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Title: Safinamide inhibits in vivo glutamate release in a rat model of Parkinson's disease

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Keywords: glutamate release; haloperidol; microdialysis; Parkinson's disease; safinamide; rasagiline.

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Abstract: To investigate whether the reversible MAO-B inhibitor and sodium channel blocker safinamide impairs glutamate release under parkinsonian conditions in vivo, and this effect is dependent on MAO-B inhibition, safinamide (and rasagiline as a comparator) were administered to 6-hydroxydopamine hemilesioned rats, a model of Parkinson's disease, and haloperidol-treated rats, a model of neuroleptic-induced parkinsonism. A microdialysis probe was implanted in the dopamine-depleted dorsolateral striatum, globus pallidus, subthalamic nucleus or substantia nigra reticulata of 6-hydroxydopamine hemilesioned rats. Glutamate and GABA release was stimulated by reverse dialysis of veratridine, and safinamide or rasagiline were acutely administered before veratridine at doses inhibiting MAO-B >50%. A microdialysis probe was implanted in the substantia nigra reticulata of naïve rats to monitor glutamate and GABA release following acute haloperidol and safinamide administration. Safinamide inhibited the veratridine-evoked glutamate release in the globus pallidus and subthalamic nucleus but not in the striatum and substantia nigra. Moreover, it reduced pallidal and nigral GABA release. Conversely, rasagiline failed to modify the veratridine-induced glutamate and GABA release in the basal ganglia. Safinamide also inhibited the haloperidol-induced nigral glutamate release. MAO-B inhibitors safinamide and rasagiline differ in their abilities to inhibit depolarization-evoked glutamate release in the basal ganglia of parkinsonian rats. The ineffectiveness of rasagiline suggests that MAO-B inhibition does not contribute to the antiglutamatergic activity of safinamide. The glutamate-inhibiting action of safinamide within the subthalamo-external pallidal loop, which shows abnormal activity in Parkinson's disease, might contribute to its therapeutic actions of improving motor performance without provoking troublesome dyskinesia

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**Prof Bruno Frenguelli**
University of Warwick,
Coventry,
England (UK)

Ferrara, January 14, 2020

Dear Prof Frenguelli

We thank you, the Editor and the Reviewers of Neuropharmacology for positive comments on our manuscript NEUROPHARM-D-19-00914 entitled "**Safinamide inhibits *in vivo* glutamate release in a rat model of Parkinson's disease**" and for suggestions to improve it.

We have followed the suggestions of the Reviewers and addressed their criticisms. All changes made have been highlighted in text. A point-to-point reply is attached.

I confirm that all Authors have contributed to the work, have read and approved the manuscript, and that the work has not been published before nor is being considered for publication in another journal.

We hope that our manuscript will be favorably considered by the Editors of **Neuropharmacology** and thank you for attention given to our work

Sincerely

Michele Morari

**In reply to the Editor**

Editor: the reviewers are favorable to this manuscript and have made relevant comments that should be addressed to improve it. Based on its antiglutamatergic activity Safinamide was reported to reduce levodopa-induced dyskinesias in parkinsonian monkeys (Gregoire et al. 2013 Park. Relat. Disord. 19:508). This reference could be added to the introduction or discussion.

*AA. At the end of Discussion, we have added a paragraph dealing with the effects of safinamide and amantadine on levodopa-induced dyskinesia, as requested by Reviewer #2. In this context, we have cited the study of Gregoire et al., 2013 in parkinsonian monkeys, as suggested by the Editor*

**In reply to Reviewer #1**

Reviewer #1: This is an interesting experimental study showing clearly the the glutamate-inhibiting action of safinamide within the subthalamic-external pallidum loop which is abnormally functioning in Parkinson's disease. The study provides also data indicating that this effect of safinamide is independent of its pharmacological properties of powerful MAO-B inhibitor, because the glutamate inhibiting action of safinamide is not reproduced when using as comparator a pure MAO-B inhibitor such as rasagiline. The methods and the results are sound and the discussion is well balanced and informative.

Just a couple of minor points to be addressed:
1.In the introduction section a typing error for repetition of GP and GPe

*AA. We have corrected the typo*

2.By the end of the introduction AA should explain why they explored the effect of safinamide on haloperidol-induced Glu release (rather than to explain it in the Results section)

*AA. We have added the rational for performing he experiments in haloperidol-treated rats at the end of Introduction, as requested.*

**In reply to Reviewer #2**

Reviewer #2: This is an interesting manuscript with experimental research which provides some more evidence that safinamide inhibits glutamate release.
Critique:
I suggest to omit the first # of the discussion which summarizes the clinical data on safinamide. Instead the authors should discuss the differentiations to amantadine, a NMDA Antagonist, which - by the way - in the retarded release form was effective against dyskinesia in PD (see Müller & Möhr 2019). One may also discuss, whether NMDA antagonism via amantadine is the relevant mode of action against dyskinesia, since amantadine has also dopamine modulating effects and anticholinergic properties. The basis for this discussion comes from the basal ganglia model, which serves as an explaination for the DBS effects. However DBS also provides a more continuous and elevated release of biogenic amines, which supports the concept of continuous dopaminergic Stimulation (see Biol Psychiatry. 2014 Apr 15;75(8):647-52. doi: 10.1016/j.biopsych.2013.06.021)similar to MAO-B Inhibition. Therefore the authors may crucially discuss their findings.

*AA. We have omitted the first paragraph of Discussion summarizing relevant clinical studies of safinamide, as requested by the Reviewer. We have repositioned it in the Introduction since we believe it contains relevant, although very condensed, information on the clinical profile of the drug. At the end of Discussion, we have added a paragraph dealing with the effects of safinamide and amantadine on levodopa-induced dyskinesia, as also requested by the Reviewer. We have compared their effects in models of levodopa-induce dyskinesia and PD patients as well as their mechanisms of action. We have added new references, among which Muller and Mohr (2019).*

The author should also discuss BMC Pharmacol 2011 Feb 21;11:2. doi: 10.1186/1471-2210-11-2, since here these authors also describe an effect of rasagiline on Glutamate neurotransmission.see also: Journal of Neural Transmission 120(5) · November 2012
These papers should be discussed in Detail.

*AA. We have reported essential details of the study of Dimpfel and Hoffman (2011) in the first paragraph of Discussion.*

## Bullet points summary

- Safinamide inhibits in vivo glutamate release in GP and STN of 6-OHDA rats.
- Rasagiline does not affect glutamate release in 6-OHDA rats
- Safinamide inhibits in vivo glutamate release in SNr of haloperidol-treated rats
- The glutamate-inhibiting action of safinamide in MAO-B independent

# Safinamide inhibits *in vivo* glutamate release in a rat model of Parkinson's disease

Clarissa A. Pisanò [1], Alberto Brugnoli [1], Salvatore Novello [1*], Carla Caccia [2], Charlotte Keywood [2], Elsa Melloni [2], Silvia Vailati [2], Gloria Padoani [2] and Michele Morari [1]

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## Abbreviations

AUC, area-under-the-curve; AP, antero-posterior; BG, basal ganglia; DA, dopamine; DLS, dorsolateral striatum; DV, dorso-ventral; GP, globus pallidus; GPe, globus pallidus externalis; GPi, globus pallidus internalis; Glu, glutamate; 6-OHDA, 6-hydroxydopamine; ML, medio-lateral; PD, Parkinson's disease; SNr, substantia nigra pars reticulata; STN, subthalamic nucleus; TH, tyrosine hydroxylase.

## Declaration of interest

Dr Clarissa Anna Pisanò is a PhD student at the University of Ferrara, and reports no disclosures
Dr Alberto Brugnoli is a postdoc at the University of Ferrara, and reports no disclosures
Dr Salvatore Novello is a postdoc at EPFL, and reports no disclosures
Dr Carla Caccia is preclinical consultant for Zambon S.p.A
Dr Charlotte Keywood is employed by Zambon S.p.A.
Dr Elsa Melloni is employed by Zambon S.p.A.
Dr Silvia Vailati is employed by Zambon S.p.A.
Dr Gloria Padoani is employed by Zambon S.p.A.
Dr Michele Morari is Associate Professor at the University of Ferrara, and received research support from Zambon SpA, and from the Italian Ministry of University for his research

## Abstract

To investigate whether the reversible MAO-B inhibitor and sodium channel blocker safinamide impairs glutamate release under parkinsonian conditions *in vivo*, and this effect is dependent on MAO-B inhibition, safinamide (and rasagiline as a comparator) were administered to 6-hydroxydopamine hemilesioned rats, a model of Parkinson's disease, and haloperidol-treated rats, a model of neuroleptic-induced parkinsonism. A microdialysis probe was implanted in the dopamine-depleted dorsolateral striatum, globus pallidus, subthalamic nucleus or substantia nigra reticulata of 6-hydroxydopamine hemilesioned rats. Glutamate and GABA release was stimulated by reverse dialysis of veratridine, and safinamide or rasagiline were acutely administered before veratridine at doses inhibiting MAO-B >50%. A microdialysis probe was implanted in the substantia nigra reticulata of naïve rats to monitor glutamate and GABA release following acute haloperidol and safinamide administration. Safinamide inhibited the veratridine-evoked glutamate release in the globus pallidus and subthalamic nucleus but not in the striatum and substantia nigra. Moreover, it reduced pallidal and nigral GABA release. Conversely, rasagiline failed to modify the veratridine-induced glutamate and GABA release in the basal ganglia. Safinamide also inhibited the haloperidol-induced nigral glutamate release. MAO-B inhibitors safinamide and rasagiline differ in their abilities to inhibit depolarization-evoked glutamate release in the basal ganglia of parkinsonian rats. The ineffectiveness of rasagiline suggests that MAO-B inhibition does not contribute to the antiglutamatergic activity of safinamide. The glutamate-inhibiting action of safinamide within the subthalamo-external pallidal loop, which shows abnormal activity in Parkinson's disease, might contribute to its therapeutic actions of improving motor performance without provoking troublesome dyskinesia

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To investigate whether the reversible MAO-B inhibitor and sodium channel blocker safinamide impairs glutamate release under parkinsonian conditions *in vivo*, and this effect is dependent on MAO-B inhibition, safinamide (and rasagiline as a comparator) were administered to 6-hydroxydopamine hemilesioned rats, a model of Parkinson's disease, and haloperidol-treated rats, a model of neuroleptic-induced parkinsonism. A microdialysis probe was implanted in the dopamine-depleted dorsolateral striatum, globus pallidus, subthalamic nucleus or substantia nigra reticulata of 6-hydroxydopamine hemilesioned rats. Glutamate and GABA release was stimulated by reverse dialysis of veratridine, and safinamide or rasagiline were acutely administered before veratridine at doses inhibiting MAO-B >50%. A microdialysis probe was implanted in the substantia nigra reticulata of naïve rats to monitor glutamate and GABA release following acute haloperidol and safinamide administration. Safinamide inhibited the veratridine-evoked glutamate release in the globus pallidus and subthalamic nucleus but not in the striatum and substantia nigra. Moreover, it reduced pallidal and nigral GABA release. Conversely, rasagiline failed to modify the veratridine-induced glutamate and GABA release in the basal ganglia. Safinamide also inhibited the haloperidol-induced nigral glutamate release. MAO-B inhibitors safinamide and rasagiline differ in their abilities to inhibit depolarization-evoked glutamate release in the basal ganglia of parkinsonian rats. The ineffectiveness of rasagiline suggests that MAO-B inhibition does not contribute to the antiglutamatergic activity of safinamide. The glutamate-inhibiting action of safinamide within the subthalamo-external pallidal loop, which shows abnormal activity in Parkinson's disease, might contribute to its therapeutic actions of improving motor performance without provoking troublesome dyskinesia.

**Keywords**: glutamate release; haloperidol; microdialysis; Parkinson's disease; safinamide; rasagiline.

## 1. Introduction

Safinamide has been approved as add-on to levodopa for the treatment of intermediate to severe forms of Parkinson's disease (PD). A randomized, double-blind, placebo controlled trial (study 016) showed that safinamide administered for 6 months to mid-to-late fluctuating PD patients treated with levodopa improved ON time without troublesome dyskinesia (Borgohain et al., 2014a). The 18-month extension of this study (study 018) demonstrated positive and long lasting effects of the drug on motor performance and activities of daily living (UPDRS part II) (Borgohain et al., 2014b). In addition, post hoc analysis suggested that safinamide had a positive effect on mood, pain and levodopa-induced dyskinesia in a subgroup of patients (Cattaneo et al., 2017; Cattaneo et al., 2015; Cattaneo et al., 2016), suggesting that safinamide action in humans might go beyond MAO-B inhibition. Indeed, beyond its MAO-B inhibiting properties, safinamide was shown to also limit depolarization-evoked glutamate (Glu) release *in vitro* as a result of its use and frequency dependent inhibition of sodium channels (Caccia et al., 2006; Morari et al., 2018; Salvati et al., 1999). Normalizing abnormal Glu transmission without interfering with the normal physiological response might provide additional therapeutic benefit to MAO-B inhibition, since different domains of PD are characterized by dysregulated Glu transmission. In particular, abnormal activity of glutamatergic neurons of the subthalamic nucleus (STN) (Faggiani and Benazzouz, 2017) and synchronous oscillatory activity between STN and the interconnected globus pallidus externalis (GPe) (Hegeman et al., 2016) is believed to contribute to parkinsonian symptoms. Excessive Glu release also contributes to nigral dopaminergic cell loss (Blandini et al., 1996; Faggiani and Benazzouz, 2017), and has been associated with levodopa-induced dyskinesia (Dupre et al., 2011; Paolone et al., 2015; Sgambato-Faure and Cenci, 2012) as well as non-motor symptoms of PD such as neuropathic pain (Quintero et al., 2011; Watson, 2016) and depression (Lener et al., 2017; Miller et al., 2014). We recently reported that safinamide inhibits depolarization-induced Glu release in the hippocampus and basal ganglia (BG) of naïve rats *in vivo* (Morari et al., 2018). We now

investigate whether the Glu-inhibiting effect of safinamide is replicated after dopamine (DA) depletion, and whether MAO-B inhibition contributes to this effect. Safinamide and the MAO-B inhibitor rasagiline (Youdim et al., 2001) were administered to 6-hydroxydopamine (6-OHDA) hemilesioned rats and the veratridine-evoked Glu release was monitored in the STN, GP, substantia nigra reticulata (SNr) and dorsolateral striatum (DLS). To further investigate the impact of safinamide on parkinsonism-associated changes of BG circuitry and Glu release, the effect of safinamide on haloperidol-induced Glu (and GABA) release in rat SNr was also explored. In fact, in this model of functional parkinsonism, elevation of Glu release can be achieved in SNr by systemic administration of a neuroleptic (haloperidol) (Mabrouk et al., 2010; Marti et al., 2004), causing blockade of striatal D2 receptors and disinhibition of the subthalamo-nigral glutamatergic projection.

## 2. Methods

### *2.1. Animal subjects*

Male Sprague-Dawley rats (Charles River Lab, Calco, Lecco; Italy) were housed in a standard facility with free access to food and water, and kept under regular lighting conditions (12 hr dark/light cycle). Experimental procedures involving the use of animals complied with the ARRIVE guidelines and the EU Directive 2010/63/EU for animal experiments, and were approved by the Ethical Committee of the University of Ferrara and the Italian Ministry of Health (license 714/2016-PR).

### *2.2. Unilateral 6-OHDA lesion*

Rats (150 g) were unilaterally injected under isoflurane anesthesia in the (right) medial forebrain bundle (stereotaxic coordinates from bregma and the dural surface: AP= -4.4 , ML= 1.2, VD= -7.8, tooth bar at -2.4 mm (Paxinos and Watson, 1986) with 12 µg 6-OHDA hydrobromide (dissolved in 0.02% ascorbate-saline) as previously described (Morari et al., 1996; Paolone et al.,

2015). Animals were pretreated with antibiotics (Synulox™, 50 µl/kg, i.p.). The wound was sutured and infiltrated with 2% lidocaine solution (Esteve™). Two weeks later, rats were screened by assessing the motor asymmetry score in two different ethological tests (the bar and drag tests) (Marti et al., 2005; Marti et al., 2007). Rats showing immobility time at the contralateral paw in the bar test >20 sec and a number of steps at the contralateral paw <3 (or alternatively a contralateral/ipsilateral paw ratio <50%) were included in the study. In fact, Western blot analysis of tyrosine hydroxylase (TH) striatal levels on a large population of 6-OHDA hemilesioned rats revealed that these thresholds were associated with striatal DA depletion >90% (see below). Microdialysis experiments were performed 4-6 weeks after 6-OHDA lesion.

**2.3.** ***Microdialysis experiments***.

In vivo microdialysis was performed as previously described (Morari et al., 2018; Morari et al., 1996; Paolone et al., 2015). Concentric microdialysis probes were constructed using AN69 (Gambro Industries, Meyzieu, France) semipermeable hollow membranes (65 kDa molecular weight cut-off, 340 µm outer diameter).

*Microdialysis in 6-OHDA hemilesioned rats.* Eighty (80) 6-OHDA rats were included in the study (four rats, one in the safinamide/GP cohort, one in the safinamide/DLS cohort and two in the rasagiline/DLS cohort, were lost due to probe clogging/rupture). One microdialysis probe was stereotactically implanted under isoflurane anesthesia in the DA-depleted DLS, or ipsilateral SNr, GP and STN. Implantation coordinates (in mm, from bregma and the dural surface) (Paxinos and Watson, 1986), and dialysis membrane lengths (in mm) were: DLS, antero-posterior (AP) +1.0, medio-lateral (ML) ±3.5, dorso-ventral (DV) -6.0 (3 mm), SNr, AP -5.5, ML ±2.2, DV -8.3 (1 mm), GP, AP -1.3, ML ±3.3, DV -6.5 (2 mm), and STN, AP -3.7, ML ±2.5, DV -8.6 (1 mm). Twenty-four hours after surgery, the probe was perfused with a modified Ringer solution ($CaCl_2$ 1.2 mM; KCl 2.7 mM; NaCl 148 mM; $MgCl_2$ 0.85 mM) at a 3 µl/min flow rate. Sample collection (every 20 min) started after 6 h rinsing. At least four baseline

samples were collected, then treatments were administered in a randomized fashion. In the safinamide cohort, saline or safinamide (Fig 1) was administered 30 min before local infusion of veratridine (10 µM, 30 min) through the probe. Safinamide was administered at the dose of 15 mg/kg (i.p.) reported to acutely inhibit brain MAO-B by >50% (Strolin Benedetti et al., 1994). At the end of veratridine infusion, sample collection continued for 80 min (safinamide group; Fig. 1). Experiments were repeated the day later (i.e. 48 h after surgery) and the treatments crossed. A similar protocol was applied to the rasagiline cohort (Fig. 2) with the differences that saline or rasagiline were given 60 min before veratridine, and sample collection continued for 90 min after the end of veratridine perfusion (Fig. 2). Also in this case, treatments were crossed in the next microdialysis session. Rasagiline was acutely administered at 0.1 mg/kg (i.p.), reported to be a MAO-B selective oral dose giving about 50% brain MAO-B inhibition (Youdim et al., 2001). The different time of administration of safinamide and rasagiline was based on the different pharmacokinetics of the two drugs in the rat: plasma Tmax≤60 and ≤120 min, respectively (Caccia et al., 2006; EMA/CHMP, 2005; Youdim et al., 2001). Within each cohort, experimenters were blinded to treatments. At the end of experiments, animals were sacrificed by an overdose of isoflurane, and the correct placement of the probes was verified histologically. Severe (>90%) dopamine depletion was randomly confirmed by post-mortem analysis of tyrosine hydroxylase (TH) levels in Western blot assay (optical density of TH band normalized over GAPDH: 0.15±0.039 vs 4.41±0.53 ipsilateral vs contralateral striatum, respectively, t=8.00 df=36, Student's t-test, two-tailed for unpaired data; n=19 rats).

*Microdialysis in haloperidol-treated rats.* Eighteen (18) male Sprague-Dawley naïve rats (270-300 g) were included in the study. One microdialysis probe (1 mm length) was stereotactically implanted under isoflurane anesthesia in SNr, according to the coordinates detailed above. Twenty-four hours after surgery, the probe was perfused with a modified Ringer solution (see above) at a 3 µl/min flow rate, and sample collection (every 20 min) started after 6 h rinsing. After collection of four baseline samples, rats were administered haloperidol (0.8 mg/kg, i.p.),

and 60 min later, were randomized to saline or safinamide (15 mg/kg, i.p.). Sample collection continued for further 100 min. A second microdialysis session was performed at 48 h after surgery, in which treatments were crossed. Experimenters were blinded to treatments. At the end of experiments, animals were sacrificed by an overdose of isoflurane and the correct placement of the probes was verified histologically.

### *2.4.Endogenous Glu and GABA analysis*

Glu and GABA in the dialysate were measured by HPLC coupled with fluorometric detection as previously described (Marti et al., 2008; Paolone et al., 2015). The limits of detection for Glu and GABA were ~1 nM and 0.5 nM.

### *2.5. TH analysis*

Rats were sacrificed by an overdose of isoflurane. Striata were solubilized and homogenized in lysis buffer (RIPA buffer, protease and phosphatase inhibitor cocktail) and centrifuged at 18,000 x g for 15 min at 4°C. Supernatants were collected and total protein levels were quantified using the bicinchoninic acid protein assay kit (Thermo Scientific). Thirty micrograms of protein per sample were separated by SDS-PAGE, transferred onto polyvinyldifluoride membrane and incubated overnight (4°C) with the rabbit anti-TH primary antibody (Merck Millipore, AB152, 1:1000). Membranes were then washed and incubated with horseradish peroxidase-linked secondary antibody (Merck Millipore, goat anti-rabbit IgG HRP-conjugate 12-348, 1:4000). Immunoreactive proteins were visualized by enhanced chemiluminescence (ECL) detection kit (Pierce™ BCA Protein Assay Kit, Thermo Scientific or ECL+, GE Healthcare). Images were acquired and quantified using the ChemiDoc MP System and the ImageLab Software (Bio-Rad). Membranes were then stripped and re-probed with rabbit anti-α-tubulin antibody (Merck-Millipore 04-1117, 1:25000). Data were analyzed by densitometry and the optical density of TH protein band was normalized to α-tubulin levels.

### *2.6. Data and statistical analysis*

Statistical analysis was performed (Graphpad Prism) using 2-way repeated measure (RM) ANOVA followed by the Bonferroni test for multiple comparisons. The Student's t-test, two-tailed for unpaired data, was used to compare area-under-the curve values (AUC; calculated in the 160-240 min range) of treatment groups in Figure 5. Alpha was set at 0.05.

### *2.7.Materials*

6-OHDA hydrobromide and rasagiline mesylate were purchased from Tocris (Bristol, UK). Safinamide methansulfonate was provided by Zambon S.p.A. All drugs were dissolved in saline (6-OHDA in ascorbate-saline) and administered at the volume of 1.0 ml/kg body weight.

## 3. Results

### 3.1. Safinamide but not rasagiline inhibited the veratridine-induced Glu release in the GP of 6-OHDA hemilesioned rats

During veratridine infusion at the 120-min time-point, Glu levels in GP (59.21±6.31 nM; n=38) doubled compared to baseline levels (200±37% of control). Glu levels peaked in the sample at 140 minutes (217±35%) and slowly returned to baseline thereafter (Fig. 1A). Safinamide (15 mg/kg) inhibited the veratridine-induced Glu release (Fig. 1A; treatment $F_{1,8}$=4.67, p=0.046; time $F_{8,128}$=6.87, p<0.0001; time vs treatment interaction $F_{8,128}$=3.36, p=0.0016), causing significant inhibition at the 120-min and 140-min time-points (p<0.05). In the rasagiline cohort, veratridine caused an increase of Glu levels similar to that observed in the safinamide cohort (Fig. 1B). However, contrary to safinamide, rasagiline (0.1 mg/kg) did not inhibit the pallidal Glu response to veratridine (Fig. 1B; treatment $F_{1,10}$=0.01, p=0.91; time $F_{10,180}$=9.45, p<0.0001; time vs treatment interaction $F_{10,180}$=0.26, p=0.98). GABA levels in GP (6.04 ± 1.00 nM; n=38) responded to veratridine in a similar fashion to Glu levels. Safinamide prevented the veratridine-induced rise in GABA (treatment $F_{1,8}$=8.39, p=0.0111; time $F_{8,120}$=2.56, p=0.0128; time vs treatment interaction $F_{8,120}$=2.39, p=0.0196; Fig. 1C). Conversely, rasagiline did not have any

effect (treatment $F_{1,10}$=0.01, p=0.99; time $F_{10,180}$=7.07, p<0.0001; time vs treatment interaction $F_{8,180}$=0.08, p=0.99; Fig. 1D).

### 3.2.Safinamide but not rasagiline inhibited the veratridine-induced Glu release in the STN of 6-OHDA hemilesioned rats

The time-course of STN Glu levels (47.52±4.78 nM; n=40) in response to veratridine (Fig. 2A-B) was similar to that observed in GP, with a maximal two-fold increase over basal levels. Safinamide (Fig. 2A) inhibited the rise in STN Glu (treatment $F_{1,8}$=1.62, p=0.21; time $F_{8,144}$=7.88, p<0.0001; time vs treatment interaction $F_{8,144}$=3.31, p=0.0017), the inhibition being significant at the 140-min time-point (p<0.05). Conversely, rasagiline (Fig. 2B) did not have any effect (treatment $F_{1,10}$=1.48, p=0.24; time $F_{10,180}$=12.54, p<0.0001; time X treatment interaction $F_{10,180}$=0.61, p=0.80). Unlike the GP (and the other nuclei examined), veratridine did not significantly elevate GABA levels in the STN (6.32 ± 0.96 nM; n=36), in either the safinamide (treatment $F_{1,8}$=0.42, p=0.52; time $F_{8,112}$=1.33, p=0.23; time vs treatment interaction $F_{8,112}$=0.66, p=0.72; Fig. 2C) or the rasagiline (treatment $F_{1,10}$=0.05, p=0.81; time $F_{10,180}$=1.72, p=0.08; time vs treatment interaction $F_{10,180}$=0.81, p=0.62; Fig. 2D) cohorts.

### 3.3.Neither safinamide nor rasagiline inhibited the veratridine-induced Glu release in the SNr of 6-OHDA hemilesioned rats

During veratridine infusion, Glu levels in SNr (36.49±3.96 nM; n=40) more than doubled baseline levels, slowly returning to baseline afterwards (Fig. 3A-B). Neither safinamide (treatment $F_{1,8}$=0.04, p=0.83; time $F_{8,144}$=12.62, p<0.0001; time X treatment interaction $F_{8,144}$=0.18, p=0.99; Fig 3A) nor rasagiline (treatment $F_{1,10}$=0.55, p=0.46; time $F_{10,180}$=9.36, p<0.0001; time vs treatment interaction $F_{10,180}$=0.61, p=0.82; Fig 3B) were able to affect veratridine-induced Glu release. Also GABA levels in SNr (5.58±0.72 nM, n=40) were transiently elevated by veratridine infusion (Fig. 3C-D). Safinamide inhibited the rise in GABA levels (treatment $F_{1,8}$=3.94, p=0.062; time $F_{8,144}$=2.44, p=0.0167; time vs treatment interaction

$F_{8,144}$=2.19, p=0.031; Fig 3C) whereas rasagiline had no effect (treatment $F_{1,10}$=0.02, p=0.87; time $F_{10,180}$=5.64, p<0.0001; time vs treatment interaction $F_{10,180}$=0.75, p=0.67; Fig 3D).

### 3.4. Neither safinamide nor rasagiline inhibited the veratridine-induced Glu release in the DLS of 6-OHDA hemilesioned rats

Glu and GABA levels in DLS (67.64±8.19 nM and 10.03±2.11 nM, respectively, n=34 both) were stimulated by veratridine (Fig. 4A). Neither safinamide (treatment $F_{1,8}$=0.07, p=0.79; time $F_{8,128}$=10.87, p<0.0001; time vs treatment interaction $F_{8,128}$=0.39, p=0.92; Fig 4A) nor rasagiline (Fig. 4B; treatment $F_{1,10}$=0.03, p=0.85; time $F_{10,140}$=13.26, p<0.0001; time vs treatment interaction $F_{10,140}$=0.41, p=0.93) affected the veratridine-induced Glu release. Likewise, neither safinamide (treatment $F_{1,8}$=0.47, p=0.50; time $F_{8,128}$=9.65, p<0.0001; time vs treatment interaction $F_{8,128}$=0.56, p=0.80; Fig 4C) nor rasagiline (Fig. 4D; treatment $F_{1,10}$=0.43, p=0.52; time $F_{10,140}$=7.91, p<0.0001; time vs treatment interaction $F_{10,140}$=0.26, p=0.98) affected the veratridine-induced GABA release.

### 3.5. Safinamide inhibited haloperidol-induced Glu release in SNr

To investigate whether the loss of effect of safinamide on Glu release in the SNr of 6-OHDA hemilesioned rats compared to naïve rats (Morari et al., 2018), was due to changes of the subthalamo-nigral Glu pathways, as a consequence of the degeneration of nigral DA neurons, we explored the effect of safinamide in the rat model of neuroleptic-induced parkinsonism (Fig. 5). In this model, acute systemic administration of haloperidol caused SNr Glu levels to rise along with akinesia (Marti et al., 2005; Marti et al., 2004), likely due to STN disinhibition. Haloperidol (0.8 mg/kg, i.p.) induced a sustained increase of nigral Glu levels and safinamide (15 mg/kg) slightly but significantly attenuated such increase, although the effect was significant only at the 180-min time-point (treatment $F_{1,9}$=7.42 p=0.0150; time $F_{9,144}$=7.81 p<0.0001 time vs treatment interaction $F_{1,144}$=1.85 p=0.0643; Fig. 5A). Nonetheless, AUC analysis also revealed a significant inhibition by safinamide (AUC in arbitrary units: 7051±529 haloperidol/saline,

5354±400 haloperidol/safinamide, t=2.55, df=16, p=0.0212, Student's t-test two-tailed for unpaired data). Haloperidol did not affect GABA release in SNr (Fig. 5B).

## 4. Discussion

In present study we provide evidence that safinamide attenuates the exocytotic Glu release in the GP and STN of 6-OHDA hemilesioned rats. The finding that rasagiline, contrary to safinamide, is ineffective on Glu release at a dose inhibiting MAO-B by ≥50%, indicates that MAO-B inhibition does not contribute to the Glu-inhibiting action of safinamide. As a matter of fact, rasagiline was shown to inhibit Glu transmission, in particular NMDA receptor signalling, in rat hippocampal slices (Dimpfel and Hoffmann, 2011). However this effect was observed at micromolar concentrations, much higher than those reached in therapy (see below), and it was shared by rasagiline aminoindan metabolite, which lacks MAO-B inhibition property, thus it appears to be MAO-B independent (Dimpfel and Hoffmann, 2011). Notably, in our experimental conditions safinamide and rasagiline were administered at doses (15 mg/kg and 0.1 mg/kg, respectively) giving rat free plasma concentrations (0.5 μM and 8 nM, respectively) corresponding to human free plasma concentrations associated with repeated clinical dose of 100 mg for safinamide (0.4 μM) and 1 mg for rasagiline (5 nM) (Campioni et al., 2010; Elgart et al., 2019; Melloni et al., 2015). The clinical relevance of these findings is further supported by a recent TMS neurophysiological study (Guerra et al., 2019) showing that two-week oral treatment of safinamide normalized the enhancement of short-interval intracortical facilitation, an index of cortical Glu neuron hyperactivity, in the primary motor cortex (M1) of PD patients manifesting levodopa-induced dyskinesia. Different from other safinamide-induced intracortical neurophysiological changes related to MAO-B inhibition, this effect was found to be dose-dependent and maximal at the higher dose of safinamide tested (i.e. 100 mg/die), ruling out MAO-B contribution (Guerra et al., 2019). This supports the notion that safinamide is capable of normalizing abnormal cortical glutamatergic activity trough non dopaminergic mechanisms.

Modulation of ionic currents is the likely mechanism through which safinamide inhibits Glu release in the BG. Early findings indicate that inhibition of Glu release from rat brain slices and synaptosomes *in vitro* might involve modulation of voltage-operated sodium ($Na_v$) and calcium ($Ca_v$) channels (Salvati et al., 1999). However, binding studies showed a weaker affinity of safinamide for $Ca_v$ channels (<25% binding at 10 μM) than $Na_v$ channels (67% binding at 10 μM, with an $IC_{50}$= 8 uM) (EMA/CHMP, 2014; Salvati et al., 1999). Different lines of evidence obtained from previous *in vivo* study in naïve rats (Morari et al., 2018) also point to a major role of $Na_v$ channels. Indeed, the effect of safinamide was not homogeneous throughout the BG, as it would be expected if the drug inhibited presynaptic N-type $Ca_v$ regulating exocytotic release, since safinamide failed to inhibit the veratridine-evoked Glu release in DLS, and GABA release in BG. Moreover, the *in vivo* action of safinamide were obtained at a dose (15 mg/kg) achieving free brain concentrations (1.89 μM) close to the affinity value for the inactivated state of $Na_v$ channels, as measured in primary cortical neurons (4.1 μM) (Morari et al., 2018). It should be remembered that other antiepileptic drugs and $Na_v$ blockers, such as carbamazepine, lamotrigine and zonisamide are capable of inhibiting cortical Glu release (Okada et al., 1998; Waldmeier et al., 1996).

How may inhibition of Glu release in STN and GP, reasonably via blockade of $Na_v$, be relevant to the antiparkinsonian action of the drug? STN is the only excitatory nucleus within the BG as it is mainly composed of glutamatergic neurons which receive glutamatergic projections from cerebral cortex (the so called hyperdirect pathway) and thalamus, dopaminergic projection from SNc/VTA, GABAergic projections from GPe, and serotonergic projections from raphe nuclei. These neurons are tonically active and provide a strong excitatory drive to the GPe, GPi and SNr. The STN and GPe form an interconnected loop, and DA loss causes a strengthening of the cortical-STN and STN-GPe connections (Ammari et al., 2011; Hegeman et al., 2016; Plenz and Kital, 1999). This causes the emergence of synchronous and oscillatory activity, most evident in the beta power frequency (13-30 Hz) (Bergman et al., 1994; Cruz et al., 2011; Hammond et al.,

2007; Nambu and Tachibana, 2014) which reverberates to the BG output nuclei (GPi/SNr) and sustains motor symptoms of PD. In fact, pharmacological inactivation of STN or GPe-STN connections as well as blockade of Glu inputs to STN, reduce abnormal STN oscillations (Nambu and Tachibana, 2014). Likewise, deep brain stimulation of the STN promotes desynchronization of STN and GP activity, and improves motor deficits, particularly bradykinesia (Kuhn et al., 2006; Weinberger et al., 2006). $Na_v$ channels play a role in these events. In fact, high frequency stimulation of STN slices silences the activity of STN neurons via inactivation of persistent NaI current (*I*NaP), which are responsible for tonic activity of glutamatergic neurons (Beurrier et al., 2000). Thus, blockade of abnormal $Na_v$ activation in STN by safinamide might act in a similar way to desynchronize abnormal STN-GPe activity and promote motor function. The fact that safinamide also blocks veratridine-stimulated GABA release in GP might confirm the view that safinamide impacts on the activity of the STN-GPe loop. In fact, GPe is mainly composed of tonically-active GABAergic neurons projecting to STN, GPi/SNr and striatum. We should recall that the effect of safinamide is use- and frequency-dependent, meaning that this drug has more affinity for the inactivated state of the channels, and is effective at higher frequency of membrane depolarization (Caccia et al., 2006; Morari et al., 2018), which makes it suitable for blockade of abnormal high-frequency firing activity, sparing the physiological one. Unexpectedly, safinamide failed to inhibit veratridine-stimulated Glu release in the DA-depleted SNr. Since safinamide inhibited the veratridine-evoked Glu release in the SNr of naïve rats (Morari et al., 2018), adaptive changes in neural circuitry following DA loss might underlie the loss of response to safinamide of nigral Glu terminals in the 6-OHDA rat. We can speculate that this might be due to different patterns of activity of STN neurons projecting to SNr (safinamide-insensitive) compared to those projecting to GP (safinamide-sensitive). Alternatively, local changes within the SNr, in particular a loss of GABA control over Glu terminals, might underlie the lack of effect of safinamide. In fact, safinamide inhibited veratridine-induced nigral GABA release, and this might lead to veratridine-induced

overstimulation of Glu release from subthalamo-nigral nerve terminals, masking the inhibitory effect of safinamide exerted at the somato-dendritic level. In favour of the hypothesis that chronic adaptive network changes following nigral DA neuron degeneration might underlie the loss of sensitivity to safinamide, safinamide was capable of inhibiting SNr Glu release in haloperidol-treated rats. Acute systemic haloperidol administration has been shown to cause an increase in burst activity in STN neurons (Degos et al., 2005) and elevation of SNr Glu release (Mabrouk et al., 2010; Marti et al., 2005; Marti et al., 2004). The finding that safinamide attenuates this effect confirms that safinamide can acutely interfere with abnormal STN activity and modulate nigral Glu release. Interestingly, also high frequency stimulation of STN is capable of attenuating neuroleptic-induced akinesia (Degos et al., 2005).

Consistent with that found in naïve rats (Morari et al., 2018), safinamide did not affect veratridine-induced striatal Glu release in 6-OHDA rats, suggesting that safinamide does not modulate exocytotic Glu release from striatal presynaptic terminals. Nonetheless, chronic safinamide was able to inhibit the rise of striatal Glu release (and the increase of GluN2A levels and GluN2A/GluN2B ratio) in dyskinetic rats acutely challenged with levodopa, indicating that in some circumstances safinamide can affect cortico/thalamo-striatal glutamatergic transmission (Gardoni et al., 2018). Although the two models cannot be compared (e.g. stimulation of striatal Glu release was induced differentially via intrastriatal perfusion with veratridine or systemic administration of levodopa), we could speculate that repeated administration of safinamide might be required to impact also veratridine-evoked striatal Glu release in 6-OHDA hemilesioned, levodopa-naïve rats. In fact, repeated administration of safinamide, acting at the STN-GP level, might be necessary to normalize dysfunctional firing activity within the cortico-BG-thalamo cortical loop. This mechanism of action clearly differentiates safinamide from another antiglutamatergic drug used in PD therapy, amantadine (for a recent review see (Muller and Mohr, 2019). Amantadine provides modest motor symptomatic benefit but marked antidyskinetic effect in all species tested, namely humans, non human primates, rats and mice

(for recent reviews see (Brigham et al., 2018; Fox et al., 2018; Stanley et al., 2018). Although the mechanism underlying the antidyskinetic effects of amantadine is not fully understood yet, different actions have been proposed to contribute, such as potentiation of dopamine transmission (increase of DA reuptake, DA release, DOPA decarboxylase activity), inhibition of cholinergic activity, increase of $K^+$ channel conductance, and weak NMDA receptor antagonism (Bido et al., 2011; Muller and Mohr, 2019). Compared to amantadine, safinamide has a milder and species-specific antidyskinetic effect, being effective in selected populations of PD patients with higher baseline dyskinesia scores (Cattaneo et al., 2015), nonhuman primates (Gregoire et al., 2013) but not rats (Gardoni et al., 2018). This might reflect the fine-tuning effect of safinamide on firing activity within the cortico-BG-thalamo-cortical loop, as discussed above, and perhaps also the prevalent role played by STN in parkinsonian akinesia rather than levodopa-induced dyskinesia in rats (Aristieta et al., 2012; Gubellini et al., 2006; Lacombe et al., 2009).

*Conclusions*

Safinamide attenuated the veratridine-induced Glu release in the STN and GP, along with GABA release in the GP (and SNr), of 6-OHDA hemilesioned rats. This effect is not shared by rasagiline, thus it is independent of MAO-B inhibition and likely relies on the ability of safinamide to inhibit $Na_v$ channels. The possibility that safinamide is capable of normalizing abnormal activity within the STN-GP loop needs to be proven in PD patients. However, the neurochemical changes observed in lesioned rats might be clinically relevant since they occurred in a free brain concentration range overlapping that estimated in PD patients at the therapeutic dose of 100 mg/die (Morari et al., 2018). Therefore, the present data suggest that this mechanism might contribute to the safinamide therapeutic actions of improving motor performance without provoking troublesome dyskinesia. Moreover, considering a wider role for STN in modulating motor, cognitive and affective functions, these data justify the study of the effect of this drug in other models of dysfunctional STN beyond PD (Bonnevie and Zaghloul, 2019; Castrioto et al., 2014).

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**Figure legends**

**Figure 1**. Safinamide but not rasagiline inhibits veratridine-induced glutamate and GABA release in the globus pallidus. Glutamate (Glu; A-B) and GABA (C-D) dialysate levels following systemic administration of saline or safinamide (15 mg/kg, i.p., arrow) (A,C), and saline and rasagiline (0.1 mg/kg, i.p.) (B,D) in combination with reverse dialysis of veratridine (10 μM, 30 min; black bar) in the globus pallidus (GP) of awake freely-moving 6-OHDA hemilesioned rats. Data are expressed as percentage of basal pre-treatment levels (calculated as the mean of the two samples preceding the treatment) and are mean ± SEM of n=9 (A), n=10 (B), n=9 (C; n=8 in the veratridine/safinamide group due to sample loss during analysis) and n=10 (D) rats per group, originating from 9 rats in the safinamide cohort and 10 rats in the rasagiline cohort. Basal Glu and GABA levels (in nM) were 46.88±4.75 (A), 64.66±8.03 (B), 7.74±1.54 (C) and 3.76±0.89 (D). *$p<0.05$ versus saline (two-way RM ANOVA followed by the Bonferroni test for multiple comparisons)

**Figure 2**. Safinamide but not rasagiline inhibits veratridine-induced glutamate release in the subthalamic nucleus. Glutamate (Glu; A-B) and GABA (C-D) dialysate levels following systemic administration of saline or safinamide (15 mg/kg, i.p., arrow) (A,C), and saline and rasagiline (0.1 mg/kg, i.p.) (B,D) in combination with reverse dialysis of veratridine (10 μM, 30 min; black bar) in the subthalamic nucleus (STN) of awake freely-moving 6-OHDA hemilesioned rats. Data are expressed as percentage of basal pre-treatment levels (calculated as the mean of the two samples preceding the treatment) and are mean ± SEM of n=10 (A), n=10 (B), n=8 (C) and n=10 (D) rats per group, originating from 10 rats in the safinamide cohort and 10 rats in the rasagiline cohort). Basal Glu and GABA levels (in nM) were 59.02±7.54 (A), 36.53±3.92 (B), 3.55±1.07 (C) and 8.55±1.32 (D). *$p<0.05$ versus saline (two-way RM ANOVA followed by the Bonferroni test for multiple comparisons)

**Figure 3**. Safinamide but not rasagiline inhibits veratridine-induced GABA release in the substantia nigra reticulata. Glutamate (Glu; A-B) and GABA (C-D) dialysate levels following systemic administration of saline or safinamide (15 mg/kg, i.p., arrow) (A,C), and saline and rasagiline (0.1 mg/kg, i.p.) (B,D) in combination with reverse dialysis of veratridine (10 μM, 30 min; black bar) in the substantia nigra reticulata (SNr) of awake freely-moving 6-OHDA hemilesioned rats. Data are expressed as percentage of basal pre-treatment levels (calculated as the mean of the two samples preceding the treatment) and are mean ± SEM of n=10 (A), n=10 (B), n=10 (C) and n=10 (D) rats per group, originating from 10 rats in the safinamide cohort and 10 rats in the rasagiline cohort. Basal Glu and GABA levels (in nM) were 38.37±4.92 (A), 34.24±6.51 (B), 6.86±1.13 (C) and 4.31±0.84 (D). *$p<0.05$ versus saline (two-way RM ANOVA followed by the Bonferroni test for multiple comparisons)

**Figure 4**. Safinamide and rasagiline do not alter veratridine-induced amino acid release in the striatum. Glutamate (Glu; A-B) and GABA (C-D) dialysate levels following systemic administration of saline or safinamide (15 mg/kg, i.p., arrow) (A,C), and saline and rasagiline (0.1 mg/kg, i.p.) (B,D) in combination with reverse dialysis of veratridine (10 μM, 30 min; black bar) in the dorsolateral striatum (DLS) of awake freely-moving 6-OHDA hemilesioned rats. Data are expressed as percentage of basal pre-treatment levels (calculated as the mean of the two samples preceding the treatment) and are mean ± SEM of n=9 (A), n=8 (B), n=9 (C) and n=8 (D) rats per group, originating from 9 rats in the safinamide cohort and 8 rats in the rasagiline cohort. Basal Glu and GABA levels (in nM) were 56.19±13.55 (A), 80.52±7.68 (B), 8.33±3.48 (C) and 11.95±2.22 (D).

**Figure 5.** Safinamide inhibits haloperidol-induced glutamate release in the substantia nigra reticulata. Glutamate (Glu; A) and GABA (B) dialysate levels in the substantia nigra reticulata (SNr) of awake, freely-moving rats following systemic administration of haloperidol (0.8 mg/kg,

i.p., arrow). Sixty (60) min after haloperidol, rats received safinamide (15 mg/kg, i.p.) or saline (arrow). Data are expressed as percentage of basal pre-haloperidol levels (calculated as the mean of the two samples preceding the treatment) and are mean ± SEM of 9 rats per group. *$p<0.05$ versus haloperidol/saline (two-way ANOVA followed by the Bonferroni test for multiple comparisons). #$p<0.05$ versus haloperidol/saline (Student t-test on AUC values, calculated in the 160-240 min range).

Figure(s)

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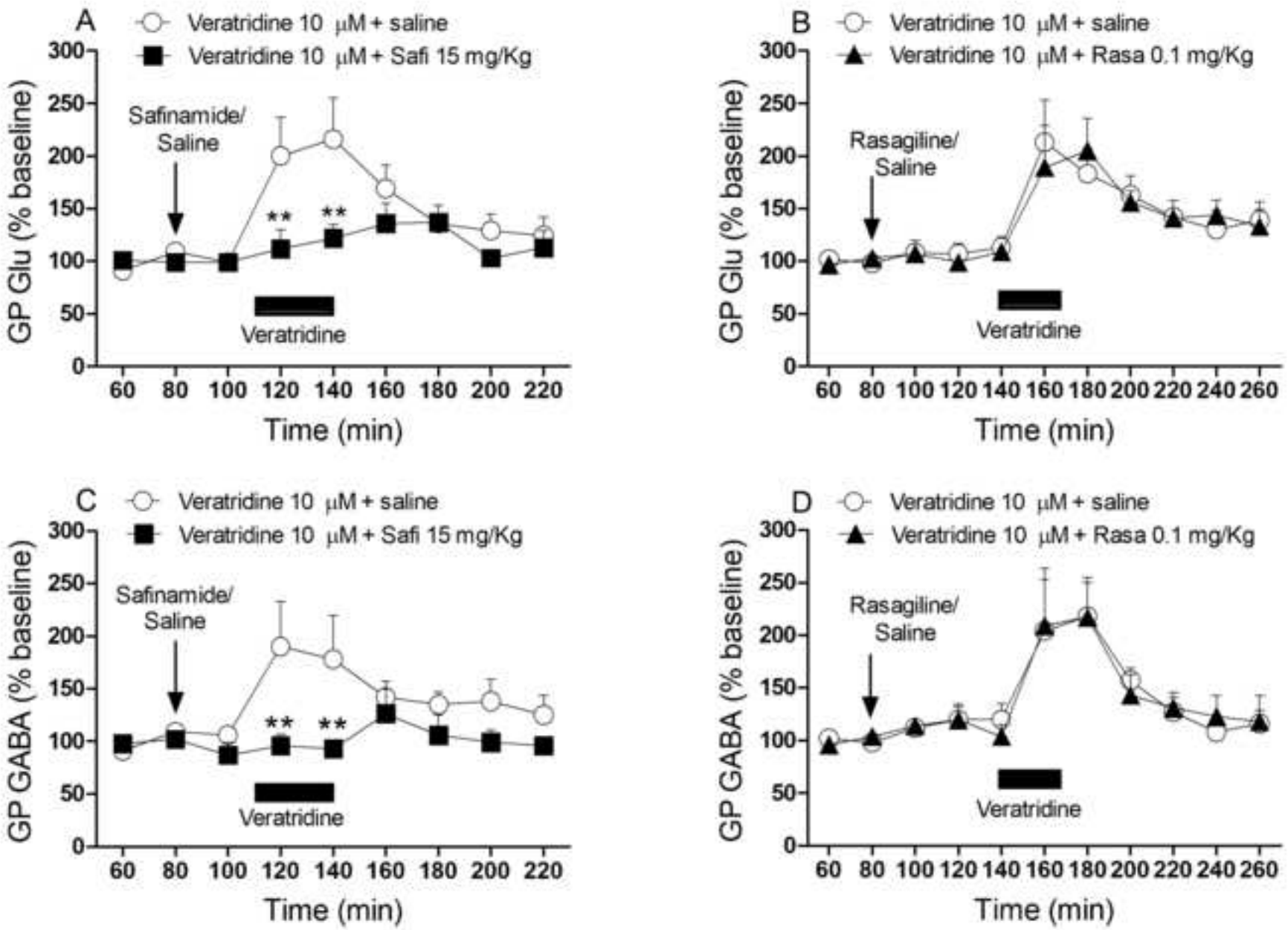

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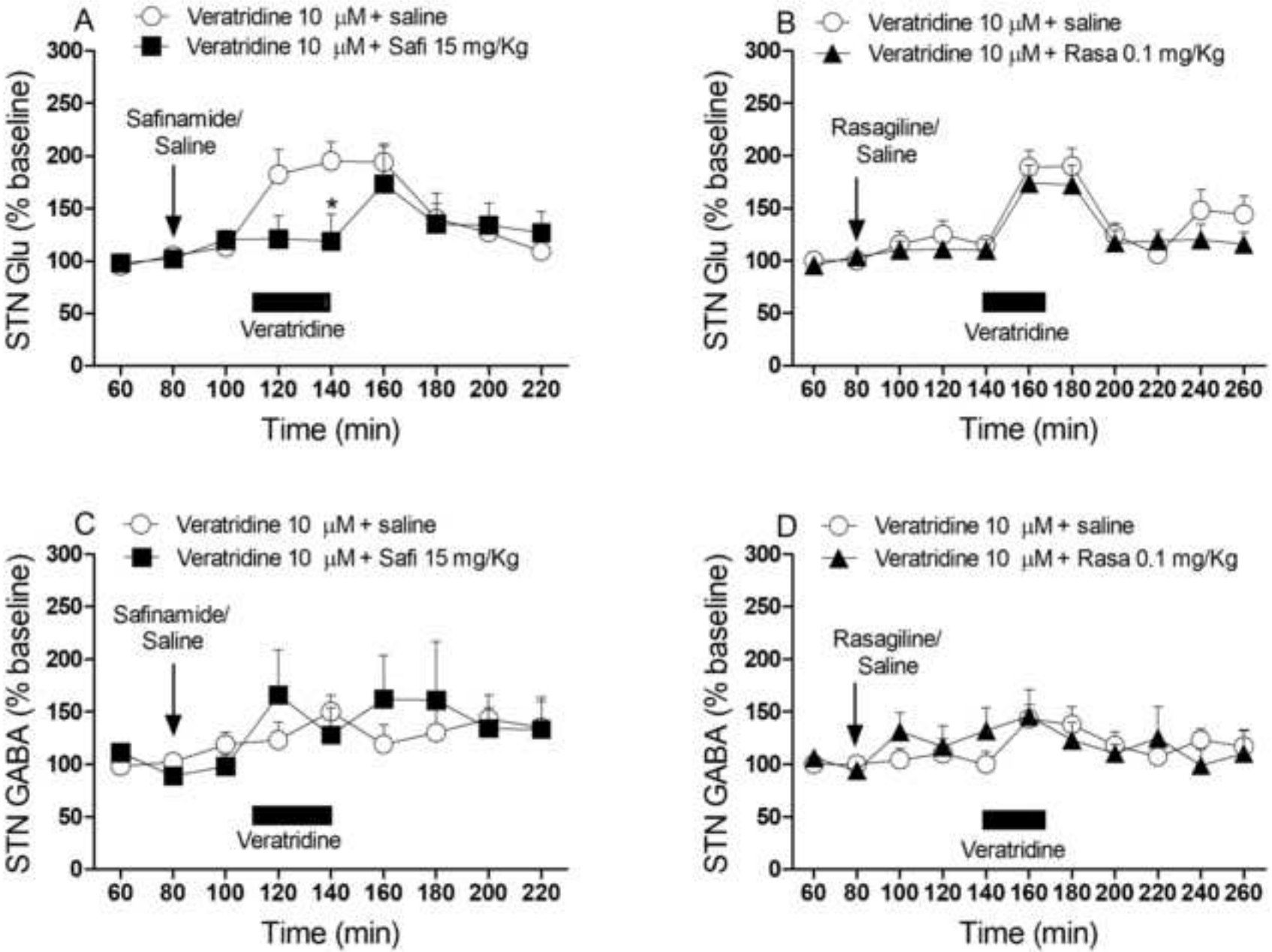

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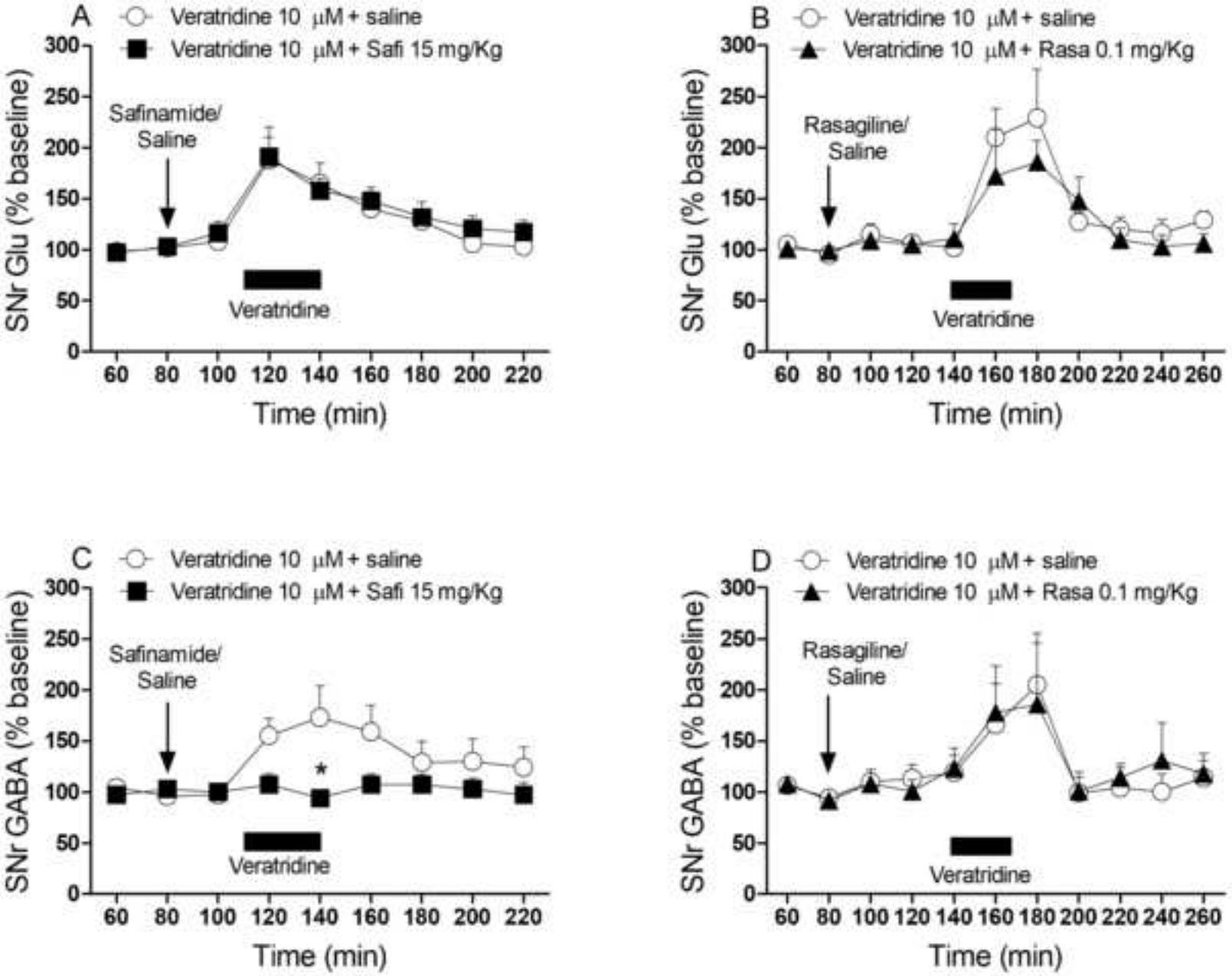

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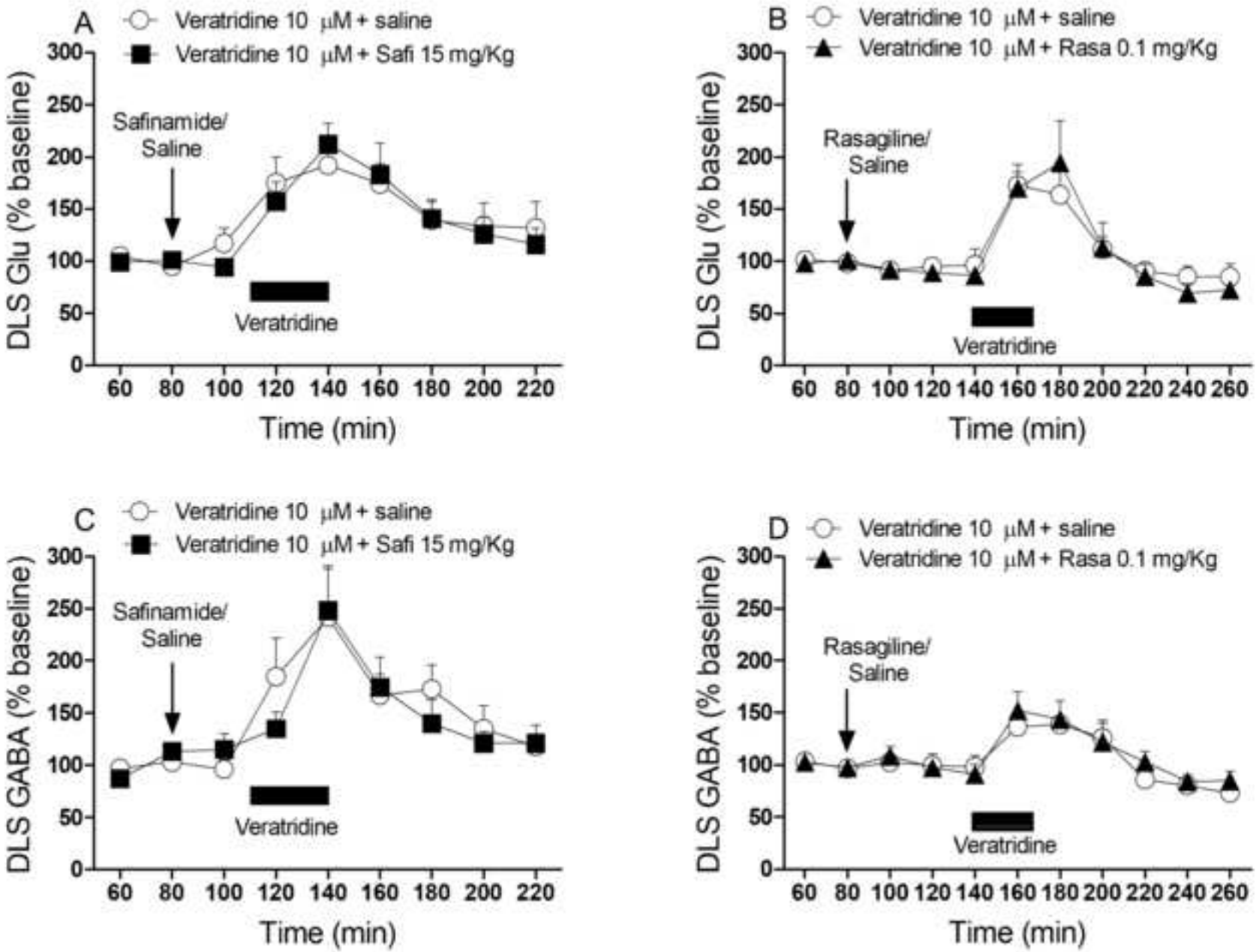

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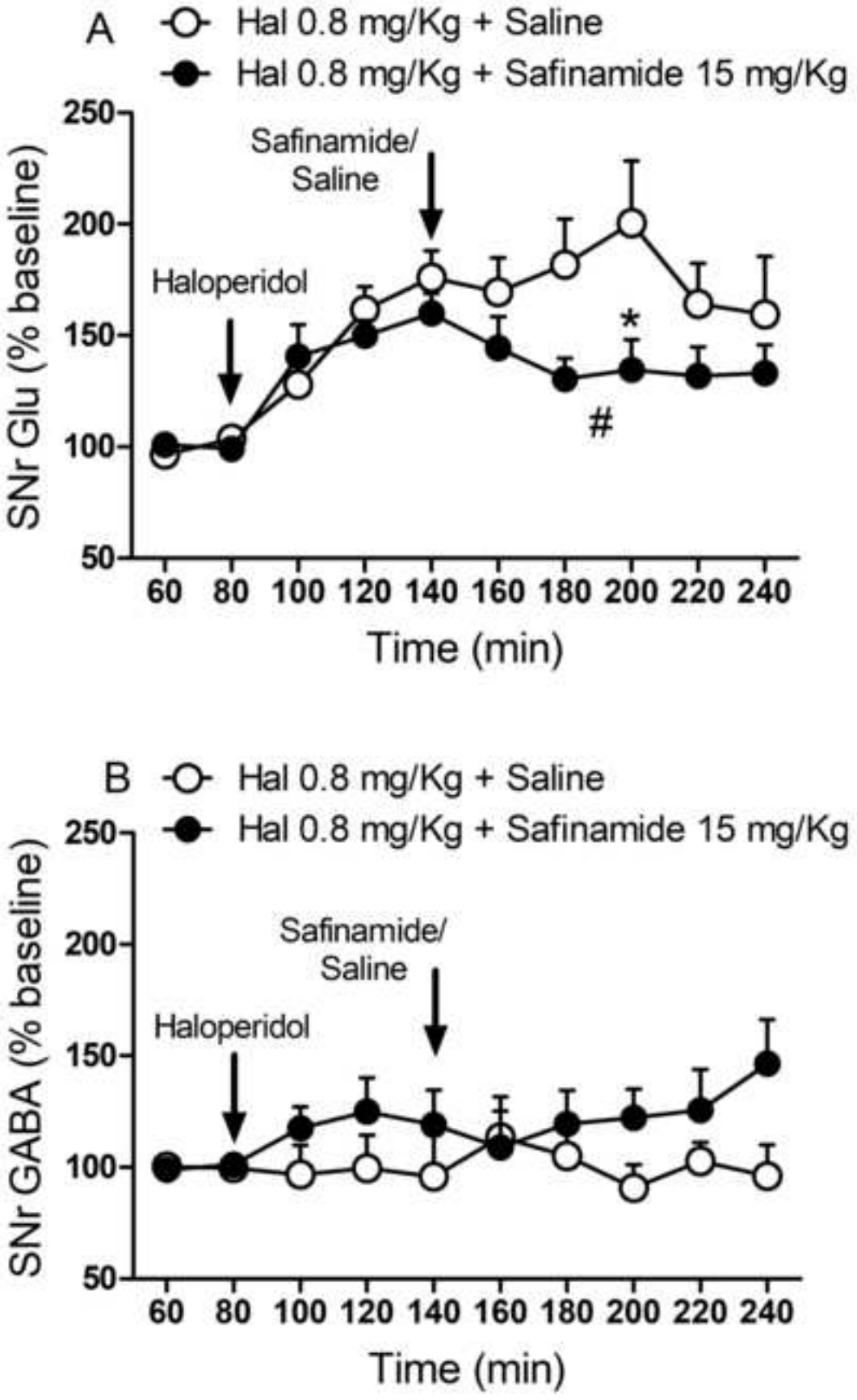

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