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Buspirone anti-dyskinetic effect is correlated with temporal normalization of dysregulated striatal DRD1 signalling in L-DOPA-treated rats

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References: 57
Figures: 5
Tables: 2

Abstract

Dopamine replacement with L-DOPA is the most effective therapy in Parkinson disease. However, with chronic treatment, half of the patients develop an abnormal motor response including dyskinesias. The specific molecular mechanisms underlying dyskinesias are not fully understood. In this study, we used a well-characterized animal model to first establish the molecular differences between rats that did and did not develop dyskinesias. We then investigated the molecular substrates implicated in the anti-dyskinetic effect of buspirone, a 5HT1A partial agonist. Striatal protein expression profile of dyskinetic animals revealed increased levels of the dopamine receptor (DR)D3, ΔFosB and phospho (p)CREB, as well as an over-activation of the DRD1 signalling pathway, reflected by elevated ratios of phosphorylated DARPP32 and ERK2. Buspirone reduced the abnormal involuntary motor response in dyskinetic rats in a dose-dependent fashion. Buspirone (4 mg/kg) dramatically reduced the presence and severity of dyskinesias (by 83%) and normalized DARPP32 and ERK2 phosphorylation ratios, while the increases in DRD3, ΔFosB and pCREB observed in dyskinetic rats were not modified. Pharmacological experiments combining buspirone with 5HT1A and DRD3 antagonists confirmed that normalization of both pDARPP32 and pERK2 is required, but not sufficient, for blocking dyskinesias. The correlation between pDARPP32 ratio and dyskinesias was significant but not strong, pointing to the involvement of convergent factors and signalling pathways. Our results suggest that in dyskinetic rats DRD3 striatal over-expression could be instrumental in the activation of DRD1-downstream signalling and demonstrate that the anti-dyskinetic effect of buspirone in this model is correlated with DRD1 pathway normalization.

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Abstract

Dopamine replacement with L-DOPA is the most effective therapy in Parkinson disease. However, with chronic treatment, half of the patients develop an abnormal motor response including dyskinesias. The specific molecular mechanisms underlying dyskinesias are not fully understood. In this study, we used a well-characterized animal model to first establish the molecular differences between rats that did and did not develop dyskinesias. We then investigated the molecular substrates implicated in the anti-dyskinetic effect of buspirone, a 5HT1A partial agonist. Striatal protein expression profile of dyskinetic animals revealed increased levels of the dopamine receptor (DR)D3, ΔFosB and phospho (p)CREB, as well as an over-activation of the DRD1 signalling pathway, reflected by elevated ratios of phosphorylated DARPP32 and ERK2. Buspirone reduced the abnormal involuntary motor response in dyskinetic rats in a dose-dependent fashion. Buspirone (4 mg/kg) dramatically reduced the presence and severity of dyskinesias (by 83%) and normalized DARPP32 and ERK2 phosphorylation ratios, while the increases in DRD3, ΔFosB and pCREB observed in dyskinetic rats were not modified. Pharmacological experiments combining buspirone with 5HT1A and DRD3 antagonists confirmed that normalization of both pDARPP32 and pERK2 is required, but not sufficient, for blocking dyskinesias. The correlation between pDARPP32 ratio and dyskinesias was significant but not strong, pointing to the involvement of convergent factors and signalling pathways. Our results suggest that in dyskinetic rats DRD3 striatal over-expression could be instrumental in the activation of DRD1-downstream signalling and demonstrate that the anti-dyskinetic effect of buspirone in this model is correlated with DRD1 pathway normalization.

Keywords: Parkinson's disease, dyskinesias, buspirone, DRD3, DARPP32 and ERK2

1. Introduction

The dopamine precursor L-3,4-dihydroxyphenylalanine (L-DOPA) is the most efficacious pharmacological treatment for Parkinson's disease (PD). However, its chronic administration results in the appearance of choreic, dystonic, and ballistic movements, collectively referred to as L-DOPA-induced dyskinesia (LID) (Obeso et al., 2000). Although the specific mechanisms underlying LID are not well understood there is vast consensus that it results from dysregulated dopamine neurotransmission depending on both presynaptic alterations and post-synaptic dopamine receptor (DR) supersensitivity in the striatum (Huot et al., 2013). Dopamine exerts its biological function by activation and signalling through two different groups of G protein-coupled receptors (GPCRs), the D1 family (DRD1 and DRD5) and the D2-class receptors (DRD2, DRD3 and DRD4). DRD1 and DRD2-like receptors are associated with opposite postsynaptic transduction mechanisms, linked, respectively, to the activation or inhibition of cyclic adenosine monophosphate (cAMP) synthesis (Beaulieu and Gainetdinov, 2011). Accumulating evidence indicates that LID develops in response to activation of sensitized DRD1 receptors located on the GABAergic medium-sized spiny neurons (MSNs) of the direct striatonigral pathway (Feyder et al., 2011; Murer and Moratalla, 2011). Chronic L-DOPA administration hyper-activates the dopamine and cAMP-regulated phosphoprotein of 32kDa (DARPP32) cascade (Picconi et al., 2003; Santini et al., 2007) but can also activate other pathways not typically related to dopamine transmission like the extracellular signal-regulated kinase (ERK1/2) (Gerfen et al., 2008; Santini et al., 2007; Westin et al., 2007). DRD2-class receptors in striatopallidal neurons also appear to be involved in LID, although they are traditionally regarded as less relevant than DRD1 receptors (Murer and Moratalla, 2011). For example, it has been reported that chronic L-DOPA administration enhances the activity

of the Akt/GSK3 pathway (Bychkov et al., 2007), a signalling cascade mediated by DRD2-class receptors in the striatum (Beaulieu et al., 2007b). Regarding DRD3, both increased expression (Aristieta et al., 2012; Bagetta et al., 2012; Bezard et al., 2003; Bordet et al., 1997) and ectopic localization of DRD3 in DRD1-expressing MSN have been described in the striatum of 6-hydroxydopamine (6-OHDA) lesioned rats after chronic L-DOPA treatment (Bordet et al., 2000).

Besides dopamine transmission, abnormalities in other neurotransmitter systems have been reported to play a role in LID. Glutamate regulates neural activity by acting on ionotropic and metabotropic G protein-coupled receptors (mGluR). The mGluR5 receptors, in particular, have received considerable attention because are major players in the excitatory drive to the subthalamic nucleus (STN) (Awad et al., 2000) an intermediate node in the indirect pathway which plays a role in LID (Aristieta et al., 2012). Serotonin (5-hydroxytryptamine, 5-HT) is another potential non-dopaminergic target, which may prove therapeutically relevant. It has been suggested that dopamine released from serotonin axon terminals acts as a false neurotransmitter and is the main pre-synaptic determinant of LID (Carta et al., 2007; Nahimi et al., 2012). Specifically, different 5-HT1A receptor agonists have shown to reduce LID, without impairing L-DOPA improvement in motor performance (Ba et al., 2007; Bibbiani et al., 2001; Dupre et al., 2007), maybe by regulating the striatal concentration of DA derived from exogenous L-DOPA (Bara-Jimenez et al., 2005).

Buspirone, a partial 5HT1-A agonist, has been shown to reduce the expression and development of LID in hemiparkinsonian rats (Aristieta et al., 2012; Dekundy et al., 2007; Eskow et al., 2007). However, the precise molecular mechanisms underlying the reduction in LID are not clear. It has been suggested that buspirone, acting as a partial agonist, binds to the 5HT1A receptors dislocating the inhibitory G-proteins, and

preventing the conversion of ATP to cAMP and the initiation of other secondary messenger signalling mechanisms (Loane and Politis, 2012). Besides the 5HT1A agonist effect, buspirone also acts as a high affinity DRD3 antagonist (Bergman et al., 2013) and the anti-dyskinetic effects of buspirone could be mediated by both 5HT1A and DRD3 receptors, as proposed for sarizotan, another 5HT1A agonist (Gerlach et al., 2011).

The present study aimed at defining the molecular differences between dyskinetic and non-dyskinetic animals in the striatum and to profile the mechanisms underlying the acute anti-dyskinetic effect of buspirone at the molecular level.

2. Material and Methods

2.1. Animals

Sprague-Dawley rats (Harlan) weighing 200-250 g at the beginning of the experiments were housed in groups of five in standard laboratory conditions (22 ± 1ºC, 55 ± 5% of relative humidity, and a 12:12 h light/dark cycle) with *ad libitum* access to food and water. Every effort was made to minimize animal suffering and to use the minimum number of animals per group and experiment. Experimental procedures were approved by the Local Ethical Committee of the University of Basque Country (UPV/EHU, CEBA/185/2011), following European (2010/63/UE) and Spanish (RD 1201/2005) regulations for the care and use of laboratory animals.

2.2. Drugs

6-OHDA (3.5 μg/μl), dissolved in MiliQ water containing 0.02% ascorbic acid, desipramine hydrochloride (25 mg/kg i.p.), amphetamine sulphate (3 mg/kg i.p.), L-DOPA (6 mg/kg i.p.), benserazide-HCL (12 mg/kg i.p.), buspirone (1, 2.5 and 4 mg/kg, i.p.), WAY-100635 (0.5 and 1 mg/kg, i.p.) from Sigma-Aldrich, were dissolved in 0.9% saline and GR103691 (1.5 mg/kg, i.p.) from Tocris, in DMSO. All drugs were prepared on the day of the experiment and administrated intraperitonealy.

2.3. Experimental design

2.3.1. Experiment 1. **Striatal protein profile of non-dyskinetic and dyskinetic rats treated chronically with L-DOPA.** Surgical operations were carried out at the beginning of the experiment and two weeks later animals were tested for amphetamine-induced rotation (see below). Animals were treated chronically (3 weeks) with saline or L-DOPA and behavioural evaluations were performed periodically. At the end of the experiment, animals were killed one hour after the last saline or L-DOPA injection. Brains were processed for histology or western blotting. For western blotting the groups

were as follows: sham L-DOPA (n = 8), 6-OHDA saline (n = 9), 6-OHDA L-DOPA non-dyskinetic (n = 6) and 6-OHDA L-DOPA dyskinetic (n = 14). An additional 2-3 animals per groups were used for immunofluorescent.

2.3.2. Experiment 2. Behavioural characterization of the effect of buspirone on AIMs. Another group of rats were lesioned and after three weeks of L-DOPA treatment, received a maintenance dose of L-DOPA treatment twice per week. Animals were scored on two consecutive days to obtain a baseline for each drug dose or combination. All drugs were injected 30 min prior to the administration of L-DOPA. After one-week washout a new baseline with L-DOPA alone was taken to verify that AIMs scored was recovered. Drug doses were chosen based on previous experiments; buspirone (Aristieta et al., 2012; Dekundy et al., 2007 ; Eskow et al., 2007), and the selective 5HT1A antagonist WAY-100635 and DRD3 antagonist GR103691(Gerlach et al., 2011). Previous studies demonstrated that at these doses buspirone does not affect locomotor behaviour (Dekundy et al., 2007; Eskow et al., 2007). Groups: Buspirone 1mg/kg (n = 6), Buspirone 1mg/kg + Way-100635 1mg/kg (n = 7), Buspirone 1mg/kg + GR103691 1.5mg/kg (n = 7), Buspirone 4mg/kg (n = 9), Buspirone 4mg/kg + Way-100635 0.5mg/kg (n = 8), Buspirone 4mg/kg + Way-100635 1mg/kg (n = 8), Buspirone 4mg/kg + GR103691 1.5mg/kg (n = 7), Way-100635 0.5mg/kg (n = 8), Way-100635 1mg/kg (n = 8) and GR103691 1.5mg/kg (n = 6).

2.3.3. Experiment 3. **Molecular changes induced by buspirone acute treatment.** Like in the previous experiments, lesioned rats were treated with L-DOPA during three weeks. Buspirone (1 and 4 mg/kg), WAY-100635 (1 mg/kg) and GR103691 (1.5 mg/kg) were injected 30 min prior to the administration of L-DOPA. After L-DOPA injection the rats were scored for 60 minutes and were then sacrificed. Brains were processed for histology or western blotting. For western blotting the groups were as

follows: Buspirone 1 mg/kg (n = 5), Buspirone 4 mg/kg (n = 6), Buspirone 4 mg/kg + WAY-100635 1 mg/kg (n = 5), WAY-100635 1 mg/kg (n = 3), GR103691 1.5 mg/kg (n = 5) and Buspirone 4 mg/kg + GR103691 1.5 mg/kg (n = 4). An additional 2-3 animals per groups were used for immunofluorescent.

2.4. 6-OHDA lesion and rotational screening

Thirty minutes before the surgery, rats were pre-treated with desipramine in order to protect noradrenergic terminals from 6-OHDA toxicity. Rats were deeply anesthetized with isoflurane (1.5-2%, Esteve) and positioned in a stereotaxic frame. 6-OHDA or an equivalent volume of vehicle (sham group) was infused (1 µl/min) using a Hamilton microsyringe. A total of 15.75 µg in 4.5 µl were injected in two sites in the right medial forebrain bundle (MFB): 2.5 µl at anteroposterior (AP) - 4.4 mm, mediolateral (ML) + 1.2 mm, and dorsoventral (DV) - 7.8 mm, relative to bregma and dura with the toothbar set at -2.4, and 2 µl at AP - 4.0 mm, ML + 0.8 mm, and DV - 8 mm, with the toothbar at +3.4 (Paxinos and Watson, 1986).

The turning behaviour after amphetamine sulphate administration was recorded 2 weeks post-surgery (Miguelez et al., 2011). Animals displaying >5 full turns per minute over 90 minutes were selected for the study and randomly assigned to one of the 6-OHDA groups.

2.5. Abnormal involuntary movement rating

AIMs were induced in 6-OHDA-lesioned rats by chronic daily injections of L-DOPA (6 mg/kg, i.p), in combination with the peripheral decarboxylase inhibitor benserazide (12 mg/kg, i.p.), over 3 weeks. AIMs were scored according to a rat dyskinesia scale previously described (Cenci and Lundblad, 2007). After three weeks of treatment, L-DOPA dosing was changed to a maintenance (twice a week) schedule (Carlsson et al., 2005). On the testing days, rats were placed individually in transparent empty plastic

cages for at least 10 min prior to drug administration. Following L-DOPA injection, each rat was observed for one full minute every 20th min, for at least 180 min. The severity of the three subtypes of dyskinetic movements (axial, limb and orolingual AIMs) and asymmetric locomotive behaviour (locomotive AIMs) were rated from 0 to 4, based on the amount of time in which the abnormal movement was present during the observation period (i.e. 0, not present to 4, continuous). In addition, the amplitude of axial, limb and orolingual AIMs was rated on a scale from 0 to 4. Axial, limb, and orolingual AIMs were analyzed separately from locomotive AIMs. Data are expressed as AIM score/session, which is calculated, multiplying the severity by the amplitude scores on each monitoring period, with all these products summed for each testing session (Lindgren et al., 2010). Scores at 60 min were used for correlation analyses.

2.6. Western blot

One hour after the last saline or L-DOPA injection animals were killed by decapitation. The brains were then removed, right and left striatum were dissected on an ice-cold surface and quickly frozen. For protein extraction (Azkona et al., 2010) each striatum was homogenized in ice-cold lysis buffer (10 mM HEPES pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1 mM $MgCl_2$, phosphate-buffered saline (PBS) 0.2% Triton X-100) and a protease inhibitor cocktail (Roche). After clearance of the lysates by centrifugation (16000xg, 30 min at 4ºC), protein quantification was performed following the DC Protein Assay (Bio-Rad Laboratories) protocol. Equal amount of protein (50 μg) for each sample was then separated in SDS-polyacrylamide gels (SDS-PAGE) and subsequently transferred to polyvinylidene difluoride (PVDF, Millipore) membranes. Blots were blocked with 5% non-fat dry milk or bovine serum albumine in Tris-buffered saline including 0.1% Tween-20 (TBS-T) and incubated overnight at 4ºC with the following primary antibodies raised in mouse: anti-TH (1:1000; Millipore), anti-

DRD3 (1:1000, Santa Cruz Biotechnology), anti-pERK1/2 (pT202/pY204, 1:1000), anti-ERK1/2 (1:1000), anti-pAkt (pS472/pS473, 1:1000), anti-Akt (1:1000), anti-GSK3 (1:1000; BD Biosciences) and anti-pGSK3 (Y279/Y216, 1:1000; ECM Bioscience), and in rabbit: anti-cFos (1:1000), anti-FosB (1:200), anti-5HT1A (1:500, Santa Cruz Biotechnology), anti-mGluR5 (1:1000; Novus Biologicals), anti-Actin (1:1000), anti-DRD1 (1:1000; Sigma-Aldrich), anti-DRD2 (1:1000; Lifespan Biosciences), anti-pDARPP32 (Thr34) (1:1000), anti-DARPP32 (1:1000), anti-pAkt (T308, 1:1000) anti-pGSK3 (S21/9, 1:1000; Cell Signaling Technology), and anti-GAPDH (V-18) HRP (1:1000; Santa Cruz Biotechnology). For detection, membranes were incubated with horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit IgG (GE Healthcare), followed by enhanced chemiluminescence (Super Signal, Thermo Scientific) detection system. Quantification was made by densitometric analysis of non-saturated films using Image J software.

2.7. Immunohistochemistry

One hour after the last saline or L-DOPA injection, animals were deeply anaesthetized and transcardially perfused with saline followed with 4% ice-cold paraformaldehyde, prepared in 0.1M phosphate buffer. Brains were removed and transferred to a 25% sucrose solution until they sank. Brains were serially cut in coronal 40 μm sections using a freezing microtome (HM 430, Microm), and kept in a cryoprotective solution at -20ºC. A standard immunofluorescence (IF) procedure for free-floating sections was followed. Sections spanning the striatum were permeabilized with 0.1% Triton X-100 in 10% donkey serum and incubated at 4ºC for 2 days with primary antibodies against TH (sheep, 1:500, Pel-Freez), NeuN (mouse, 1:200, Millipore) and phosphoCreb Ser133 (rabbit, 1:200, Neuromics); after washing in PBS the sections were incubated with the fluorophore-conjugated specific secondary antibodies for 2 hours at room temperature.

Sections were washed, counterstained with Hoechst, cover-slipped with Vectashield and examined under a confocal microscope (LSM 510 Meta, Zeiss). Images were acquired from the striatum at the level of the anterior commisure from both hemispheres.

2.8. Statistical analysis of data

Experimental data were analyzed using GraphPad Prism software (v. 5.01, GraphPad Software, Inc). AIM scores were evaluated using two-way repeated measures (RM) ANOVA. We used paired t-test to compare the AIM scores of the same animal in 2 consecutive sessions (baseline and experimental) and unpaired t-tests for the analysis of the effect of different pharmacological combinations. Western blot data were normalized to loading control. We analyzed protein expression in the lesioned (right) and unlesioned (left) sides and expressed the WB results as right to left ratios, to enable comparison between different groups. Unilateral effects were supervised to ensure that this normalization (R/L) did not mask any significant contralateral or bilateral changes. Group comparisons were performed using one-way ANOVA followed by Bonferroni *post-hoc* test. For correlation between AIMs and molecular data we used Spearman's rho. The level of statistical significance was set at $p < 0.05$. Data are presented as group mean ± standard error of the mean (S.E.M.).

3. Results

3.1. Characterization of L-DOPA-induced involuntary movements in 6-OHDA lesioned rats

Chronic L-DOPA administration (6 mg/kg plus benserazide 12 mg/kg) induced AIMs in 6-OHDA lesioned rats. In our hands over 80% of hemi-parkinsonian animals develop LID with this regime. A group of lesioned rats treated with L-DOPA did not develop AIMs (Global score = 0), and were thus classified as non-dyskinetic animals (n = 6). After a single injection of L-DOPA we observed that the AIMs peaked at around 60 minutes (Fig 1A-D). We verified that all 6-OHDA lesioned rats showed a significant reduction of TH expression in the lesioned striatum by western blot ($F_{(3,34)} = 54.43$, $p < 0.0001$, Fig 1E). Importantly, there were no significant differences between dyskinetic and non-dyskinetic animals in TH protein content by WB (0.33 ± 0.05 vs. 0.30 ± 0.03; $p = 0.59$), nor in the extent of the DA denervation by immunofluorescence (Fig 1F). We analyzed the expression of cFos and did not observe any significant differences in the expression of this indirect marker of neuronal activity (Fig 1G). ΔFosB/FosB ratio (Andersson et al., 1999) was significantly increased in dyskinetic animals compared to non-dyskinetic and control groups ($F_{(3,34)} = 10.04$, $p < 0.0001$; Fig 1H).

3.2. Striatal expression of receptors implicated in L-DOPA induced dyskinesia

Non-dyskinetic animals provide the opportunity to investigate the molecular profile underlying AIMs. Since AIMs peak at about 60 min after L-DOPA administration in our paradigm, we analysed the animals at this time point. We first determined the expression of the DA receptors, DRD1, DRD2 and DRD3 in the striatum. The results showed that there were no significant differences in the expression of either DRD1 (Fig 2A) or DRD2 (Fig 2B), whereas DRD3 expression was significantly different ($F_{(3,34)} = 4.36$, $p < 0.01$; Fig 1C), showing higher levels in dyskinetic rats (1.17 ± 0.04) compared

with non-dyskinetic (0.89 ± 0.07; $p < 0.05$) and 6-OHDA saline (0.93 ± 0.05, $p < 0.05$) animals. In this model, 5HT1A agonists have been shown to reduce AIMs, thus we analysed the expression of this serotonergic receptor. We did not observe significant differences in striatal expression levels in any of the groups analysed (Fig. 2D). We also examined the expression of mGluR5, as mGluR5 antagonists have been reported to have anti-dyskinetic effects. The results showed that the expression was significantly different among the groups ($F_{(3,34)} = 3.68$, $p < 0.05$), being higher in the group of 6-OHDA saline (1.19 ± 0.08) than in non-dyskinetic (0.93 ± 0.03; $p < 0.05$) and dyskinetic (0.90 ± 0.07; $p < 0.05$) animals (Fig 2E). These data confirm our previous results (Aristieta et al., 2012) and suggest that chronic L-DOPA treatment normalizes mGluR5 expression in the DA denervated striatum independently of dyskinetic status.

3.3. Phosphorylation profile of key proteins involved in DRD1 and DRD2 signalling pathways

It has been suggested that AIMs are associated with changes downstream DRD1 activation. Our results confirmed an increased phosphorylation of DARPP32 on Thr34 ($F_{(3,34)} = 7.04$, $p < 0.001$, Fig 3A) in the dyskinetic group (1.35 ± 0.11) compared with both non-dyskinetic (0.85 ± 0.09; $p < 0.01$) and saline (0.85 ± 0.07; $p < 0.01$) groups. ERK2 phosphorylation data followed a similar pattern ($F_{(3,34)} = 4.92$, $p < 0.01$, Fig 3B), with an increase in the dyskinetic group (1.17 ± 0.07) compared with non-dyskinetic (0.84 ± 0.07; $p < 0.01$) and saline (0.94 ± 0.03; $p < 0.05$) animals.

We analyzed pCREB expression in the striatum by IF (Fig 3C) and observed that non-dyskinetic animals presented fewer pCREB positive neurons, compared with dyskinetic animals in the motor striatum.

On the other hand, it has been reported that chronic L-DOPA treatment engages the DRD2 pathway through the Akt/GSK3 signalling cascade (Bychkov et al., 2007). In this regard we found that, despite an increase in the phosphorylation of Akt on Ser473 and Thr308 in all L-DOPA treated animals (Fig 3D), there were no significant differences between dyskinetic and non-dyskinetic animals. Next, we analyzed the phosphorylation state of GSK3β in two residues, Tyr216, which increases the kinase activity and Ser9, which inhibits it (Morissette et al., 2010). Again, we could not detect significant differences across groups (Fig 3E) although non-dyskinetic animals showed a trend towards lower levels of pSer9.

In summary, compared with non-dyskinetic animals, dyskinetic rats presented an overexpression of DRD3 and an increased expression of pDARPP32, pERK2 and pCREB protein levels.

3.4. Behavioural analysis of buspirone mediated anti-dyskinetic effect

Buspirone reduced AIMs in a dose-dependent manner (Fig 4A-C) without a significant impact on overall locomotor activity, which was only slightly reduced with the highest dose (Table 1). The global AIMs score decreased by 33, 71 and 83% respectively for 1, 2.5 and 4 mg/kg of buspirone ($F_{(2,20)} = 14.78$, $p < 0.0001$, Fig 4D). The time course analysis of individual AIMs (axial, limb and orolingual), showed the same pattern. Despite the remarkable reduction in the AIMs severity, there was a perceptible increase in the duration in the limb scores.

Given that buspirone can act as a 5HT1A agonist (Eskow et al., 2007) we used a 5HT1A antagonist, WAY-100635, to outline the contribution of this pathway. Buspirone also acts on DRD3 (and DRD2) receptors as an antagonist (Bergman et al., 2013; Gerlach et al., 2011), but because L-DOPA already activates the DRD3 we used a DRD3 antagonist (GR103691) here to indirectly assess the contribution of this receptor

to the anti-dyskinetic effect of buspirone. Behavioural results are summarized in Table 1. Administration of WAY-100635 (0.5 and 1mg/kg) did not modify or tended to increase AIMs score and, in co-administration, efficiently attenuated the anti-dyskintetic effect of buspirone (Fig 4E) in a dose-dependent manner (see % AIMs score value in Table 1). The DRD3 antagonist GR103691 slightly reduced global AIMs (~15%, $p < 0.05$) and co-administration with buspirone did not modify buspirone anti-dyskinetic effect (Fig 4F). Global limb, axial and orolingual AIMs score were similarly affected and no differences were observed between buspirone 1 mg/kg or 4 mg/kg alone and in combination with GR103691. The combined administration of GR and buspirone significantly reduced the locomotor score (which can indicate a negative effect on L-DOPA anti-parkinsonian efficacy by this combination).

3.5. Molecular changes associated with buspirone treatment

Animals treated with buspirone presented a striatal receptor expression pattern identical with dyskinetic animals, including a significant increase in DRD3 (Table 2).

We first analyzed the AIMs scores at the time of sacrifice for biochemical analysis (60 min; Fig 5A). As for cumulative scores (Fig 3 and Table 1), scores at 60 min were significantly different across groups ($F_{(5,23)} = 9.34$, $p < 0.0001$) despite larger variability within groups. Buspirone 4 mg/kg significantly reduced AIMs compared with the lower dose (1 mg/kg). This effect was blocked by WAY-100635, but not by GR103691 co-administration. At this time point, administration of WAY-100635 or GR103691 alone did not modify the AIMs scores.

Western blot analyses showed that DARPP32 phosphorylation ratio was significantly different across groups ($F_{(9,56)} = 4.54$, $p < 0.001$; Fig 5B). Post-hoc analysis revealed that the higher dose of buspirone significantly reduced the phosphorylation of DARPP32 (0.94 ± 0.08) compared with the 1 mg/kg dose (1.40 ± 0.16; $p < 0.05$), and

with untreated dyskinetic animals (1.35 ± 0.10; $p < 0.05$). Intriguingly, the reduction in DARPP32 phosphorylation was not significantly blocked by co-administration of WAY-100635 (1.02 ± 0.07). WAY-100635 alone did not affect the elevated levels of pDARPP32 (1.5 ± 0.14) in the lesioned striatum of dyskinetic rats. The DRD3 antagonist, GR103691, significantly reduced pDARPP32 levels (0.92 ± 0.12) compared to non-treated dyskinetic animals ($p < 0.05$), in spite of having no significant behavioural effect at 60 min –and only a marginal effect on the cumulative scores (>15% decrease). Nonetheless, taken together all groups pDARPP32 ratios were significantly correlated with global AIMs scores (Spearman's rho= 0.415, $p < 0.05$).

Regarding ERK2 phosphorylation, there were significant differences among the treatment groups ($F_{(9,56)} = 4.31$, $p < 0.001$, Figure 5C). The higher dose of buspirone significantly reduced ERK2 phosphorylation (0.81 ± 0.09), compared with the lower dose (1.44 ± 0.26; $p < 0.05$) and with untreated dyskinetic group (1.17 ± 0.07; $p < 0.01$). Normalization of pERK2 ratio was also achieved by co-administration of buspirone and WAY-100635, but note that WAY-100635 alone also reduced ERK2 phosphorylation (0.96 ± 0.06), as previously described (Crane et al., 2007). Regarding GR103691, co-administration with buspirone significantly reduced pERK2 (0.96 ± 0.1, $p < 0.05$) while GR103691 treated animals showed significantly higher phosphorylation ratios (1.66 ± 0.12). pERK2 ratios were not significantly correlated with AIMs scores at 60 min ($p = 0.09$). However, multiple regression analysis showed that pERK2 contributed to the AIMs variance (multiple R= 0.447, F= 6.48, $p < 0.01$).

Finally, we analyzed CREB phosphorylation by IF in buspirone 4 mg/kg treated animals with and without WAY-100635 (1 mg/kg) and GR103691 (1.5 mg/kg). The results showed that buspirone did not induce changes in pCREB immunoreactivity in the striatum (Fig 5D). Buspirone in combination with WAY-100635 increased, and, in

combination with GR103691 reduced the expression of pCREB, as did GR103691 alone (data not shown). Additionally, we examined phosphorylation sites in GSK3β, even if there were no significant differences in dyskinetic animals (Fig 3). The expression of the two phosphorylated residues, Tyr216 and Ser9 (1.32 ± 0.09 and 0.95 ± 0.11, respectively; data not shown) was no different from either non-dyskinetic or dyskinetic animals, indicating that buspirone does not affect this pathway, at least at the time examined, and acts predominantly on the DRD1 pathway.

Taken together these results, we can conclude that buspirone exerts its anti-dyskinetic effect through a transient normalization of the phosphorylation of DARPP32 and ERK2 without modifying the underlying dyskinetic profile (DRD3, ΔFosB, pCREB).

4. Discussion

In this study we first carried out a systematic characterization of the molecular differences between rats that did and did not develop AIMs using a well-established model (Cenci et al., 1998). We identified two differences between non-dyskinetic and dyskinetic rats, which have not been previously reported in the same animals, an over-expression of the DRD3 and an over-activation of the DRD1 signalling pathway. In the second part of this study we show that buspirone not only reduced, in a dose dependent manner AIMs score, but it also reversed the over-activation of the DRD1 signalling pathway, while DRD3, ΔFos and pCREB protein levels remained unmodified. Moreover, there was a direct correlation between normalization of DR1 signalling cascades and the suppression of AIMs.

It has been estimated that ~50% of PD patients develop dyskinesias and other motor complications after 5 years of L-DOPA treatment (Roos et al., 1990; Smith et al., 2012), increasing to 60% after 10 years (Suwijn et al., 2013). A young age at disease onset, disease severity (reflecting the extent of putaminal DA denervation), and high doses of L-DOPA (Schrag and Quinn, 2000) are all correlated with a higher prevalence of dyskinesias. Why some patients do not develop LID is not known. It is also noteworthy that dyskinesias can develop in the absence of nigrostriatal denervation (Pons et al., 2013). In our study age and striatal denervation were equivalent, as was L-DOPA dose. Unlike patients, few 6-OHDA-lesioned rats (≤ 20 % in our experience) do not develop this motor complication. It has been proposed that differences in blood brain barrier permeability may underlie this difference in the 6-OHDA model because following L-DOPA administration striatal concentrations of L-DOPA (Carta et al., 2006) or dopamine (Lindgren et al., 2010) were increased only in dyskinetic animals.

DRD1 expression level was not modified in dyskinetic rats. However, increased phosphorylated levels of DARPP32 and ERK2 revealed an over-activation of this pathway. In dyskinetic rats DRD1 is abnormally localized at the membrane of MSNs (Berthet et al., 2009). This redistribution appears to be actively maintained by the DRD3 anchoring the DRD1 to the membrane. *In vitro* and *in vivo* studies reported DRD1-DRD3 heteromers that are functionally active (Fiorentini et al., 2010; Marcellino et al., 2008). Furthermore, the ectopic over-expression of DRD3 in the motor striatum of dyskinetic rats seen with chronic L-DOPA (Aristieta et al., 2012; Bagetta et al., 2012; Berthet et al., 2009; Bezard et al., 2003; Bordet et al., 1997; Guillin et al., 2001) could facilitate the cross-talk between both receptors co-activating the DRD1 direct pathway, as proposed by previous works (Bagetta et al., 2012; Berthet et al., 2009). Striatal activation of DRD1 receptors coupled to adenyl cyclase increases cAMP production stimulating the PKA-catalyzed phosphorylation of DARPP-32 on Thr34. In different PD animal models L-DOPA induced activation of this cascade has been consistently associated with AIMs (Lebel et al., 2010; Picconi et al., 2003; Santini et al., 2010; Santini et al., 2007). This activation can be mediated through sensitized DRD3 receptors, as previously observed in dyskinetic rats and primates (Berthet et al., 2009; Sanchez-Pernaute et al., 2007). DRD1 stimulation also induces ERK1/2 phosphorylation in the DA-depleted brain (Gerfen et al., 2008), and elevation of pERK has also been correlated with AIMs (Darmopil et al., 2009; Pavon et al., 2006; Santini et al., 2007; Westin et al., 2007). Thus, our results suggest that the over-expression of DRD3 is functionally relevant and could be instrumental in the activation of DRD1-downstream signalling and the development of dyskinesias. However, in vivo imaging studies would be necessary to address the contribution of these receptors in PD patients.

In this study we did not observe significant changes in the DRD2 pathway. It has been reported that DA can function through the Akt/GSK3 signalling cascade involving DRD2-class receptors (for review see (Beaulieu et al., 2007a) and that chronic L-DOPA treatment exacerbates imbalances in the Akt pathway caused by the loss of DA (Bychkov et al., 2007). Although we observed a slight increase of pAkt in the lesioned striatum, this was not significant. Methodological differences between these studies include the dose (6 vs. 25 mg/kg) and the time of analysis. In Bychkov´s study animals were killed 24h after the last L-DOPA injection, whereas in the present work we sacrificed the rats 1h after L-DOPA injection. This could indicate that while the Akt/GSK3 pathway may be involved in the long-term action of L-DOPA, it may not be critical at the peak of dyskinesias.

A number of studies have implicated the serotonin system in LID (Carta et al., 2007; Huot et al., 2013; Nahimi et al., 2012). Serotonin modulates DA release and can also store and release DA from L-DOPA. We did not observe changes in the expression of 5-HT1A receptors in any of the experimental groups. In this regard, published results are inconsistent since increments, decrements and unchanged 5-HT1A expression in parkinsonian and dyskinetic animals have all been reported (Frechilla et al., 2001; Huot et al., 2013; Mo et al., 2008; Wang et al., 2009).

In addition, 5HT1A partial agonists, like buspirone, can reduce AIMs in 6-OHDA lesioned rats, without modifying motor improvement induced by L-DOPA (Dekundy et al., 2007; Eskow et al., 2007; Paquette et al., 2009). Our behavioural characterization showed that the dose-dependent effect of buspirone could be primarily due to its action on 5HT1A receptors, because co-treatment with WAY-100635 significantly reduced buspirone anti-dyskinetic effect.

Besides the 5HT1A partial agonist effect, buspirone also acts as a high affinity DRD3 antagonist (Bergman et al., 2013) and the anti-dyskinetic effects of buspirone could therefore be mediated by both 5HT1A and DRs, as recently proposed for other 5HT1A partial agonists (Dupre et al., 2013; Gerlach et al., 2011). Moreover, DRD3 antagonists and partial agonists have been reported to exert a potent anti-dyskinetic effect in rats and primates (Bezard et al., 2003; Bordet et al., 1997; Kumar et al., 2009; Visanji et al., 2009) perhaps by releasing DRD1 from the membrane (Berthet et al., 2009). If buspirone effect was mediated by DRD3 antagonism, GR103691 should mimic it. In our study DRD3 antagonist caused only a marginal reduction of AIMs and did not modify the anti-dyskinetic effect of buspirone. Thus, a putative antagonism at the DRD3 is less likely to have contributed to buspirone effects in our paradigm. A possible caveat of our study is that we did all drug administration 30 min prior to L-DOPA based on our previous experiments with buspirone. It is possible that this is not the optimal regime for testing GR103691 efficacy. For example, for another DRD3 antagonist PG01037, the efficacy increased when administered 15 min after L-DOPA (Kumar et al., 2009).

At the molecular level, the higher dose of buspirone, that efficiently blocked AIMs, normalized pDARPP32 and pERK2 expression, indicating that it regulates the DRD1-pathway, as proposed for other 5HT1A agonists (Dupre et al., 2013). However, our molecular analyses demonstrate that while normalization of both pDARPP32 and pERK2 is necessary, it is not sufficient to block the appearance of the AIMs. This is not completely surprising given that DARPP32 knock-out mice do develop dyskinesia (Santini et al., 2007). As mentioned above, the co-treatment with WAY-100635 (1 mg/kg) blocked the anti-dyskinetic effect of buspirone (4 mg/kg). However, at the molecular level the expression of the two markers, pDARPP32 and pERK2, was not

significantly modified. On the other hand, GR103691 caused only a marginal reduction of AIMs, in spite of a robust decrease of pDARPP32 and pCREB. These data illustrate the complexity of this disorder and suggest that the normalization of the DRD1-pathway produced by buspirone could be mediated by co-activation of different pathways.

In fact, buspirone can also act as a DRD2 antagonist and, in a model of graft-induced dyskinesias, the anti-dyskinetic effect of buspirone was mimicked by eticlopride, a DRD2 antagonist (Shin et al., 2012). Nevertheless, the mechanisms operating in that model are most probably quite different because buspirone effect was not affected by either the removal of the endogenous 5HT system (presynaptic 5HT1A) or the administration of WAY-100635 (post-synaptic 5HT1A) (Shin et al., 2012).

If buspirone is acting through a negative modulation of the serotonin system it is reasonable that is more efficient in animal models than in patients, as reported (Bonifati et al., 1994; Kleedorfer et al., 1991). Like buspirone, sarizotan and other 5-HT1A receptor agonists have been shown to reduce LID in animal models, without impairing L-DOPA improvement of motor performance (Ba et al., 2007; Bibbiani et al., 2001; Dupre et al., 2007). However, in PD patients saritozan failed to improve LID (25%) and exacerbated parkinsonism (Dupre et al., 2013; Goetz et al., 2008), probably due to a concomitant impairment of 5HT neurons by the disease process (Kish et al., 2008). Indeed, in patients (and also in MPTP-treated macaques) striatal binding for the serotonin transporter was found to be reduced (Rylander et al., 2010). In contrast, a striatal serotonergic hyperinnervation has been documented in rats chronically treated with L-DOPA (Rylander et al., 2010; Shin et al., 2012). Imaging studies in dyskinetic patients would help clarify the involvement of this and other neurotransmitter systems.

In summary, while there is ample evidence of a modulatory role of 5HT on DA function and LID in the rat model, the mechanisms involved are complex and, could be partly

different from those operating in PD patients. Nevertheless, our study highlights the loss of balance between DRD1 and DRD2 signalling pathways in the striatum and demonstrates that buspirone anti-dyskinetic effect is correlated with temporal normalization of the DRD1 signalling cascade. Understanding these mechanisms at the molecular level provides the ground for the development of rational pharmacological approaches for the management of LID.

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Statement of Interest

None.

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Figure Legends

Figure 1. Characterization of hemi-parkinsonian rats with L-DOPA induced dyskinesia. Last testing session evaluation of the time course (180 min) of the AIMs after a single injection of L-DOPA showing (A) the sum of AIM score severity, (B) axial, (C) limb and (D) orolingual scores. The peak was reached between 40-80 mins. Note that animals in the sham L-DOPA and 6-OHDA saline groups did not develop any AIMs. Interestingly 6 of the 6-OHDA L-DOPA group rats did not develop any AIMs and they were classified as non-dyskinetic. In the graphs the scores of these three groups appear superimposed to the X axis (AIMS = 0). (E) Western blot representative images (upper panel) and quantification (lower panel) of TH expression showing significant reduction in all the 6-OHDA lesioned groups striatum. (F) Representative confocal images of TH immunofluorescence in the striatum showed a similar level of denervation in dyskinetic and non-dyskinetic animals. Scale bar 100µm. (G-H) Western blot representative images (upper panel) and quantification (lower panel) of Fos protein expression showing no significant differences among groups and ΔFosB/FosB ratio showing a significant increase in 6-OHDA L-DOPA dyskinetic group. Groups: sham L-DOPA (n =8), 6-OHDA saline (n = 9), 6-OHDA L-DOPA non-dyskinetic (n = 6) and 6-OHDA L-DOPA dyskinetic (n = 14). R = right, ipsilateral to sham or 6-OHDA injection; L = left, contralateral to sham or 6-OHDA injection. Data are expressed as mean ± S.E.M. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Figure 2. **Receptor expression in the striatum of hemiparkinsonian rats with and without L-DOPA induced dyskinesia.** Western blot representative images (upper panel) and quantification (lower panel) of dopamine receptor expression. There were no significant differences between groups in (A) DRD1 and (B) DRD2 expression. (C) DRD3 expression was significantly lower in non-dyskinetic animals than in dyskinetic

animals. (D) The expression of the serotonergic receptor 5HT1A was not different among groups. (E) mGLUR5 receptor expression was significantly higher in the 6-OHDA saline control group than in 6OHDA L-DOPA treated groups. Groups: sham L-DOPA (n =8), 6-OHDA saline (n = 9), 6-OHDA L-DOPA non-dyskinetic (n = 6) and 6-OHDA L-DOPA dyskinetic (n = 14). R = right, ipsilateral to 6-OHDA injection; L = left, contralateral to 6-OHDA injection. Data are expressed as mean ± S.E.M. * $p < 0.05$.

Figure 3. DRD1 and DRD2 pathways in non-dyskinetic and dyskinetic rats. Western blot representative images (upper panel) and quantification (lower panel). (A) pDARPP32/DARPP32 and (B) pERK2/ERK2 showing significantly higher ratios in dyskinetic animals than in non-dyskinetic animals. (C) Representative confocal images of immunofluorescence on coronal striatal sections showing that less neurons (labelled with NeuN, green) displayed nuclear pCREB Ser133 (red) in non-dyskinetic animals than in dyskinetic animals. In both groups the distribution of $pCREB^{+}$ neurons was broader in the right striatum (ipsilateral to the 6-OHDA lesion). Scale bar: 50µm. Downstream DRD2 we found no differences in (D) Akt phosphorylation in Ser473 (upper panel), or Thr308 (lower panel) and in (E) GSK3 phosphorylation in Tyr216 (upper panel), or Ser9 (lower panel). Groups: sham L-DOPA (n =8), 6-OHDA saline (n = 9), 6-OHDA L-DOPA non-dyskinetic (n = 6) and 6-OHDA L-DOPA dyskinetic (n = 14). R = right, ipsilateral to 6-OHDA injection; L = left, contralateral to 6-OHDA injection. Data are expressed as mean ± S.E.M. * $p < 0.05$ and ** $p < 0.01$.

Figure 4. Pharmacological effect of buspirone on AIMs. Time course of axial, limb and orolingual AIMs after a single injection of L-DOPA (empty symbols) and after its co-administration with (A) buspirone 1 mg/kg, (B) 2.5 mg/kg and (C) 4 mg/kg 30 min before L-DOPA (black symbols). (D) Dose-response effect of buspirone on AIMs. To

determine whether buspirone anti-dyskinetic is mediated by its agonist effect on 5HT1A receptors or antagonist effect on DRD3, we co-administered buspirone 4 mg/kg with 1 mg/kg of the 5HT1A antagonist, WAY-100636 and with (E) 1.5 mg/kg of the DRD3 antagonist GR103691. Empty symbols: basal AIMs, and Black symbols: AIMs after treatment (buspirone, WAY-100636, GR103691 or them combination). Points represent the mean ± S.E.M of 7-8 trials.

Figure 5. Molecular correlates of buspirone acute pharmacological effects. (A AIMs score at minute 60 after L-DOPA administration on the day of the sacrifice. Buspirone 4 mg/kg alone and in combination with GR103691 significantly reduced AIMs score while all other groups were dyskinetic at this time. (B) Western blot representative images (upper panel) and quantification (lower panel) of pDARPP32 on Thr34. Reference dotted lines mark the average levels of dyskinetic and non-dyskinetic animals (see Fig. 3A). Buspirone at 4 mg/kg, but not at 1 mg/kg, significantly reduced pDARPP32/DARPP32 ratio, even in co-administration with the 5HT1A antagonist WAY-100635 (1 mg/kg). GR103691 also reduced the ratio to levels observed in non-dyskinetic animals. (C) Western blot representative images (upper panel) and quantification (lower panel) of pERK2/ERK2 ratios. Dotted lines mark the average levels of dyskinetic and non-dyskinetic animals (see Fig. 3B). Buspirone at 4 mg/kg, but not at 1 mg/kg, significantly reduced pERK2/ERK2 ratio and this effect was not modified by co-administration of either WAY-100635 or GR10369. Groups: Buspirone 1 mg/kg (n =5), Buspirone 4 mg/kg (n = 6), Buspirone 4 mg/kg + WAY-100635 1 mg/kg (n = 5), WAY-100635 1 mg/kg (n = 3), GR103691 1.5 mg/kg (n = 5) and Buspirone 4 mg/kg + GR103691 1.5 mg/kg (n = 4). R = right, ipsilateral to sham or 6-OHDA injection; L = left, contralateral to sham or 6-OHDA injection. Data are expressed as mean ± S.E.M. * $p < 0.05$. (D) Confocal images showing pCREB (red)

expression in the lesioned striatum of animals treated with different drugs. Treatment with buspirone 4mg/kg together with the 5HT1A antagonist resulted in widespread activation of pCREB, while in animals receiving buspirone with GR103691 very few cells showed pCREB nuclear signal. The activation was more pronounced in the medial than in the lateral striatum. Scale bars: 100μm.

Table 1. Pharmacological effects on abnormal involuntary movements (AIM) scores.

Treatment	Gb ALO AIM	Limb AIM	Axial AIM	Orolingual AIM	Locomotor score
Buspirone 1mg/kg (n=6)	67 ± 11 *	78 ± 12	66 ± 11 *	41 ± 9 *	80 ± 10
Buspirone 1mg/kg + **Way-100635** 1mg/kg (n=7)	68 ± 9 *	72 ± 10 *	68 ± 9 *	63 ± 13	90 ± 10
Buspirone 1mg/kg + **GR103691** 1.5mg/kg (n=7)	59 ± 8 *	63 ± 10 *	61 ± 9 *	44 ± 21 *	72 ± 13 *
Buspirone 4mg/kg (n=9)	17 ± 3 *	28 ± 6 *	15 ± 3 *	1 ± 1 *	71 ± 11 *
Buspirone 4mg/kg + **Way-100635** 0.5mg/kg (n=8)	44 ± 11 * #	44 ± 12 *	48 ± 12 * #	36 ± 24 *	128 ± 33
Buspirone 4mg/kg + **Way-100635** 1mg/kg (n=8)	56 ± 5 * #	73 ± 8 * #	50 ± 6 * #	59 ± 14 * #	86 ± 19
Buspirone 4mg/kg + **GR103691** 1.5mg/kg (n=7)	16 ± 7 *	17 ± 7 *	15 ± 6 *	5 ± 3 *	40 ± 16 *
Way-100635 0.5mg/kg (n=8)	98 ± 14	84 ± 16	128 ± 40	65 ± 15	118 ± 32
Way-100635 1mg/kg (n=8)	98 ± 8	108 ± 11	98 ± 8	89 ± 18	89 ± 16
GR103691 1.5mg/kg (n=6)	87 ± 3 *	92 ± 4	85 ± 4 *	92 ± 6	111 ± 15

AIMs cumulative scores for the global axial, limb and orolingual (Gb ALO) and for each individual subtype. Data are expressed as % (average ± SEM) of the respective baseline score obtained in the same animals treated with L-DOPA alone (100 %) on the preceding testing session. The locomotor score is included as an indicator of pharmacological effect on motor activity.

* $p < 0.05$ vs basal in the previous testing day (paired t test).

# $p < 0.05$ buspirone 4 mg/kg + WAY-100635 0.5 mg/kg and buspirone 4 mg/kg + WAY-100635 1 mg/kg vs buspirone 4 mg/kg (unpaired t test).

Table 2. Protein expression of dyskinetic rats treated with buspirone 4 mg/kg. Western blot analysis showed that buspirone treated animals presented the same striatal protein profile as dyskinetic animals. Data are expressed as mean ± S.E.M. (n = 6)

Protein	Right/Left Ratio	Statistics
TH	0.32 ± 0.03*	$p < 0.001$ vs Sham + L-DOPA
cFos	0.92 ± 0.02	NS
ΔFosB/FosB	1.39 ± 0.06*	$p < 0.01$ vs Sham + L-DOPA $p < 0.001$ vs 6-OHDA + Saline **$p < 0.001$ vs Non-dysk**
DRD1	0.99 ± 0.10	NS
DRD2	1.16 ± 0.05	NS
DRD3	1.20 ± 0.10*	$p < 0.05$ vs 6-OHDA + Saline **$p < 0.05$ vs Non-dysk**
5HT1A	0.90 ± 0.20	NS
mGluR5	0.90 ± 0.07*	$p < 0.05$ vs 6-OHDA + Saline

Figure 1

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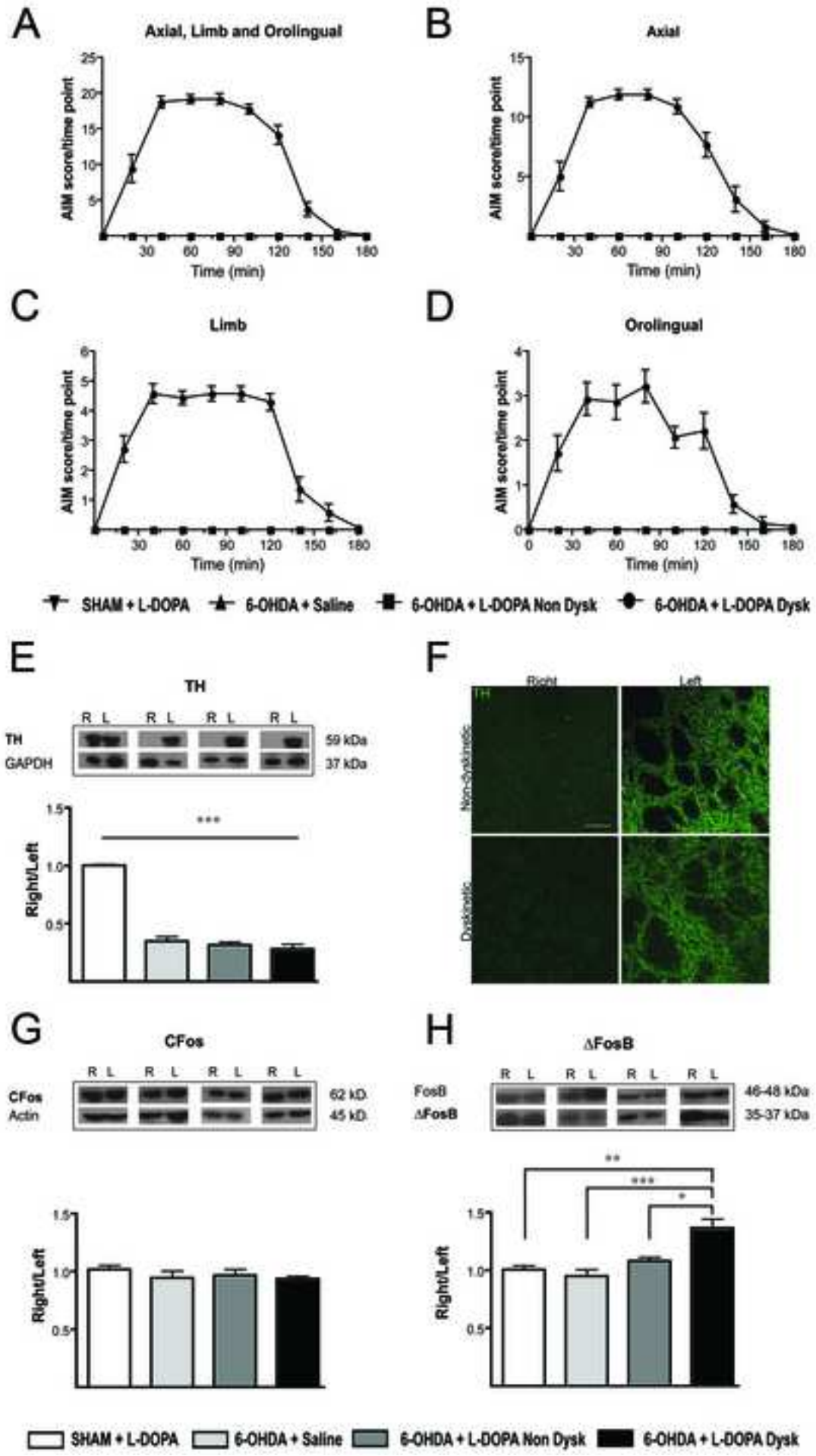

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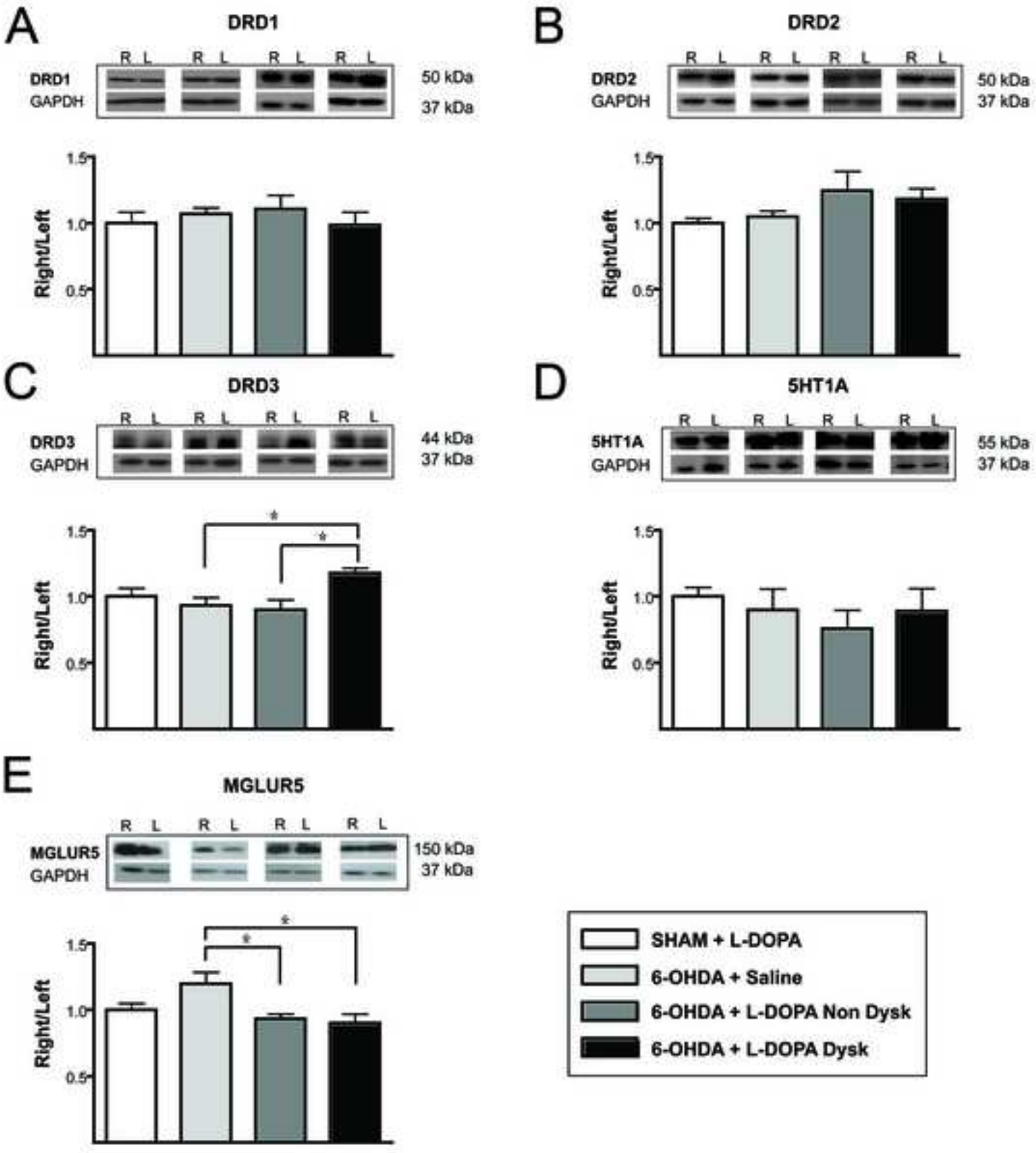

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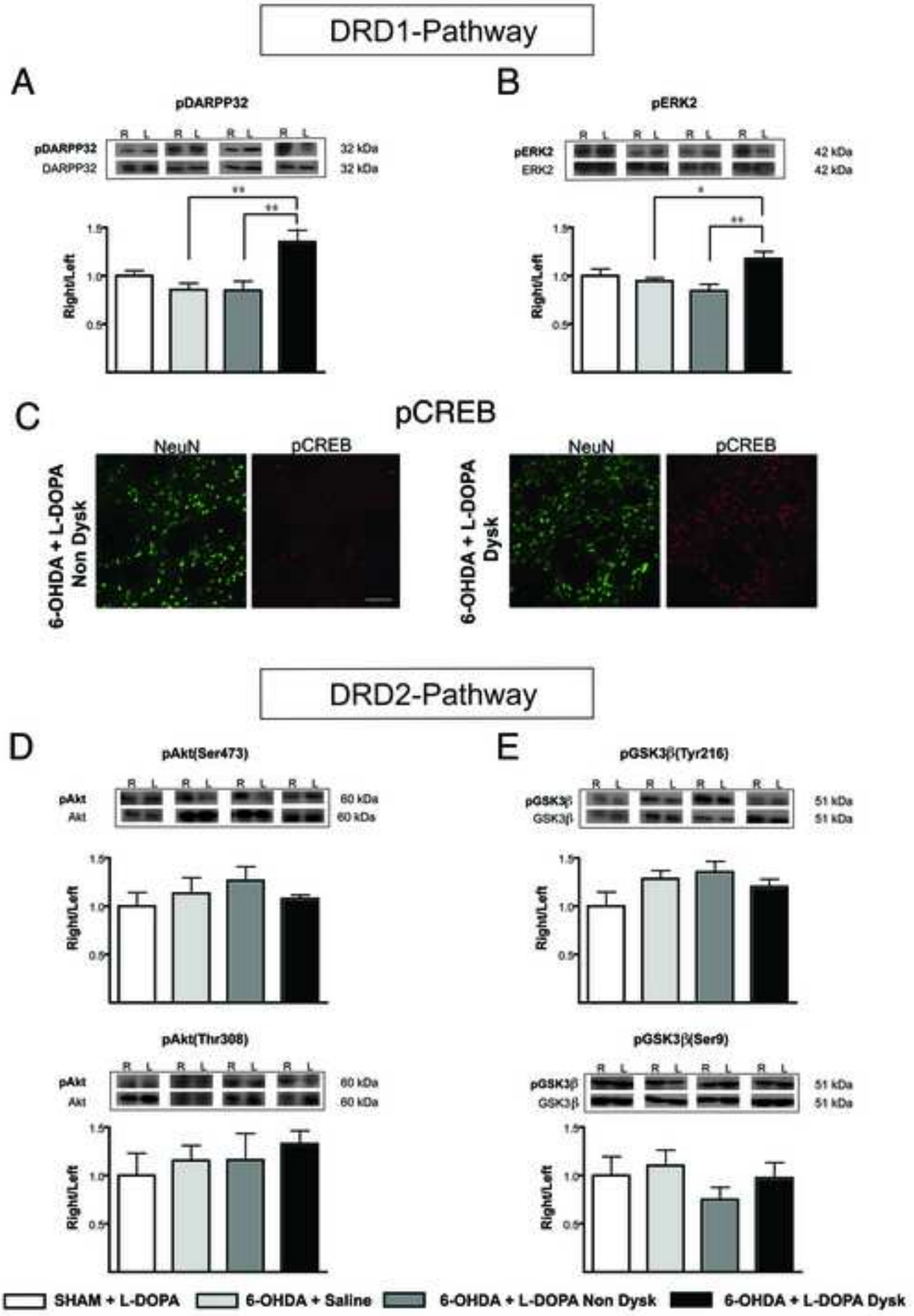

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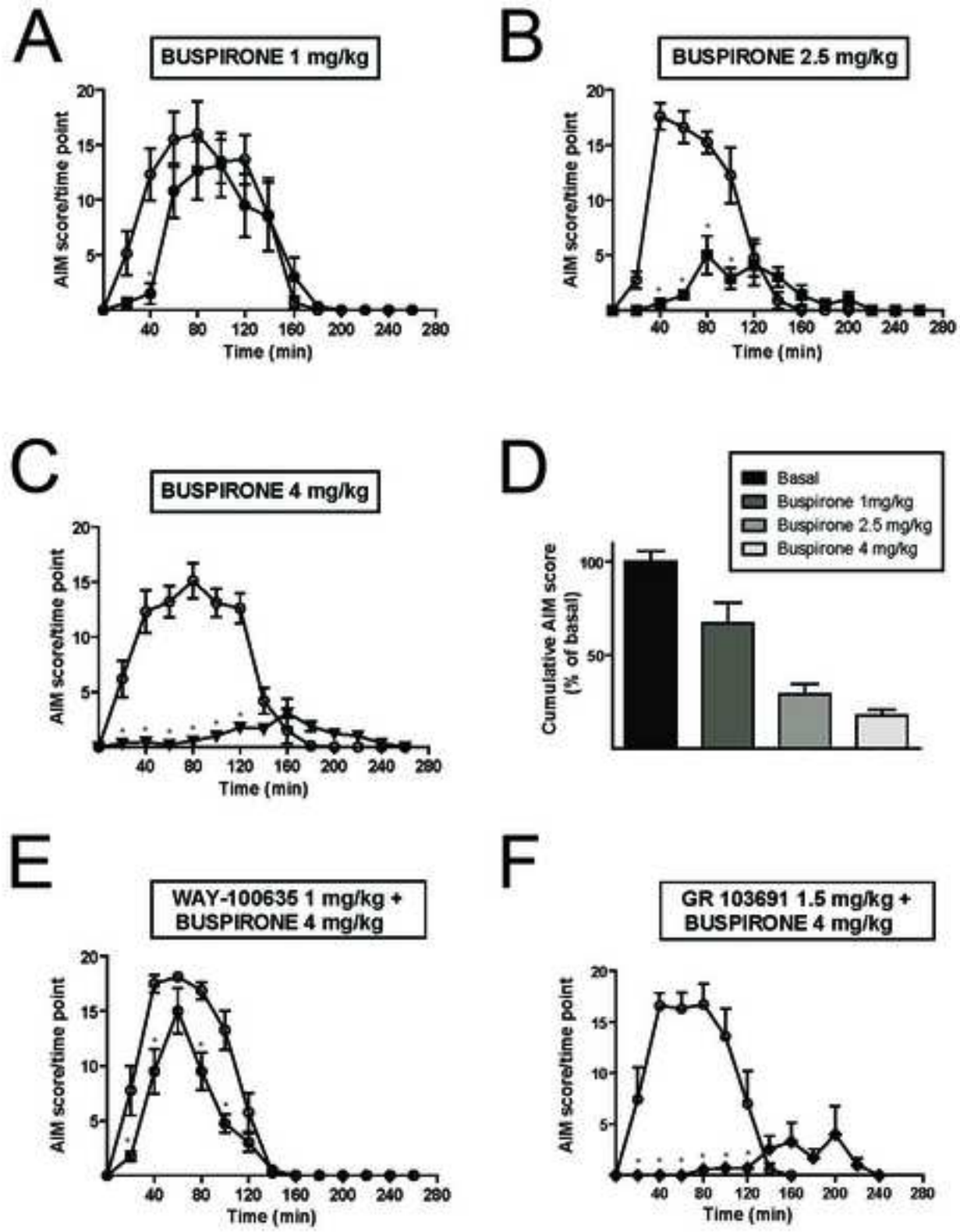

Figure 5

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