 0.4–1.2 μM range (Fariello et al., 1998).

Previous in vitro studies in rat cortical neurons reported that safinamide, in addition to sodium channels, also blocks voltage-operated calcium channels of the L- and N-types (Caccia et al., 2006); however, 50% inhibition of calcium currents was achieved only at concentrations ≥20 μM (Caccia et al., 2006), which are higher than those achieved in the present in vivo study. Moreover, we reason that if the drug acted on N-type calcium channels, it should inhibit neurotransmitter release evenly across the areas investigated, which was not the case.

In this respect, the ineffectiveness of safinamide on striatal Glu release might be due to the lack of safinamide-sensitive sodium channels on the membranes of cortico/thalamo-striatal glutamatergic terminals. In fact, relative Nav subtype expression in cortical, striatal, and hippocampal areas has been reported (Westphalen et al., 2010); however, safinamide does not show relevant selectivity (<5-fold) for any Nav channel subtypes (Nav1.1–1.8) (http://wwwemaeuropaeu/ema/indexjsp?curl=pages/medicines/human/medicines/002396/human_med_001847jsp&mid=WC0b01ac058001d124). Moreover, other anticonvulsants acting on voltage-operated sodium channels, such as lamotrigine and carbamazepine, share with safinamide the ability of inhibiting veratridine-induced hippocampal but not striatal Glu release in vivo (Waldmeier et al., 1996). Therefore, the differential effect of safinamide on Glu

release might reflect a preferential action at the somatodendritic level (on firing) rather than at the terminal level (on exocytosis). Thus, local perfusion with veratridine in the hippocampus would drive the firing of intrahippocampal glutamatergic and GABAergic neurons, and pretreatment with safinamide would prevent their overexcitation and, eventually, Glu and GABA release. In striatum, which does not contain Glu interneurons, veratridine would stimulate only axon terminals, inducing a different pattern of discharge, insensitive to the use- and frequency-dependent action of safinamide. GP and SNr also do not contain Glu interneurons; however, both GP and SNr receive massive glutamatergic projections from STN, and STN neurons discharge at much higher frequency (5–30 Hz) (Barraza et al., 2009; Sagarduy et al., 2016) than do pyramidal glutamatergic corticostriatal (and cortico-STN) neurons (1.7 Hz) (Degos et al., 2013), which might favor the use-dependent action of safinamide. Therefore, we can hypothesize that the Glu-inhibiting action of safinamide on pallidal and nigral Glu release might be due to a preconditioning effect of safinamide on the excitability of tonically active STN neurons. This view might be supported by the finding that at the same dose attenuating pallidal and nigral Glu release, safinamide prevented the veratridine-induced Glu release in STN. In STN, neuronal Glu derives either from cortical afferents (which, however, makes up only of 15% of whole afferents to the STN) or from intrinsic axon collaterals of efferent projections, which do not leave the nucleus and innervate other STN neurons (~50% of STN neurons send intranuclear axon collaterals) (Kita et al., 1983). Therefore, veratridine-induced Glu release might reflect overactivity of STN neurons. Finally, in favor of a safinamide effect on STN neuron excitability, this drug selectively prevented Glu release without simultaneously affecting GABA release in STN and projection areas.

Concluding Remarks. Safinamide is a new antiparkinsonian drug endowed with a dual dopaminergic and nondopaminergic mechanism of action. In this respect, we now provide evidence that safinamide differentially inhibits the veratridine-induced Glu and GABA release in the hippocampus and basal ganglia of naïve awake rats, at free brain concentrations effective in blocking voltage-dependent sodium channels. Interestingly, sodium channel blockade and veratridine-stimulated Glu release inhibition occurred within the free brain concentration range estimated in PD patients. Since the unbound brain-to-plasma ratio ($K_{p,uu}$) for those molecules, as safinamide (http://wwwemaeuropaeu/ema/indexjsp?curl=pages/medicines/human/medicines/002396/human_med_001847jsp&mid=WC0b01ac058001d124), that are not substrates of transporters is generally preserved across species, from the rat $K_{p,\ uu}$ an estimated human $K_{p,\ uu}$ of ~3 was inferred. Therefore, from the free plasma concentrations observed in patients treated with safinamide at the recommended clinical doses of 50 and 100 mg/day (Campioni et al., 2010), the free brain concentrations (median and 5^{th}–95^{th} percentile) were estimated to be 0.51 μM (0.21–0.84) and 1.02 μM (0.45–1.92), respectively (Melloni et al., 2015). Therefore, from a clinical perspective, the effect of safinamide on abnormal Glu release may be optimal at a dose of 100 mg/day.

Sodium-channel inhibition is an established anticonvulsant mechanism in animal models and epileptic patients (Catterall, 1999; Rogawski and Loscher, 2004) and might underlie the anticonvulsant activity of safinamide observed in a broad spectrum of preclinical models of epilepsy (e.g., in rat maximal electroshock test, ED_{50} = 7 mg/kg i.p.) and in a pilot study in epileptic patients (Fariello et al., 1998). Use-dependent sodium channel inhibition might be also relevant in PD. Indeed, degeneration of nigral DA neurons cause a (slight) increase in the firing rate of STN neurons and (dramatic) changes in their discharge pattern (burst activity, interneuronal synchrony, and oscillatory activity), both in animal models and in PD patients (Lopez-Azcarate et al., 2010; Wichmann et al., 2011). In animal models of PD, an increase in STN activity is associated with an increase in Glu levels in the basal ganglia output nuclei. Indeed, we showed that striatal D2 receptor blockade after systemic neuroleptic administration induces akinesia and a simultaneous, sustained rise in nigral Glu release, most likely as a consequence of striatopallidal MSN (i.e., the "indirect" pathway) activation and STN disinhibition (Marti et al., 2004; Mabrouk et al., 2010). Consistent with a causal role of nigral Glu in sustaining akinesia, compounds able to normalize neuroleptic- or reserpine-evoked nigral Glu levels improved akinesia (Marti et al., 2004; Austin et al., 2010; Mabrouk et al., 2010; Volta et al., 2010). In line with these findings, conditional ablation of the VGlut2-expressing population of STN neurons caused an increase in locomotor activity that was associated with a reduction in EPSC in slices of STN target areas (Schweizer et al., 2014). This finding confirms numerous studies (reviewed in Baunez and Gubellini (2010) reporting that lesion or pharmacologic inactivation of STN causes hyperkinesia in intact animals and improves motor functions in parkinsonian animals. More recently, optogenetic inactivation of STN was found to improve forelimb akinesia in 6-OHDA hemilesioned rats (Yoon et al., 2014). These lines of evidence suggest that safinamide might improve PD symptoms not only via MAO-B inhibition in the striatal complex but also via normalization of abnormal glutamatergic transmission in STN and its target areas.

The beneficial effect of safinamide in PD patients might also extend beyond the control of motor symptoms. In fact, overactive glutamatergic transmission plays a role in nigral DA neuron loss (Rodriguez et al., 1998; Ambrosi et al., 2014); nonmotor symptoms, such as cognitive impairment, depression, and pain (Finlay and Duty, 2014); and motor complications (wearing-off and dyskinesia) induced by L-dopa pharmacotherapy (Chase et al., 2000; Sgambato-Faure and Cenci, 2012). Preliminary evidence that safinamide improves L-dopa-induced dyskinesia in human (Cattaneo et al., 2015) and nonhuman (Gregoire et al., 2013) primates, as well as pain or depression (Cattaneo et al., 2017) in PD patients, has been presented.

In conclusion, the present neurochemical study provides the first evidence that safinamide selectively inhibits Glu release in STN and its projection areas (GP and SNr) but not in striatum, consistent with an action at the STN level. These neurochemical changes might be clinically relevant since they occur in a free brain concentration range overlapping that estimated in PD patients. Although these neurochemical data need to be replicated in animal models of PD, they provide novel insights into the antiparkinsonian mechanism of action of safinamide, offering a preliminary support for the non-dopaminergic aspects of its action.

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Authorship Contributions

Participated in research design: Morari, Caccia, Melloni, Padoani, Vailati, Sardina.

Conducted experiments: Brugnoli, Pisanò, Novello.

Performed data analysis: Morari, Brugnoli, Pisanò, Novello, Caccia.

Wrote or contributed to the writing of the manuscript: Morari, Caccia.

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