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L-DOPA promotes striatal dopamine release through D1 receptors and reversal of dopamine transporter

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Abstract:	Highlights D1 and D2 receptors oppositely modulate synaptosomal dopamine release. Low L-DOPA stimulates exocytotic dopamine release via D1 receptors. Low L-DOPA stimulates D1 receptors independent of its conversion to dopamine. High L-DOPA stimulates dopamine release through the reversal of dopamine transporter.

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Cover Letter

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Prof Barry Waterhouse
Brain Research
Senior Editor

Ferrara, June 8, 2021

Dear Prof Waterhouse,

We thank the Editorial Board for the positive evaluation of our manuscript BRAINRES-D-21-00086 entitled "**L-DOPA promotes striatal dopamine release through D1 receptors and reversal of dopamine transporter**". In this revised version we have tried to address all the concerns raised by the Reviewers. A point-to-point reply has been detailed on a separate sheet. Changes have been highlighted in text.
We therefore hope that our manuscript will now be considered suitable for publication in **Brain Research** and thank you for the attention given to our work.

Sincerely

Michele Morari

In reply to Reviewer #1

Point 1: *I have no specific comments except one. I'm not convinced that D1 receptors can play a powerful role as autoreceptors. I would appreciate if the authors could balance a little bit more their conclusion or bring up solid anatomical arguments that D1 receptors are expressed by dopamine neurons and exported at terminals (Wu's paper 2006 is not convincing enough and they invited us to look at noradrenergic terminals). How do the authors ensure that they are solely measuring the activity of dopaminergic terminals when they are stimulating with K+? I don't know how they can guarantee that 3H-dopamine was only uploaded in dopamine synaptosomes. Thus, I think that they can remove (auto) from the title and conclusion and discuss the possibility that the reported effect could correspond to D1 autoreceptors.*

Reply. We have mitigated our statement that L-Dopa stimulated [3H]DA release through presynaptic D1 autoreceptors, and discussed this possibility as one of the possible mechanisms involved. We have therefore removed the term "auto" from the title, highlights, and the conclusions of the abstract. Regarding Wu's 2006 paper, the Authors often refer to TH positive terminals as "predominantly dopaminergic", probably because the noradrenergic innervation to the striatum is much weaker compared to the dopaminergic one and therefore the nerve terminals sampled in the study are more likely dopaminergic than noradrenergic. Nonetheless, in Discussion we have better described the Wu's study and indicated that TH staining marks both dopaminergic and noradrenergic nerve terminals.

In reply to Reviewer #2

Major points:

Point 1: *The results in Fig 1 show a ki value of SCH23390 and SKF38393 of 0.42 nM and 29.1 nM, respectively. But neither in the Results section nor in the Discussion section do the authors comment on their values in relation to values reported in the bibliography.*

Reply. In the Discussion section (p 6), we have commented our binding values in relation with previous studies. This sentence was added: "In our preparation, Ki values of SCH233390 (0.42 nM) and SKF 38393 (29.1 nM) were in line with those previously reported in the rodent brain (SCH23390, 0.12-0.80 nM; SKF 38393, 18-41 nM) (Andersen, 1988; Neumeyer et al., 2003; Qandil et al., 2003; Watts et al., 1993)".

Point 2: *Usually, SKF38393 shows a biphasic competition curve of the [3H]SCH23390 radioligand, which is characteristic of the binding of this agonist to two D1R populations or of negative cooperativity on the D1Rs.*

Reply. The data obtained in our preparation agree with those in the abovementioned studies showing a monophasic curve for SKF38393, with a single Ki value.

Points 3-7: *The authors do not report the Ki or IC50 values of L-DOPA deduced from the dissociation curves in Fig 3C. Compare these values with those reported in the bibliography, if any. Why doesn't L-DOPA displace 100% radioligand like the agonist SKF38393 does? It does not appear to be a truncated dissociation curve due to the lack of higher concentrations of L-DOPA in the experiment, since the curve seems to*

stabilize after 40% displacement of the radioligand. If L-DOPA does not displace 100% of the radioligand, it would mean that they are not competing for the same binding site. The most common interpretation of this curve is that L-DOPA binds to an allosteric site of D1R and not to the orthosteric site like dopamine, SKF38393 or SCH23390 does. Therefore, it could be a negative allosteric effect of L-DOPA on the binding of the radioligand or a biphasic orthosteric displacement curve where the second fall does not appear in the graph because it occurs at very high concentrations of L-DOPA. But, in the latter case, why does the SKF38393 agonist not show the typical biphasic competition curve? Radioligand binding kinetic experiments have been reported to distinguish when the competition curve is due to allosteric or orthosteric binding, such as those of May et al., 2007 (Annu. Rev. Pharmacol. Toxicol. 47, 1-51).

Reply. The finding that L-DOPA does not completely displace SCH23390 binding indicates a mechanism of displacement different from classical competitive orthosteric binding. In fact, Toll and coworkers (Toll et al., 1998) showed that L-DOPA did not displace SCH23390 binding up to 10 uM in D1 receptor-transfected cells, ruling out a significant interaction with the orthosteric site. The L-DOPA profile is reminiscent of allosteric negative modulation, as suggested by the Reviewer (May et al., 2007) although the possibility that L-DOPA behaves as a negative allosteric modulator does not easily reconcile with its facilitatory effect on [^{3}H]DA release. It is also possible that the SCH23390-sensitive D1 receptor identified in our preparation does not belong to the classical D1 receptor subtype (i.e. the $D1_A$), since more than one D1 receptor subtype has been cloned (Tiberi et al., 1991) and D1 receptors can heteromerize with DA and non-DA receptors (Casado-Anguera et al., 2019; Fuxe et al., 2015) or couple with different G-proteins (Wang et al., 1995) displaying unique pharmacological profiles (Undie et al., 1994). However, the most parsimonious explanation for the early stabilization of the curve relative to L-DOPA is probably represented by the occurrence of a saturation effect due to the competition of endogenous DA formed by the L-DOPA conversion and DAT reversal. In fact, the maximal displacement of L-Dopa is rescued by GBR12783 and benserazide. However, it is also possible, as speculated by the Reviewer, that higher concentrations might reveal a biphasic profile, although these concentrations would be biologically meaningless.
It is out of the scope of the study to analyse in depth the binding kinetics of [^{3}H]SCH23390 in the presence of L-Dopa, also considering the very high (and biologically meaningless) concentrations at which L-Dopa modulates SCH23390 binding, and the possible indirect and confounding effects related to endogenous DA release.

Point 8: *The Results section only contains 3 figures, and the Discussion section is too long for the Results reported in the manuscript. Discussion is also too speculative. Shorten the Discussion section and long speculative comments.*
Reply. As suggested by the Reviewer, we have shortened the Discussion (to less than three pages) in the hope of making it more concise and stringent.

Point 9: *Distinguish more clearly in the Discussion section when bibliographic experiments are discussed and when manuscript experiments are discussed.*
Reply. In the Discussion, we have tried to highlight when a comment is related to findings obtained in the present study or a literature citation.

Minor points:
Point 1: *Fist and third line of Results: check flux units: pmol mg protein/min is incorrect.*
Reply. We apologize for the typos: the correct definition is "pmol/mg tissue/min".

Point 2: *Second line of Results: define FR.*
Reply. We have defined FR in the *Data and statistical analysis* paragraph of Methods. For the sake of clarity, as suggested by the Reviewer, we have added this definition also in Results *"...fractional Release (FR, i.e. the tritium efflux expressed as percentage of the tritium content in the filter at the onset of the corresponding collection period)..."*

Point 3. *Second paragraph of Results: change "being effective only at 10 uM and 100 uM" by "being effective at concentrations higher than 1 uM".*
Reply. We have changed the sentence as suggested by the Reviewer.

Point 4. *Discussion section, second paragraph, third line: "in this monolayer synaptosome preparation…" What is "this"? bibliographic experiments or experiments of the present manuscript.*
Reply. We have removed the sentence to shorten the Discussion.

Point 5: *In the Discussion section the authors comment that D1Rs can heteromerize with other receptors. Update the bibliographic reference with a more recent one, such as that of Casadó-Anguera et al., 2019 (Expert Opin. Drug Discov. 14, 1297-1312).*
Reply: We have incorporated in Discussion the reference indicated by the Reviewer.

Point 6: *Add the reference of Cheng & Prusoff.*
Reply. We have incorporated in Methods the reference of the classical study of Cheng and Prusoff (Cheng, Y., Prusoff, W.H., Biochem. Pharmacol. 22, 3099-108,1973.).

Point 7: *In the Materials section, the authors point out that drugs were dissolved in Krebs solution, but was the addition of DMSO not necessary to dissolve them?*
Reply. In our conditions, drugs were soluble in Krebs solution.

Point 8: *Caption of Fig 2: * and ** are differences from washout, but at what time?*
Reply. We have chosen to statistically analyse the effects of L-DOPA on spontaneous tritium efflux using ANOVA on area-under-the curve data. In fact, the long-lasting response of tritium efflux to prolonged application of L-DOPA were well suited for such analysis. We have specified that we used ANOVA on area-under-the-curve data in Methods.

Point 9-10: *In section 4.4 and in caption of Fig 3 the expression "The data are expressed as mean+-SEM of 4 independent experiments" probably is incorrect. Delete and add "Curves are representative of 4 independent experiments performed in…"*
Reply: We have corrected the sentence and specified that "Binding curves are representative of 4 independent experiments performed in duplicate".

Point 11: *Fourth Highlight: add "High L-DOPA stimulates…"*
Reply. We have modified highlight #4, as suggested. To better differentiate the effect at low and high concentrations of L-DOPA we have modified highlight #2 and #3 by adding "Low" at the beginning of the sentence.

Point 12: *Fifth Highlight: change High by Low, and put as fourth Highlight.*
Reply. We have removed the fifth highlight to tune down our statement that L-DPOA binds to D1 receptors. In fact, the finding that ability of L-DOPA to displace [^{3}H]SCH23390 was halved in the presence GBR12783 and benserazide suggests that endogenous DA formed by L-DOPA and released via DAT is likely responsible for the binding to D1 receptors. The displacement of [^{3}H]SCH23390 binding by L-DOPA still observed in the presence of GBR12783 and benserazide occurs at very high, and biologically meaningless concentrations.

Title Page

L-DOPA promotes striatal dopamine release through D1 receptors and reversal of dopamine transporter

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Declaration of interests

None

Abbreviations

AADC, aromatic L-amino acid decarboxylase; DA, dopamine; DAT, dopamine transporter; L-DOPA, levodopa; PD, Parkinson's disease.

Highlights

- D1 and D2 receptors oppositely modulate synaptosomal dopamine release.
- Low L-DOPA stimulates exocytotic dopamine release via D1 receptors.
- Low L-DOPA stimulates D1 receptors independent of its conversion to dopamine.
- High L-DOPA stimulates dopamine release through the reversal of dopamine transporter.

L-DOPA promotes striatal dopamine release through D1 receptors and reversal of dopamine transporter

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Declaration of interests

None

Abbreviations

AADC, aromatic L-amino acid decarboxylase; DA, dopamine; DAT, dopamine transporter; L-DOPA, levodopa; PD, Parkinson's disease.

Abstract

Previous studies have pointed out that L-DOPA can interact with D1 or D2 receptors independent of its conversion to endogenous dopamine. The present study was set to investigate whether L-DOPA modulates dopamine release from striatal nerve terminals, using a preparation of synaptosomes preloaded with [^{3}H]DA. Levodopa (1 μM) doubled the K^+-induced [^{3}H]DA release whereas the D2/D3 receptor agonist pramipexole (100 nM) inhibited it. The L-DOPA-evoked facilitation was mimicked by the D1 receptor agonist SKF38393 (30-300 nM) and prevented by the D1/D5 antagonist SCH23390 (100 nM) but not the DA transporter inhibitor GBR12783 (300 nM) or the aromatic L-amino acid decarboxylase inhibitor benserazide (1 μM). Higher L-DOPA concentrations (10 and 100 μM) elevated spontaneous [^{3}H]DA efflux. This effect was counteracted by GBR12783 but not SCH23390. Binding of [^{3}H]SCH23390 in synaptosomes (in test tubes) revealed a dense population of D1 receptors (2105 fmol/mg protein). Both SCH23390 and SKF38393 fully inhibited [^{3}H]SCH23390 binding (Ki 0.42 nM and 29 nM, respectively). L-DOPA displaced [^{3}H]SCH23390 binding maximally by 44% at 1 mM. This effect was halved by addition of GBR12935 and benserazide. We conclude that L-DOPA facilitates exocytotic [^{3}H]DA release through SCH23390-sensitive D1 receptors, independent of its conversion to DA. It also promotes non-exocytotic [^{3}H]DA release, possibly via conversion to DA and reversal of DA transporter. These data confirm that L-DOPA can directly interact with dopamine D1 receptors and might extend our knowledge of the neurobiological mechanisms underlying L-DOPA clinical effects.

Keywords

Dopamine release, D1 receptors, dopamine transporter, L-DOPA, SCH-23390, synaptosomes.

1. Introduction

Replacement therapy with levodopa (L-DOPA) remains the most effective treatment for Parkinson's disease (PD). L-DOPA therapeutic action relies on its enzymatic conversion to dopamine (DA) by aromatic L-amino acid decarboxylase (AADC), which occurs in dopaminergic and non-dopaminergic striatal terminals or striatal neurons (Arai et al., 1994; Hefti et al., 1981; Ng et al., 1970). Nonetheless, different lines of evidence suggest that, in addition to serving as DA precursor, endogenous L-DOPA may act as a neurotransmitter or neuromodulator (Misu and Goshima, 1993). Indeed, neurons containing L-DOPA as end-product (i.e. positive for tyrosine hydroxylase while not expressing AADC) have been detected in the rat brain (Kitahama et al., 1988; Mons et al., 1989; Tison et al., 1989) and neurotransmitter-like release of L-DOPA has been found (Goshima et al., 1988; Misu et al., 1990). Consistently, L-DOPA has been found to stimulate D1, D2 or beta-adrenergic receptors without being converted to DA (Aceves et al., 1991; Goshima et al., 1986; Goshima et al., 1991; Nakamura et al., 1994; Silva et al., 2006). Although these effects of L-DOPA have been registered both at pre- and postsynaptic levels, a significant number of studies have demonstrated that L-DOPA modulates DA and noradrenaline (Chang and Webster, 1995; Goshima et al., 1986; Misu et al., 1986), glutamate (Goshima et al., 1993) and GABA (Aceves et al., 1991) release in the brain, independent of its conversion to DA and purportedly through activation of presynaptic receptors. Nonetheless, one main limitation of these studies is that they were conducted in brain slices thus making quite difficult to dissect out presynaptic actions from network (polysynaptic) effects. For this reason, we set to investigate the neurochemical effects of L-DOPA in a preparation of superfused striatal synaptosomes preloaded with [^{3}H]DA (Longo et al., 2017; Marti et al., 2003b; Mercatelli et al., 2019). The superfusion conditions emphasize L-DOPA effects on presynaptic receptors, minimizing indirect effects due to endogenous DA formed through L-DOPA uptake and decarboxylation into nerve terminals. In this preparation, we analyzed both spontaneous (Ca^{2+}-independent) and K^+-stimulated (Ca^{2+}-dependent) [^{3}H]DA release (Marti et al., 2003b), comparing the effect of L-DOPA with those of the D2/D3 receptor agonist pramipexole and D1 receptor agonist SKF38393. Moreover, since neurochemical data suggested that L-DOPA stimulated D1 receptors, a binding study in a synaptosomal preparation was conducted.

2. Results

Basal synaptosomal [^{3}H]DA efflux was 0.021 ± 0.001 pmol/ mg tissue /min (n=68) and corresponded to a fractional release (FR; tritium efflux expressed as percentage of the tritium

content in the filter at the onset of the corresponding collection period) of 6.79 ± 0.15%. A 2 min pulse of 10 mM K^+ evoked a tritium overflow of 0.006 ± 0.001 pmol/ mg tissue/min (n=24), which was attenuated by ~70% in the absence of Ca^{2+} (Fig. 1A). Firstly, the effect of striatal D2 receptor activation was evaluated using the D2/D3 receptor agonist pramipexole ($W_{4,12.10}$=67.02, p<0.0001; Fig. 1A). Pramipexole (100 nM) halved the K^+-induced tritium overflow and this effect was prevented by pre-treatment with the D2/D3 receptor antagonist amisulpride (100 nM), which was per se ineffective. In parallel, the effect of D1 receptor activation was also assessed ($W_{5,13.68}$=8.57, p<0.0001; Fig. 1B). The D1 receptor agonist SKF38393 (30-300 nM) increased [^{3}H]DA overflow, and pre-treatment with SCH23390 (100 nM), ineffective per se, suppressed this modulation. Superfusion of synaptosomes with L-DOPA qualitatively replicated SKF38393 profile ($W_{4,11.53}$=11.30, p=0.0006; Fig. 1C). In fact, L-DOPA (1 μM) doubled tritium overflow, an effect prevented by SCH23390 (100 nM). The DA transporter (DAT) inhibitor GBR12783 was per se ineffective ($W_{5,13.44}$=20.46, p<0.0001; Fig. 1D) and did not attenuate the increase of [^{3}H]DA overflow evoked by L-DOPA (1 μM). This effect was unaltered also in the presence of the AADC inhibitor, benserazide, indicating it was not due to the conversion of L-DOPA into DA by AADC.

In order to more thoroughly evaluate the contribution of L-DOPA to synaptosomal DA release, L-DOPA effect on spontaneous [^{3}H]DA efflux was also monitored. Striatal [^{3}H]DA efflux in our preparation was previously shown to be essentially unaffected by Ca^{2+} removal and tetrodotoxin application (Marti et al., 2003b). L-DOPA increased tritium efflux in a concentration-dependent manner ($W_{3,9.41}$=24.97, p<0.0001; Fig. 2A) being effective at concentrations higher than 1 μM. GBR12783 (300 nM) prevented the response to 10 μM L-DOPA ($W_{5,12.73}$=29.38, p<0.0001; Fig. 2B) and attenuated that to 100 μM L-DOPA ($W_{5,13.06}$=21.11, p<0.0001; Fig. 2C) while SCH23390 (1 μM) was ineffective. GBR12783 and SCH23390 alone did not affect spontaneous tritium efflux at the concentrations tested.

The SCH23390-sensitive effect of L-DOPA (1 μM) on stimulated tritium overflow suggested an interaction of L-DOPA with D1/D5 receptors. To confirm this interaction, binding experiments were performed. Saturation binding experiments of [^{3}H]SCH23390 were performed in mouse striatal synaptosomes to investigate the affinity (K_D) and density (Bmax) of D1/D5 dopamine receptors. The saturation curve of [^{3}H]SCH23390 and the relative Scatchard plot revealed a K_D value of 0.72 ± 0.05 nM and a receptor density of 2105 ± 69 fmol/mg protein (Fig. 3A-B). Competition binding experiments were performed in mouse striatal synaptosomes to evaluate the possible interaction of L-DOPA with D1 receptors in comparison with the D1/D5 antagonist SCH23390 and the D1/D5 agonist SKF38393 as

reference compounds. As expected, SCH23390 showed a good affinity towards D1/D5 receptors with a Ki value of 0.42 ± 0.02 nM (Fig. 3C). The D_1 agonist SKF38393 inhibited [^{3}H]SCH23390 binding with a Ki of 29.1 ± 2.0 nM. Interestingly, L-DOPA was able to partially displace [^{3}H]SCH23390 with a 44% maximal inhibition of specific binding at 1 mM (Fig. 3C). Since binding experiments were performed in test tubes, the possibility was investigated that endogenous DA could contribute to SCH23390 displacement from D1 receptors. Therefore, displacement curve was retested in the presence of GBR12783 (to block DAT) and benserazide (to block AADC). Under these conditions, L-DOPA still displaced [^{3}H]SCH23390 binding, although less effectively and at higher concentrations than in the absence of DAT and AADC inhibitors.

3. Discussion

Previous studies in striatal slices showed that L-DOPA concentration-dependently facilitated spontaneous and stimulus-evoked DA release (Chang and Webster, 1995; Misu et al., 1986). Interestingly, when slices were treated with an AADC inhibitor the L-DOPA profile became biphasic, low concentrations (30 nM) facilitating and higher ones (1 μM) inhibiting impulse-evoked DA release (Misu et al., 1986). In the present study, pramipexole inhibited impulse-evoked synaptosomal DA release via amisulpride-sensitive D2 receptors whereas L-DOPA elevated it via SCH23390-sensitive D1 receptors. Therefore, it could be speculated that beside the well-characterized D2 autoreceptors, dopaminergic terminals are endowed also with facilitatory D1 autoreceptors. Indeed, rare presynaptic D1 receptors have been detected on dopaminergic striatal nerve terminals (Hersch et al., 1995), and D1 receptors have been found to co-localize not only with choline acetyltransferase-positive (i.e. cholinergic) and glutamic acid decarboxylase-positive (i.e. GABAergic) but, less intensely, also with striatal tyrosine hydroxylase-positive (i.e. dopaminergic and noradrenergic) striatal nerve terminals (Wu et al., 2006). Moreover, activation of presynaptic D1 receptors stimulated Ca^{2+} levels in striatal synaptosomes in a SCH23390-sensitive manner (Wu et al., 2006). However, it is well known that striatal D1 receptors are mainly postsynaptic or located presynaptically on non-dopaminergic nerve terminals (heteroreceptors) (Fremeau et al., 1991; Hersch et al., 1995; Wu et al., 2006), thus a significant contribution of postsynaptic D1 receptors would account for the dense D1 receptor binding observed in our synaptosomal preparation. Indeed, in this synaptosomal preparation postsynaptic membranes often remain attached to the "active site" of presynaptic terminals (Gulyassy et al., 2020; Miklosi et al., 2018), suggesting that postsynaptic D1 receptors might be involved in the L-DOPA-induced [^{3}H]DA release.

Interestingly, stimulation of postsynaptic D1 receptors on medium-sized spiny neurons elevated striatal glutamate release via inhibition of retrograde endocannabinoid release (Andre et al., 2010). Whether this is also the case for DA release in our preparation remains to be determined. Therefore, although we cannot dissect out whether pre- or postsynaptic SCH23390-sensitive D1 receptors mediated the effect of L-DOPA, it clearly appears that L-DOPA can directly interact with D1 receptors without its conversion to dopamine. Whether such an effect contributes to the therapeutic and side-effects of L-DOPA is controversial. The effective concentrations of L-Dopa stimulating exocytotic DA release in the present study are similar to those (1 μM) measured in the CSF of PD patients after oral (Olanow et al., 1991) or i.v. (Woodward et al., 1993) L-DOPA administration, and very close to those measured in the striatum of marmosets (442 nM) orally administered with a therapeutic dose of L-DOPA (Zhang et al., 2003). However, previous studies showed that L-DOPA-mediated behaviors are inconsistently affected by AADC blockers. In some studies, L-DOPA-mediated contralateral circling (Melamed et al., 1984; Treseder et al., 2000) or dyskinesia (Buck and Ferger, 2008) were markedly reduced (albeit not suppressed) by blockade of central AADC whereas in others L-DOPA-induced turning was unaffected (Alachkar et al., 2010; Nakamura et al., 1994; Nakazato and Akiyama, 1989; Treseder et al., 2001). Moreover, L-DOPA was reported to induce dyskinesia independently of modulation of striatal DA levels (Navailles et al., 2011; Nevalainen et al., 2011; Porras et al., 2014), possibly via stimulation of somato-dendritic and presynaptic D1 receptors at striato-nigral medium-sized spiny neurons, leading to enhanced firing and/or nigral GABA release (Mela et al., 2012; Robertson and Robertson, 1989; Yamamoto et al., 2006). In fact, L-DOPA stimulation of [^{3}H]GABA release (EC_{50} 1 μM) from nigral slices of DA-denervated rats was observed in the presence of an AADC inhibitor, i.e. independent of its conversion to DA (Aceves et al., 1991).

Binding experiments in our synaptosomal preparation, however, revealed, a negligible binding of L-DOPA to D1 receptors, in agreement with previous study in D1-transfected cells (Toll et al., 1998). In our preparation, Ki values of SCH233390 (0.42 nM) and SKF 38393 (29.1 nM) were in line with those previously reported in the rodent brain (SCH23390, 0.12-0.80 nM; SKF 38393, 18-41 nM) (Andersen, 1988; Neumeyer et al., 2003; Qandil et al., 2003; Watts et al., 1993). L-DOPA displaced [^{3}H]SCH23390 binding but, different from classical D1 receptor ligands, only partially (40%). This profile is reminiscent of allosteric negative modulation (May et al., 2007) although the possibility that L-DOPA behaves as a negative allosteric modulator does not easily reconcile with its facilitatory effect on [^{3}H]DA release. It is also possible that the SCH23390-sensitive D1 receptor identified in our

preparation does not belong to the classical $D1_A$ receptor subtype, since more than one D1 receptor subtype has been cloned (Tiberi et al., 1991) and D1 receptors can heteromerize with DA and non-DA receptors (Casado-Anguera et al., 2019; Fuxe et al., 2015) or couple with different G-proteins (Wang et al., 1995) generating receptor entities with unique pharmacological profiles (Undie et al., 1994). Nonetheless, we must consider that release and binding experiments were conducted under substantially different experimental conditions. Specifically, L-DOPA binding to D1 receptors was assessed in test tubes, where binding might be modulated by other molecules released and accumulated in the extracellular milieu. In fact, the [^{3}H]SCH23390 binding displacement induced by L-DOPA was halved in the presence of DAT and AADC inhibitors, suggesting that occupation of D1 receptors is partly mediated by endogenous DA formed from L-DOPA and released from DA terminals through the reversal of DAT. Indeed, DAT reversal of spontaneous [^{3}H]DA efflux by L-DOPA was observed in the present synaptosomal preparation. Different from stimulus-evoked tritium overflow, spontaneous [^{3}H]DA efflux is Ca^{2+}-insensitive and only slightly (~15%) tetrodotoxin-sensitive (Marti et al., 2003b), suggesting it mainly reflects non vesicular release and leakage from synaptosomes. Misu and coworkers (Misu et al., 1986) showed that, different from the stimulus-evoked DA release, the increase in spontaneous efflux induced by L-DOPA was prevented by an AADC inhibitor, i.e. relied on L-DOPA conversion to DA. Consistently, we found that GBR12783 was ineffective on stimulus-evoked [^{3}H]DA overflow but prevented the elevation of [^{3}H]DA efflux induced by high L-DOPA concentrations. These data support the view that L-DOPA is taken up into nerve terminals, decarboxylated to DA by AADC, and released via DAT reversal, confirming the in vivo evidence that L-DOPA can promote DA release through non vesicular mechanisms (De Deurwaerdere et al., 2017). Specifically, in vivo microdialysis showed that reverse dialysis of 5 μM L-DOPA into SN increased local DA levels via non vesicular mechanisms (Thorre et al., 1998), and systemic administration of L-DOPA (3-12 mg/Kg) elevated in vivo hippocampal and prefronto-cortical DA also after removal of Ca^{2+} from the perfusion medium, i.e. under conditions of impaired vesicular release (Miguelez et al., 2016). Altogether, these findings indicate that DAT reversal might occur in vivo at therapeutic L-DOPA concentrations, causing DA to stimulate D1 receptors.

In conclusion, this study presents the first evidence that low L-DOPA concentrations facilitate synaptosomal exocytotic DA release through direct stimulation of D1 receptors. Higher concentrations L-DOPA stimulate non vesicular DA release via L-DOPA conversion to DA and DAT reversal. These data add to previous evidence that L-DOPA can act as a

neurotransmitter, and may offer new insights into the neurobiological mechanisms underlying L-DOPA therapeutic and side-effects, in particular L-DOPA-induced dyskinesia (Bastide et al., 2015; De Deurwaerdere et al., 2017).

4. Experimental procedure

4.1. Animal subjects

Young adult (8-10-week-old) male C57BL/6J mice (20-25 g) obtained from a colony set at the LARP facility of the University of Ferrara were used. Mice were housed with free access to food and water and kept under environmentally controlled conditions (12-h light/dark cycle with light on between 07:00 and 19:00). The experiments complied with the ARRIVE guidelines, were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and were approved by the Italian Ministry of Health and Ethical Committee of the University of Ferrara (license #ECE79.2.EXT.3). All efforts were made to minimize the number of animals used and their suffering.

4.2. Synaptosome preparation and [^{3}H]DA analysis

Mice were anesthetized and sacrificed via cervical dislocation, and striatum was quickly excised to prepare synaptosomes, as previously described (Marti et al., 2001; Marti et al., 2003b; Morari et al., 1998). Striatum was homogenized in ice-cold 0.32 M sucrose (pH 7.4) with a Teflon-glass homogenizer and centrifuged at 800 x g for 10 min at 4°C. The supernatant was then centrifuged at 11,000 x g for 20 min at 4°C, the pellet resuspended in 1.5 mL oxygenated (95% O_2, 5% CO_2) Krebs solution (mM: NaCl 118.5, KCl 4.7, $CaCl_2$ 1.2, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 10) containing ascorbic acid (0.05 mM) and disodium EDTA (0.03 mM). Synaptosomes were incubated at 37°C with 50 nM [^{3}H]-DA (specific activity 40 Ci/mmol; Perkin-Elmer, Boston, MA, USA) for 25 min, after which 12 mL of pre-oxygenated Krebs were added (Longo et al., 2017; Marti et al., 2003a). One millilitre aliquots of the suspension (~0.35 mg protein) were slowly injected into nylon syringe filters (outer diameter 13 mm, 0.45 µM pore size, internal volume ~100 µL; Teknokroma, Barcelona, Spain), maintained at 36.5 °C in a thermostatic bath and superfused (0.4 mL/min) with a pre-carbogenated Krebs solution. Under these experimental conditions, spontaneous [^{3}H]DA efflux was essentially unaffected by reuptake. Filters were washed for 20 min, after which sample collection was started (every 3 min). The effect of drugs was evaluated on both spontaneous and K^+-stimulated neurotransmitter outflow. In this case, drugs were added to the perfusion medium 6 (agonist) or 9 (antagonist) min before a 10 mM K^+ pulse (120 sec) and maintained until the end of the experiment. [^{3}H]DA levels in the samples

were measured by liquid scintillation spectrophotometry. Sample superfusate (1.2 ml/sample) and filter retained (dissolved with 1 ml of 1 M NaOH followed by 1 M HCl) were opportunely mixed with Ultima Gold XR scintillation fluid (Packard Instruments B.V., Groningen, The Netherlands) and radioactivity in the samples and in the filters was measured using a Perkin Elmer Tri Carb 2810 TR scintillation counter.

4.3. Saturation and competition binding experiments

Saturation binding experiments to D_1 dopamine receptors were carried out by using [^{3}H]SCH23390 as radioligand (specific activity 84.3 Ci/mmol) (Trampus et al., 1991). Mouse striatal synaptosomes were incubated for 60 minutes at 30°C with different concentrations (0.1 nM-10 nM) of [^{3}H]-SCH23390 in 50 mM Tris-HCl pH 7.4, 5 mM $MgCl_2$. Non-specific binding was determined in the presence of 1 μM SCH23390 (Tocris, Bristol, UK) and was always <10% of the total binding. Competition binding experiments were performed incubating mouse striatal synaptosomes with 1 nM of [^{3}H]SCH23390 in 50 mM Tris-HCl pH 7.4, 5 mM $MgCl_2$ for 60 minutes at 30°C in the presence of increasing concentrations of SCH23390 (0.01 nM-1 μM), SKF38393 (0.1 nM-10 μM) or L-DOPA (1 nM-1 mM). Non-specific binding was determined in the presence of 1 μM SCH 23390 and was always <10% of the total binding. At the end of the incubation time, bound and free radioactivity was separated by filtering the assay mixture through Whatman GF/B glass fiber filters using a Brandel cell harvester (Brandel Instruments, Unterföhring, Germany). The filter bound radioactivity was counted using a Perkin Elmer Tri Carb 2810 TR scintillation counter. The protein concentration was determined according to a Bio-Rad method with bovine albumin as standard reference. Inhibitory binding constant values, Ki were calculated from the IC_{50} values according to the Cheng & Prusoff equation $Ki = IC_{50}/(1+[C^*]/K_D^*)$, where [C*] is the concentration of the radioligand and K_D* its dissociation constant (Cheng and Prusoff, 1973).

4.4. Data and statistical analysis

Data, means ± SEM of 6 determinations per group, were expressed as absolute content (pmol/mg tissue/min), percent of basal tritium efflux (Fig. 2) or K^+-evoked tritium overflow (Fig. 1). Tritium efflux was calculated as fractional release (FR, i.e. tritium efflux expressed as percentage of the tritium content in the filter at the onset of the corresponding collection period) whereas K^+-evoked tritium overflow was calculated as net FR, i.e. tritium overflow as percent of the tritium content in the filter at the onset of the corresponding collection period. All values displayed a normal distribution (Kolmogorov-Smirnov test) but violate the assumption of homogeneity of variance (Bartlett's test). Therefore, statistical analysis was performed (Prism software; San Diego, CA, USA) by Welch's ANOVA on percent (Fig. 1) or

area-under-the curve (AUC; Fig. 2) values followed by the Dunnett's T3 test for multiple comparisons (Dunnett, 1980). Binding curves are representative of 4 independent experiments performed in duplicate. P values <0.05 were considered statistically significant.

4.5. Materials

Amisulpride, benserazide, GBR12783 dihydrochloride, L-DOPA, SCH23390 hydrochloride and SKF38393 were purchased from Tocris Bioscience (Bristol, UK). Pramipexole hydrochloride was purchased from McTony Bio&Chem (Vancouver, Canada), [^{3}H]DA and [^{3}H]SCH23390 from Perkin Elmer (Boston, MA, USA). All drugs were freshly dissolved in Krebs solution just prior to use.

Figure captions

Figure 1. Pramipexole, SKF38393 and L-DOPA differentially modulated K^+-evoked DA release from synaptosomes. The D2/D3 receptor agonist pramipexole (100 nM) inhibited (**A**) while the D1 receptor agonist SKF38393 (30-300 nM, **B**) and L-DOPA (0.1-1 μM, **C**) elevated the $[^3H]$DA overflow evoked by a 2 min pulse of 10 mM KCl from a preparation of striatal synaptosomes in superfusion. The inhibition induced by pramipexole was prevented by the D2/D3 receptor antagonist amisulpride (**A**) while the stimulation induced by SKF38393 and L-DOPA was prevented by the D1/D5 receptor antagonist SCH23390 (**B-C**). GBR12783, alone or combined with benserazide, did not block the L-DOPA effect (**D**). Pramipexole, SKF38393 and L-DOPA were administered 6 min before KCl whereas antagonists 3 min before agonists. Dots represent 6 determinations per group and were expressed as percentage of control (i.e. the K^+-evoked tritium overflow). $^*p<0.05$, $^{**}p<0.01$ different from control; $^{\#}p<0.05$, $^{\#\#}p<0.01$ different from pramipexole, SKF38393 or L-DOPA 1 μM alone (Welch's ANOVA followed by the Dunnett's T3 test).

Figure 2. L-DOPA increased spontaneous tritium efflux from synaptosomes. L-DOPA (1-100 μM) elevated spontaneous tritium efflux from a preparation of striatal synaptosomes in superfusion pre-loaded with $[^3H]$-DA (**A**). The DAT blocker GBR12783 but not the D1/D5 receptor selective antagonist SCH23390 prevented the elevation induced by L-DOPA (10 μM; **B**) and attenuated that induced by L-DOPA (100 μM; **C**). GBR12783 and SCH23390 were given 3 min before L-DOPA and maintained until the end of experiments. Data are means ± SEM of 6 determinations per group and were expressed as percentage of basal tritium efflux (calculated as the mean between the two samples before L-DOPA). $^*p<0.05$, $^{**}p<0.01$ different from washout; $^{\#}p<0.05$ different from L-DOPA alone (Welch's ANOVA followed by the Dunnett's T3 test performed on AUC values).

Figure 3. L-DOPA displaced $[^3H]$-SCH23390 binding in synaptosomes. Saturation curve (**A**) and Scatchard plot (**B**) of $[^3H]$-SCH23390 in mouse striatal synaptosomes. **C**. Inhibition curves of $[^3H]$-SCH23390 binding in mouse striatal synaptosomes by the D1/D5 receptor antagonist SCH23390 (0.01 nM-1 μM), the D1/D5 receptor agonist SKF38393 (0.1 nM-10 μM) or the dopamine precursor L-DOPA (1 nM-1 mM), both in the absence and in the presence of the DOPA decarboxylase inhibitor benserazide (1 μM) and the DAT inhibitor GBR12935. Curves are representative of 4 independent experiments performed in duplicate.

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L-DOPA promotes striatal dopamine release through D1 receptors and reversal of dopamine transporter

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Declaration of interests

None

Abbreviations

AADC, aromatic L-amino acid decarboxylase; DA, dopamine; DAT, dopamine transporter; L-DOPA, levodopa; PD, Parkinson's disease.

Abstract

Previous studies have pointed out that L-DOPA can interact with D1 or D2 receptors independent of its conversion to endogenous dopamine. The present study was set to investigate whether L-DOPA modulates dopamine release from striatal nerve terminals, using a preparation of synaptosomes preloaded with [^{3}H]DA. Levodopa (1 μM) doubled the K^+-induced [^{3}H]DA release whereas the D2/D3 receptor agonist pramipexole (100 nM) inhibited it. The L-DOPA-evoked facilitation was mimicked by the D1 receptor agonist SKF38393 (30-300 nM) and prevented by the D1/D5 antagonist SCH23390 (100 nM) but not the DA transporter inhibitor GBR12783 (300 nM) or the aromatic L-amino acid decarboxylase inhibitor benserazide (1 μM). Higher L-DOPA concentrations (10 and 100 μM) elevated spontaneous [^{3}H]DA efflux. This effect was counteracted by GBR12783 but not SCH23390. Binding of [^{3}H]SCH23390 in synaptosomes (in test tubes) revealed a dense population of D1 receptors (2105 fmol/mg protein). Both SCH23390 and SKF38393 fully inhibited [^{3}H]SCH23390 binding (Ki 0.42 nM and 29 nM, respectively). L-DOPA displaced [^{3}H]SCH23390 binding maximally by 44% at 1 mM. This effect was halved by addition of GBR12935 and benserazide. We conclude that L-DOPA facilitates exocytotic [^{3}H]DA release through SCH23390-sensitive D1 receptors, independent of its conversion to DA. It also promotes non-exocytotic [^{3}H]DA release, possibly via conversion to DA and reversal of DA transporter. These data confirm that L-DOPA can directly interact with dopamine D1 receptors and might extend our knowledge of the neurobiological mechanisms underlying L-DOPA clinical effects.

Keywords

Dopamine release, D1 receptors, dopamine transporter, L-DOPA, SCH-23390, synaptosomes.

1. Introduction

Replacement therapy with levodopa (L-DOPA) remains the most effective treatment for Parkinson's disease (PD). L-DOPA therapeutic action relies on its enzymatic conversion to dopamine (DA) by aromatic L-amino acid decarboxylase (AADC), which occurs in dopaminergic and non-dopaminergic striatal terminals or striatal neurons (Arai et al., 1994; Hefti et al., 1981; Ng et al., 1970). Nonetheless, different lines of evidence suggest that, in addition to serving as DA precursor, endogenous L-DOPA may act as a neurotransmitter or neuromodulator (Misu and Goshima, 1993). Indeed, neurons containing L-DOPA as end-product (i.e. positive for tyrosine hydroxylase while not expressing AADC) have been detected in the rat brain (Kitahama et al., 1988; Mons et al., 1989; Tison et al., 1989) and neurotransmitter-like release of L-DOPA has been found (Goshima et al., 1988; Misu et al., 1990). Consistently, L-DOPA has been found to stimulate D1, D2 or beta-adrenergic receptors without being converted to DA (Aceves et al., 1991; Goshima et al., 1986; Goshima et al., 1991; Nakamura et al., 1994; Silva et al., 2006). Although these effects of L-DOPA have been registered both at pre- and postsynaptic levels, a significant number of studies have demonstrated that L-DOPA modulates DA and noradrenaline (Chang and Webster, 1995; Goshima et al., 1986; Misu et al., 1986), glutamate (Goshima et al., 1993) and GABA (Aceves et al., 1991) release in the brain, independent of its conversion to DA and purportedly through activation of presynaptic receptors. Nonetheless, one main limitation of these studies is that they were conducted in brain slices thus making quite difficult to dissect out presynaptic actions from network (polysynaptic) effects. For this reason, we set to investigate the neurochemical effects of L-DOPA in a preparation of superfused striatal synaptosomes preloaded with [^{3}H]DA (Longo et al., 2017; Marti et al., 2003b; Mercatelli et al., 2019). The superfusion conditions emphasize L-DOPA effects on presynaptic receptors, minimizing indirect effects due to endogenous DA formed through L-DOPA uptake and decarboxylation into nerve terminals. In this preparation, we analyzed both spontaneous (Ca^{2+}-independent) and K^+-stimulated (Ca^{2+}-dependent) [^{3}H]DA release (Marti et al., 2003b), comparing the effect of L-DOPA with those of the D2/D3 receptor agonist pramipexole and D1 receptor agonist SKF38393. Moreover, since neurochemical data suggested that L-DOPA stimulated D1 receptors, a binding study in a synaptosomal preparation was conducted.

2. Results

Basal synaptosomal [^{3}H]DA efflux was 0.021 ± 0.001 pmol/ mg tissue /min (n=68) and corresponded to a fractional release (FR; tritium efflux expressed as percentage of the tritium

content in the filter at the onset of the corresponding collection period) of 6.79 ± 0.15%. A 2 min pulse of 10 mM K^+ evoked a tritium overflow of 0.006 ± 0.001 pmol/ mg tissue/min (n=24), which was attenuated by ~70% in the absence of Ca^{2+} (Fig. 1A). Firstly, the effect of striatal D2 receptor activation was evaluated using the D2/D3 receptor agonist pramipexole ($W_{4,12.10}$=67.02, p<0.0001; Fig. 1A). Pramipexole (100 nM) halved the K^+-induced tritium overflow and this effect was prevented by pre-treatment with the D2/D3 receptor antagonist amisulpride (100 nM), which was per se ineffective. In parallel, the effect of D1 receptor activation was also assessed ($W_{5,13.68}$=8.57, p<0.0001; Fig. 1B). The D1 receptor agonist SKF38393 (30-300 nM) increased [^{3}H]DA overflow, and pre-treatment with SCH23390 (100 nM), ineffective per se, suppressed this modulation. Superfusion of synaptosomes with L-DOPA qualitatively replicated SKF38393 profile ($W_{4,11.53}$=11.30, p=0.0006; Fig. 1C). In fact, L-DOPA (1 μM) doubled tritium overflow, an effect prevented by SCH23390 (100 nM). The DA transporter (DAT) inhibitor GBR12783 was per se ineffective ($W_{5,13.44}$=20.46, p<0.0001; Fig. 1D) and did not attenuate the increase of [^{3}H]DA overflow evoked by L-DOPA (1 μM). This effect was unaltered also in the presence of the AADC inhibitor, benserazide, indicating it was not due to the conversion of L-DOPA into DA by AADC.

In order to more thoroughly evaluate the contribution of L-DOPA to synaptosomal DA release, L-DOPA effect on spontaneous [^{3}H]DA efflux was also monitored. Striatal [^{3}H]DA efflux in our preparation was previously shown to be essentially unaffected by Ca^{2+} removal and tetrodotoxin application (Marti et al., 2003b). L-DOPA increased tritium efflux in a concentration-dependent manner ($W_{3,9.41}$=24.97, p<0.0001; Fig. 2A) being effective at concentrations higher than 1 μM. GBR12783 (300 nM) prevented the response to 10 μM L-DOPA ($W_{5,12.73}$=29.38, p<0.0001; Fig. 2B) and attenuated that to 100 μM L-DOPA ($W_{5,13.06}$=21.11, p<0.0001; Fig. 2C) while SCH23390 (1 μM) was ineffective. GBR12783 and SCH23390 alone did not affect spontaneous tritium efflux at the concentrations tested.

The SCH23390-sensitive effect of L-DOPA (1 μM) on stimulated tritium overflow suggested an interaction of L-DOPA with D1/D5 receptors. To confirm this interaction, binding experiments were performed. Saturation binding experiments of [^{3}H]SCH23390 were performed in mouse striatal synaptosomes to investigate the affinity (K_D) and density (Bmax) of D1/D5 dopamine receptors. The saturation curve of [^{3}H]SCH23390 and the relative Scatchard plot revealed a K_D value of 0.72 ± 0.05 nM and a receptor density of 2105 ± 69 fmol/mg protein (Fig. 3A-B). Competition binding experiments were performed in mouse striatal synaptosomes to evaluate the possible interaction of L-DOPA with D1 receptors in comparison with the D1/D5 antagonist SCH23390 and the D1/D5 agonist SKF38393 as

reference compounds. As expected, SCH23390 showed a good affinity towards D1/D5 receptors with a Ki value of 0.42 ± 0.02 nM (Fig. 3C). The D_1 agonist SKF38393 inhibited [^{3}H]SCH23390 binding with a Ki of 29.1 ± 2.0 nM. Interestingly, L-DOPA was able to partially displace [^{3}H]SCH23390 with a 44% maximal inhibition of specific binding at 1 mM (Fig. 3C). Since binding experiments were performed in test tubes, the possibility was investigated that endogenous DA could contribute to SCH23390 displacement from D1 receptors. Therefore, displacement curve was retested in the presence of GBR12783 (to block DAT) and benserazide (to block AADC). Under these conditions, L-DOPA still displaced [^{3}H]SCH23390 binding, although less effectively and at higher concentrations than in the absence of DAT and AADC inhibitors.

3. Discussion

Previous studies in striatal slices showed that L-DOPA concentration-dependently facilitated spontaneous and stimulus-evoked DA release (Chang and Webster, 1995; Misu et al., 1986). Interestingly, when slices were treated with an AADC inhibitor the L-DOPA profile became biphasic, low concentrations (30 nM) facilitating and higher ones (1 μM) inhibiting impulse-evoked DA release (Misu et al., 1986). In the present study, pramipexole inhibited impulse-evoked synaptosomal DA release via amisulpride-sensitive D2 receptors whereas L-DOPA elevated it via SCH23390-sensitive D1 receptors. Therefore, it could be speculated that beside the well-characterized D2 autoreceptors, dopaminergic terminals are endowed also with facilitatory D1 autoreceptors. Indeed, rare presynaptic D1 receptors have been detected on dopaminergic striatal nerve terminals (Hersch et al., 1995), and D1 receptors have been found to co-localize not only with choline acetyltransferase-positive (i.e. cholinergic) and glutamic acid decarboxylase-positive (i.e. GABAergic) but, less intensely, also with striatal tyrosine hydroxylase-positive (i.e. dopaminergic and noradrenergic) striatal nerve terminals (Wu et al., 2006). Moreover, activation of presynaptic D1 receptors stimulated Ca^{2+} levels in striatal synaptosomes in a SCH23390-sensitive manner (Wu et al., 2006). However, it is well known that striatal D1 receptors are mainly postsynaptic or located presynaptically on non-dopaminergic nerve terminals (heteroreceptors) (Fremeau et al., 1991; Hersch et al., 1995; Wu et al., 2006), thus a significant contribution of postsynaptic D1 receptors would account for the dense D1 receptor binding observed in our synaptosomal preparation. Indeed, in this synaptosomal preparation postsynaptic membranes often remain attached to the "active site" of presynaptic terminals (Gulyassy et al., 2020; Miklosi et al., 2018), suggesting that postsynaptic D1 receptors might be involved in the L-DOPA-induced [^{3}H]DA release.

Interestingly, stimulation of postsynaptic D1 receptors on medium-sized spiny neurons elevated striatal glutamate release via inhibition of retrograde endocannabinoid release (Andre et al., 2010). Whether this is also the case for DA release in our preparation remains to be determined. Therefore, although we cannot dissect out whether pre- or postsynaptic SCH23390-sensitive D1 receptors mediated the effect of L-DOPA, it clearly appears that L-DOPA can directly interact with D1 receptors without its conversion to dopamine. Whether such an effect contributes to the therapeutic and side-effects of L-DOPA is controversial. The effective concentrations of L-Dopa stimulating exocytotic DA release in the present study are similar to those (1 μM) measured in the CSF of PD patients after oral (Olanow et al., 1991) or i.v. (Woodward et al., 1993) L-DOPA administration, and very close to those measured in the striatum of marmosets (442 nM) orally administered with a therapeutic dose of L-DOPA (Zhang et al., 2003). However, previous studies showed that L-DOPA-mediated behaviors are inconsistently affected by AADC blockers. In some studies, L-DOPA-mediated contralateral circling (Melamed et al., 1984; Treseder et al., 2000) or dyskinesia (Buck and Ferger, 2008) were markedly reduced (albeit not suppressed) by blockade of central AADC whereas in others L-DOPA-induced turning was unaffected (Alachkar et al., 2010; Nakamura et al., 1994; Nakazato and Akiyama, 1989; Treseder et al., 2001). Moreover, L-DOPA was reported to induce dyskinesia independently of modulation of striatal DA levels (Navailles et al., 2011; Nevalainen et al., 2011; Porras et al., 2014), possibly via stimulation of somato-dendritic and presynaptic D1 receptors at striato-nigral medium-sized spiny neurons, leading to enhanced firing and/or nigral GABA release (Mela et al., 2012; Robertson and Robertson, 1989; Yamamoto et al., 2006). In fact, L-DOPA stimulation of [^{3}H]GABA release (EC_{50} 1 μM) from nigral slices of DA-denervated rats was observed in the presence of an AADC inhibitor, i.e. independent of its conversion to DA (Aceves et al., 1991).

Binding experiments in our synaptosomal preparation, however, revealed, a negligible binding of L-DOPA to D1 receptors, in agreement with previous study in D1-transfected cells (Toll et al., 1998). In our preparation, Ki values of SCH233390 (0.42 nM) and SKF 38393 (29.1 nM) were in line with those previously reported in the rodent brain (SCH23390, 0.12-0.80 nM; SKF 38393, 18-41 nM) (Andersen, 1988; Neumeyer et al., 2003; Qandil et al., 2003; Watts et al., 1993). L-DOPA displaced [^{3}H]SCH23390 binding but, different from classical D1 receptor ligands, only partially (40%). This profile is reminiscent of allosteric negative modulation (May et al., 2007) although the possibility that L-DOPA behaves as a negative allosteric modulator does not easily reconcile with its facilitatory effect on [^{3}H]DA release. It is also possible that the SCH23390-sensitive D1 receptor identified in our

preparation does not belong to the classical $D1_A$ receptor subtype, since more than one D1 receptor subtype has been cloned (Tiberi et al., 1991) and D1 receptors can heteromerize with DA and non-DA receptors (Casado-Anguera et al., 2019; Fuxe et al., 2015) or couple with different G-proteins (Wang et al., 1995) generating receptor entities with unique pharmacological profiles (Undie et al., 1994). Nonetheless, we must consider that release and binding experiments were conducted under substantially different experimental conditions. Specifically, L-DOPA binding to D1 receptors was assessed in test tubes, where binding might be modulated by other molecules released and accumulated in the extracellular milieu. In fact, the [^{3}H]SCH23390 binding displacement induced by L-DOPA was halved in the presence of DAT and AADC inhibitors, suggesting that occupation of D1 receptors is partly mediated by endogenous DA formed from L-DOPA and released from DA terminals through the reversal of DAT. Indeed, DAT reversal of spontaneous [^{3}H]DA efflux by L-DOPA was observed in the present synaptosomal preparation. Different from stimulus-evoked tritium overflow, spontaneous [^{3}H]DA efflux is Ca^{2+}-insensitive and only slightly (~15%) tetrodotoxin-sensitive (Marti et al., 2003b), suggesting it mainly reflects non vesicular release and leakage from synaptosomes. Misu and coworkers (Misu et al., 1986) showed that, different from the stimulus-evoked DA release, the increase in spontaneous efflux induced by L-DOPA was prevented by an AADC inhibitor, i.e. relied on L-DOPA conversion to DA. Consistently, we found that GBR12783 was ineffective on stimulus-evoked [^{3}H]DA overflow but prevented the elevation of [^{3}H]DA efflux induced by high L-DOPA concentrations. These data support the view that L-DOPA is taken up into nerve terminals, decarboxylated to DA by AADC, and released via DAT reversal, confirming the in vivo evidence that L-DOPA can promote DA release through non vesicular mechanisms (De Deurwaerdere et al., 2017). Specifically, in vivo microdialysis showed that reverse dialysis of 5 μM L-DOPA into SN increased local DA levels via non vesicular mechanisms (Thorre et al., 1998), and systemic administration of L-DOPA (3-12 mg/Kg) elevated in vivo hippocampal and prefronto-cortical DA also after removal of Ca^{2+} from the perfusion medium, i.e. under conditions of impaired vesicular release (Miguelez et al., 2016). Altogether, these findings indicate that DAT reversal might occur in vivo at therapeutic L-DOPA concentrations, causing DA to stimulate D1 receptors.

In conclusion, this study presents the first evidence that low L-DOPA concentrations facilitate synaptosomal exocytotic DA release through direct stimulation of D1 receptors. Higher concentrations L-DOPA stimulate non vesicular DA release via L-DOPA conversion to DA and DAT reversal. These data add to previous evidence that L-DOPA can act as a

neurotransmitter, and may offer new insights into the neurobiological mechanisms underlying L-DOPA therapeutic and side-effects, in particular L-DOPA-induced dyskinesia (Bastide et al., 2015; De Deurwaerdere et al., 2017).

4. Experimental procedure

4.1. Animal subjects

Young adult (8-10-week-old) male C57BL/6J mice (20-25 g) obtained from a colony set at the LARP facility of the University of Ferrara were used. Mice were housed with free access to food and water and kept under environmentally controlled conditions (12-h light/dark cycle with light on between 07:00 and 19:00). The experiments complied with the ARRIVE guidelines, were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and were approved by the Italian Ministry of Health and Ethical Committee of the University of Ferrara (license #ECE79.2.EXT.3). All efforts were made to minimize the number of animals used and their suffering.

4.2. Synaptosome preparation and [^{3}H]DA analysis

Mice were anesthetized and sacrificed via cervical dislocation, and striatum was quickly excised to prepare synaptosomes, as previously described (Marti et al., 2001; Marti et al., 2003b; Morari et al., 1998). Striatum was homogenized in ice-cold 0.32 M sucrose (pH 7.4) with a Teflon-glass homogenizer and centrifuged at 800 x g for 10 min at 4°C. The supernatant was then centrifuged at 11,000 x g for 20 min at 4°C, the pellet resuspended in 1.5 mL oxygenated (95% O_2, 5% CO_2) Krebs solution (mM: NaCl 118.5, KCl 4.7, $CaCl_2$ 1.2, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 10) containing ascorbic acid (0.05 mM) and disodium EDTA (0.03 mM). Synaptosomes were incubated at 37°C with 50 nM [^{3}H]-DA (specific activity 40 Ci/mmol; Perkin-Elmer, Boston, MA, USA) for 25 min, after which 12 mL of pre-oxygenated Krebs were added (Longo et al., 2017; Marti et al., 2003a). One millilitre aliquots of the suspension (~0.35 mg protein) were slowly injected into nylon syringe filters (outer diameter 13 mm, 0.45 μM pore size, internal volume ~100 μL; Teknokroma, Barcelona, Spain), maintained at 36.5 °C in a thermostatic bath and superfused (0.4 mL/min) with a pre-carbogenated Krebs solution. Under these experimental conditions, spontaneous [^{3}H]DA efflux was essentially unaffected by reuptake. Filters were washed for 20 min, after which sample collection was started (every 3 min). The effect of drugs was evaluated on both spontaneous and K^+-stimulated neurotransmitter outflow. In this case, drugs were added to the perfusion medium 6 (agonist) or 9 (antagonist) min before a 10 mM K^+ pulse (120 sec) and maintained until the end of the experiment. [^{3}H]DA levels in the samples

were measured by liquid scintillation spectrophotometry. Sample superfusate (1.2 ml/sample) and filter retained (dissolved with 1 ml of 1 M NaOH followed by 1 M HCl) were opportunely mixed with Ultima Gold XR scintillation fluid (Packard Instruments B.V., Groningen, The Netherlands) and radioactivity in the samples and in the filters was measured using a Perkin Elmer Tri Carb 2810 TR scintillation counter.

4.3. Saturation and competition binding experiments

Saturation binding experiments to D_1 dopamine receptors were carried out by using [^{3}H]SCH23390 as radioligand (specific activity 84.3 Ci/mmol) (Trampus et al., 1991). Mouse striatal synaptosomes were incubated for 60 minutes at 30°C with different concentrations (0.1 nM-10 nM) of [^{3}H]-SCH23390 in 50 mM Tris-HCl pH 7.4, 5 mM $MgCl_2$. Non-specific binding was determined in the presence of 1 μM SCH23390 (Tocris, Bristol, UK) and was always <10% of the total binding. Competition binding experiments were performed incubating mouse striatal synaptosomes with 1 nM of [^{3}H]SCH23390 in 50 mM Tris-HCl pH 7.4, 5 mM $MgCl_2$ for 60 minutes at 30°C in the presence of increasing concentrations of SCH23390 (0.01 nM-1 μM), SKF38393 (0.1 nM-10 μM) or L-DOPA (1 nM-1 mM). Non-specific binding was determined in the presence of 1 μM SCH 23390 and was always <10% of the total binding. At the end of the incubation time, bound and free radioactivity was separated by filtering the assay mixture through Whatman GF/B glass fiber filters using a Brandel cell harvester (Brandel Instruments, Unterföhring, Germany). The filter bound radioactivity was counted using a Perkin Elmer Tri Carb 2810 TR scintillation counter. The protein concentration was determined according to a Bio-Rad method with bovine albumin as standard reference. Inhibitory binding constant values, Ki were calculated from the IC_{50} values according to the Cheng & Prusoff equation $Ki = IC_{50}/(1+[C^*]/K_D^*)$, where [C*] is the concentration of the radioligand and K_D^* its dissociation constant (Cheng and Prusoff, 1973).

4.4. Data and statistical analysis

Data, means ± SEM of 6 determinations per group, were expressed as absolute content (pmol/mg tissue/min), percent of basal tritium efflux (Fig. 2) or K^+-evoked tritium overflow (Fig. 1). Tritium efflux was calculated as fractional release (FR, i.e. tritium efflux expressed as percentage of the tritium content in the filter at the onset of the corresponding collection period) whereas K^+-evoked tritium overflow was calculated as net FR, i.e. tritium overflow as percent of the tritium content in the filter at the onset of the corresponding collection period. All values displayed a normal distribution (Kolmogorov-Smirnov test) but violate the assumption of homogeneity of variance (Bartlett's test). Therefore, statistical analysis was performed (Prism software; San Diego, CA, USA) by Welch's ANOVA on percent (Fig. 1) or

area-under-the curve (AUC; Fig. 2) values followed by the Dunnett's T3 test for multiple comparisons (Dunnett, 1980). Binding curves are representative of 4 independent experiments performed in duplicate. P values <0.05 were considered statistically significant.

4.5. Materials

Amisulpride, benserazide, GBR12783 dihydrochloride, L-DOPA, SCH23390 hydrochloride and SKF38393 were purchased from Tocris Bioscience (Bristol, UK). Pramipexole hydrochloride was purchased from McTony Bio&Chem (Vancouver, Canada), $[^{3}H]$DA and $[^{3}H]$SCH23390 from Perkin Elmer (Boston, MA, USA). All drugs were freshly dissolved in Krebs solution just prior to use.

Figure captions

Figure 1. Pramipexole, SKF38393 and L-DOPA differentially modulated K^+-evoked DA release from synaptosomes. The D2/D3 receptor agonist pramipexole (100 nM) inhibited (**A**) while the D1 receptor agonist SKF38393 (30-300 nM, **B**) and L-DOPA (0.1-1 μM, **C**) elevated the $[^3H]$DA overflow evoked by a 2 min pulse of 10 mM KCl from a preparation of striatal synaptosomes in superfusion. The inhibition induced by pramipexole was prevented by the D2/D3 receptor antagonist amisulpride (**A**) while the stimulation induced by SKF38393 and L-DOPA was prevented by the D1/D5 receptor antagonist SCH23390 (**B-C**). GBR12783, alone or combined with benserazide, did not block the L-DOPA effect (**D**). Pramipexole, SKF38393 and L-DOPA were administered 6 min before KCl whereas antagonists 3 min before agonists. Dots represent 6 determinations per group and were expressed as percentage of control (i.e. the K^+-evoked tritium overflow). $*p<0.05$, $**p<0.01$ different from control; $^{\#}p<0.05$, $^{\#\#}p<0.01$ different from pramipexole, SKF38393 or L-DOPA 1 μM alone (Welch's ANOVA followed by the Dunnett's T3 test).

Figure 2. L-DOPA increased spontaneous tritium efflux from synaptosomes. L-DOPA (1-100 μM) elevated spontaneous tritium efflux from a preparation of striatal synaptosomes in superfusion pre-loaded with $[^3H]$-DA (**A**). The DAT blocker GBR12783 but not the D1/D5 receptor selective antagonist SCH23390 prevented the elevation induced by L-DOPA (10 μM; **B**) and attenuated that induced by L-DOPA (100 μM; **C**). GBR12783 and SCH23390 were given 3 min before L-DOPA and maintained until the end of experiments. Data are means ± SEM of 6 determinations per group and were expressed as percentage of basal tritium efflux (calculated as the mean between the two samples before L-DOPA). $*p<0.05$, $**p<0.01$ different from washout; $^{\#}p<0.05$ different from L-DOPA alone (Welch's ANOVA followed by the Dunnett's T3 test performed on AUC values).

Figure 3. L-DOPA displaced $[^3H]$-SCH23390 binding in synaptosomes. Saturation curve (**A**) and Scatchard plot (**B**) of $[^3H]$-SCH23390 in mouse striatal synaptosomes. **C**. Inhibition curves of $[^3H]$-SCH23390 binding in mouse striatal synaptosomes by the D1/D5 receptor antagonist SCH23390 (0.01 nM-1 μM), the D1/D5 receptor agonist SKF38393 (0.1 nM-10 μM) or the dopamine precursor L-DOPA (1 nM-1 mM), both in the absence and in the presence of the DOPA decarboxylase inhibitor benserazide (1 μM) and the DAT inhibitor GBR12935. Curves are representative of 4 independent experiments performed in duplicate.

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Figure 1

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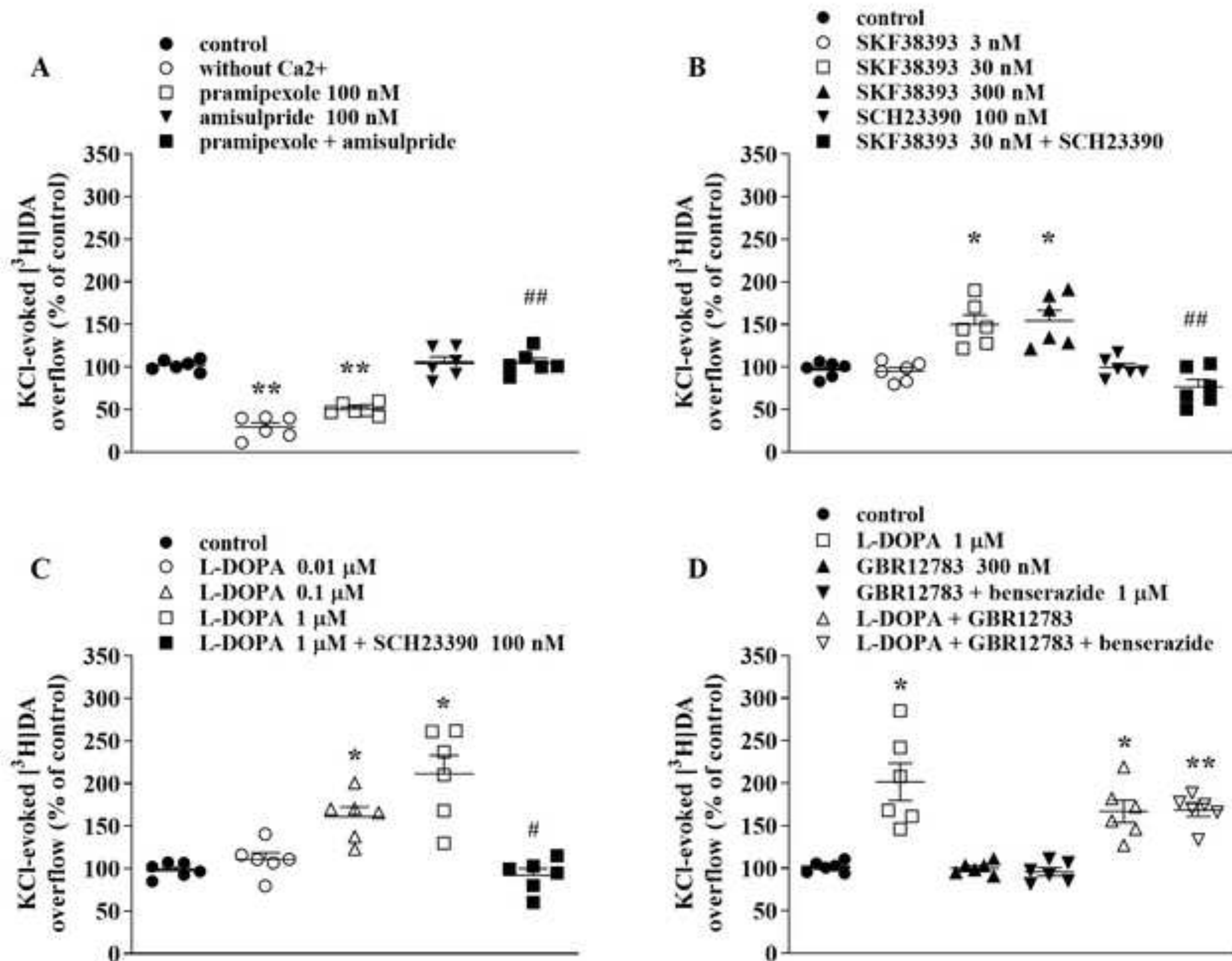

Figure 2

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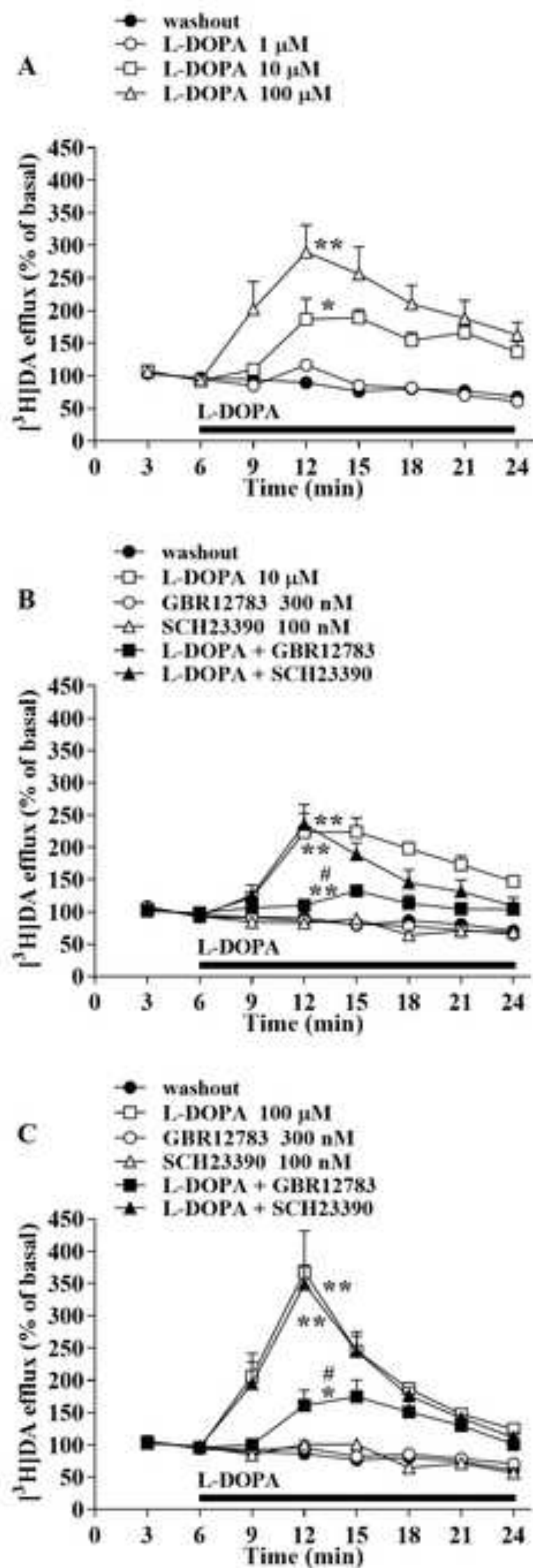

Figure 3

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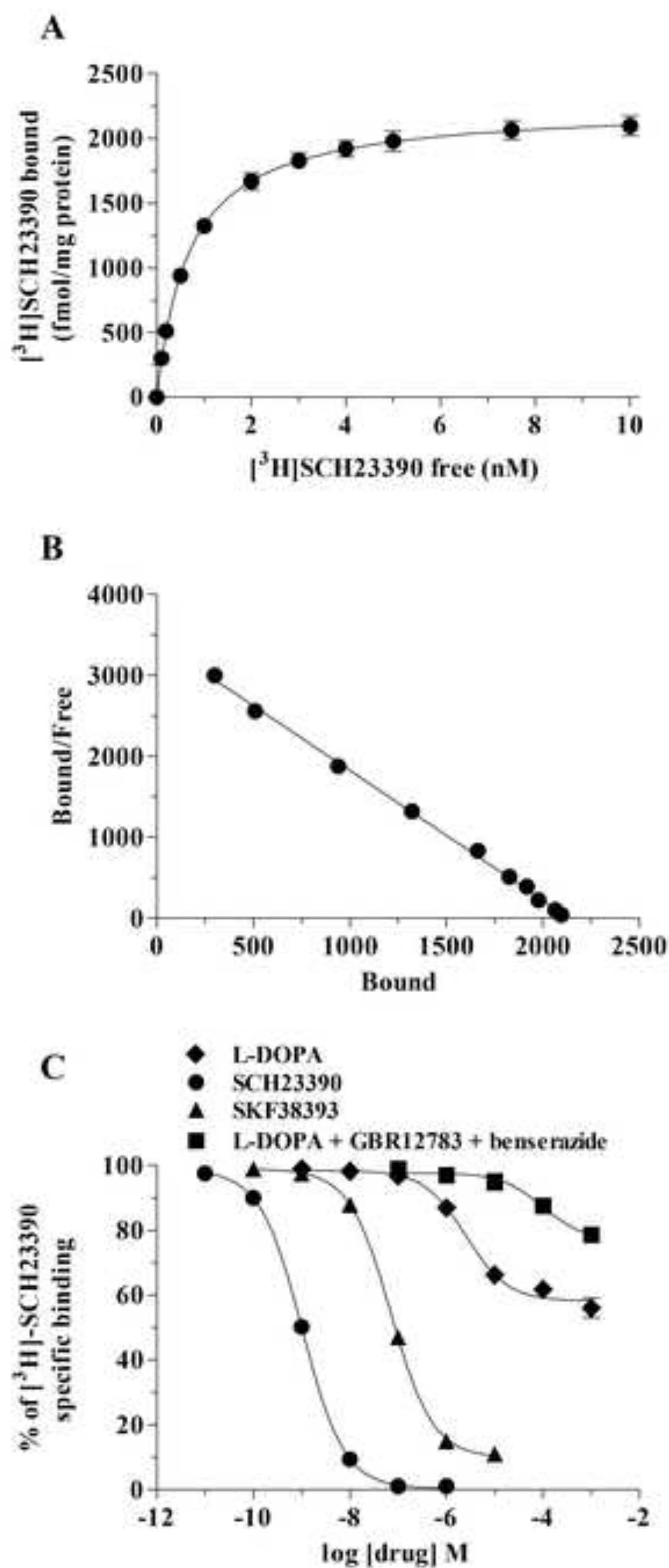

CRediT authorship contribution statement

RV: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Software; Supervision; Validation; Visualization; Roles/Writing - original draft; Writing - review & editing. **FL**: Data curation; Formal analysis; Investigation; Methodology; Software; Validation. **FV**: Data curation; Formal analysis; Investigation; Methodology; Software; Validation. **KV**: Data curation; Formal analysis; Investigation; Methodology; Software; Validation. **MM**: Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Resources; Software; Supervision; Validation; Visualization; Roles/Writing - original draft; Writing - review & editing.