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Pharmacological antagonism of histamine H2R ameliorated L-DOPA–induced dyskinesia via normalization of GRK3 and by suppressing FosB and ERK in PD

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ABSTRACT

Parkinson's disease (PD) is often managed with L-3,4-dihydroxyphenylalanine (L-DOPA), which is still the gold standard to relieve the clinical motor symptoms of PD. However, chronic use of L-DOPA leads to significant motor complications, especially L-DOPA–induced dyskinesia (LID), which limit the therapeutic benefit. Few options are available for the pharmacological management of LID partly due to the inadequacy of our mechanistic understanding of the syndrome. We focused on the role of the histamine (HA) H2 receptor (H2R) in the striatum, which others have shown to be involved in the development of LID. We generated LID in a hemiparkinsonian mouse model and tested the signaling effects of ranitidine, an H2R antagonist. We used histidine decarboxylase deficient mice (Hdc-Ko) which lacks HA to study the role of G-protein-coupled receptor kinases (GRKs) in HA deficiency. Loss of HA in Hdc-Ko mice did not result in the downregulation of GRKs, especially GRK3 and GRK6, which were previously found to be reduced in hemiparkinsonian animal models. Ranitidine, when given along with L-DOPA, normalized the expression of GRK3 in the dopamine-depleted striatum thereby inhibiting LID in mice. The extracellular signal regulated kinase and ΔFosB signaling pathways were attenuated in the lesioned striatum when ranitidine was combined with L-DOPA than L-DOPA alone. These results demonstrate that ranitidine inhibits LID by normalizing the levels of GRK3, extracellular signal regulated kinase activation, and FosB accumulation in the dopamine-depleted striatum via HA H2R antagonism.

1. Introduction

Parkinson's disease (PD) is a neurodegenerative condition caused by the death of dopaminergic neurons in the basal ganglia. The clinical symptoms of PD are commonly managed by daily oral intake of the dopamine (DA) precursor L-3,4-dihydroxyphenylalanine (L-DOPA). Chronic intake of L-DOPA leads to the development of purposeless movements, a syndrome known as L-DOPA–induced dyskinesia (LID). LID is thought to result from the development of supersensitivity of DA receptors which belong to the super family of G-protein-coupled receptors (GPCRs) and of the downstream signaling cascades to which they are coupled (Francardo and Cenci, 2014; Gainetdinov et al., 2003; Gerfen, 2003; Prieto et al., 2011). Activation of DA receptors leads to desensitization, which are mediated by G-protein-coupled receptor kinases (GRKs) and arrestins. GRK-mediated phosphorylation of GPCRs leads to high-affinity binding by arrestins and subsequent endocytosis of activated receptors via coated pits (Gurevich and Gurevich 2006a, b; Gurevich and Gurevich, 2008; Gurevich et al., 2008; Shenoy and Lefkowitz, 2005; Thomsen et al., 2016). DA depletion and subsequent L-DOPA therapy causes longlasting changes in the expression of GRKs, arrestins, and signaling cues such as extracellular signal regulated kinase (ERK) and FosB proteins in the striatum (Ahmed et al., 2008a; Gurevich et al., 2016). In the hemiparkinsonian rodent models of PD, there is selective downregulation of GRK3 and GRK6 (Ahmed et al., 2008a, 2010, 2015b). GRK downregulation may reduce DA receptor desensitization upon agonist stimulation contributing to receptor hypersensitivity and to LID. Consistent with

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this model, virus-mediated expression of GRKs in the striatum ameliorates LID in hemiparkinsonian animal models (Ahmed et al., 2010, 2015a, 2015b).

In the striatum, chronic pulsatile DA receptor stimulation leads to alterations in cholinergic, histaminic, glutamatergic, and adrenergic function and its signaling pathways. Acetylcholine is essential to striatal functioning. The cholinergic interneurons in the striatum are modulated by DA and the striatal DA release is regulated by disynaptic pathway involving cortical and thalamic glutamatergic inputs to the cholinergic interneurons with stimulation of nicotinic acetylcholine receptors on nigrostriatal axons (Kosillo et al., 2016). Histamine H2 receptor (H2R) located within the striatum could also play an important role in LID by modulation of acetylcholine (Lim et al., 2015).

Previous studies have examined the role of histamine (HA) and its receptors (H1R-H4R) in movement disorders (Arias-Montano et al., 2001; Ferrada et al., 2008; Garcia-Ramirez et al., 2004; Moreno et al., 2011). The H2R antagonists famotidine (Johnston et al., 2010) and ranitidine (Cui et al., 2014; Shi et al., 2015) can reduce LID in animal models of PD. H2R excites cholinergic interneurons (CINs), and this effect is enhanced in LID (Lim et al., 2015); one plausible mechanism for the mitigating effect of H2R antagonists in LID models, therefore, is the downregulation of HA-regulated ERK activity in striatal CINs (Ding et al., 2011).

The neuroplastic changes that lead to LID after chronic L-DOPA therapy are not well understood (Garcia-Ramirez et al., 2004), and we examined the potential role of HA dysregulation in this process. HA modulates striatal function in numerous ways; it activates both medium spiny neurons (MSNs) and CINs via the H2R receptor and inhibits the release of GABA from striatonigral terminals and may regulate striatal DA release (Schlicker et al., 1993). We used HA-H2R antagonism as a pharmacological approach using ranitidine with L-DOPA to combat LID in mouse models. GRKs are essential in striatal signaling and play vital role in GPCR stimulation and desensitization modulating the ERK, protein kinase B (Akt), and FosB expression (Ahmed et al., 2010, 2015a, 2015b); however, there are no significant studies on GRKs in light of histaminergic stimulation related to LID. Given the fact that there is a switch in ERK activation from MSNs to cholinergic interneurons in LID (Ding et al., 2011) and the enhanced activation of HA-H2Rs in cholinergic interneurons, we hypothesized that ranitidine would effectively counteract HA H2R-mediated excitation and mitigate ERK activation, FosB accumulation by enhancing GRK expression thereby reducing LID.

2. Materials and methods

The sequence of manipulations and studies is shown schematically in Fig. 1A.

2.1. The hemiparkinsonian mouse model

C57Bl/6 mice from Jackson laboratories, age 12 weeks, were used for all studies, with the exception of histidine decarboxylase deficient mice (Hdc-Ko) studies, for which mice backcrossed extensively onto the C57Bl/6 background were bred in-house; wild-type siblings were used as controls. Adult males were housed in a Stanford University animal facility with 12/12 hour light/dark cycle and had free access to water and food. All procedures followed National Institute of Health guidelines and were approved by the Institutional Administrative Panel on Laboratory Animal Care.

Mice were anesthetized with 5% isoflurane (Isothesia, Henry Schein Animal Health, Dublin, OH) in 100% oxygen with a delivery rate of 5 L/min until loss of righting reflex and mounted on a stereotaxis. The anesthesia was maintained with 1%–1.5% isoflurane throughout the surgical procedure. Body temperature was maintained using heating pads; respiration was monitored every 10 minutes. Mice were treated with desipramine HCl (25 mg/kg i/p) 30 minutes before the infusion of 6-hydroxydopamine (6-OHDA). 2 μL (2 μg/μL) of 6-OHDA (Santa Cruz Biotechnology, Santa Cruz, CA) was unilaterally infused at the rate of 0.5 μL/min into the median forebrain bundle at coordinates AP = 1.1, ML = −1.3, DV = 5.02 from the bregma to target dopaminergic projection neurons. Animals were allowed to recover from anesthesia. Four weeks after lesion surgery, animals were tested for behavior.

2.2. Drug formulations

Gelatinized formulations of ranitidine, dihydroxyphenylalanine (DOPA), DOPA+ ranitidine were used for the studies. The peripheral DOPA decarboxylase inhibitor, benserazide, was added and spray-dried according to a standard protocol using a Buchi spray dryer. The following drug formulations were used:

(1)Ranitidine (500 mg) + benserazide (500 mg).
(2)L-DOPA (500 mg) + benserazide (500 mg).
(3)L-DOPA (500 mg) + benserazide (500 mg) + ranitidine (2 grams).

The DOPA decarboxylase inhibitor, benserazide, was added to all the drug combinations to maintain uniformity among the formulations. In the ranitidine and DOPA-alone groups, a pharmaceutical filler substance was used to compensate for the additional weight. All the formulations were then mixed with 500 mg of gelatin before the spray drying and the weights of the formulations were normalized to the recovered spray-dried powder. All the formulations were given as subcutaneous injections after dissolving in normal saline.

2.3. Behavioral studies

The cylinder test was used to study the DOPA-mediated amelioration of akinesia (Brooks and Dunnett, 2009). Mice were treated with a single dose of saline, ranitidine (20 mg/kg), L-DOPA/benserazide combination (5 and 5 mg/kg, respectively, by s/c), or the DOPA + ranitidine formulations described previously (dose equivalent to 5 mg/kg of L-DOPA and 20 mg/kg of ranitidine was used) and immediately placed in a 500 mL clean glass beaker, partially filled with bedding material. Video recording was started 2 minutes after and continued for 10 minutes. Touches of the cylinder wall with all digits clearly extended were quantified for the affected and control side. The experimenter and the analyzer of the video tapes were blind to lesion and treatment assignment. Animals were tested twice, once with ranitidine, DOPA, or DOPA + ranitidine formulation and once with saline, in counterbalanced order, with 1 week between.

AIMs were quantified after chronic DOPA administration in hemiparkinsonian mice, following previously reported methods (Ahmed et al., 2010, 2015b). In the pretesting sessions, mice were given daily injections of DOPA/benserazide (5/5 mg/Kg s/c) for 9 days; AIMs were assessed every 20 minutes for 3 hours after injection every third day of DOPA administration. Total duration of contralateral abnormal movements of the tongue-orofacial muscles and forelimb, dystonia, and locomotor behavior were scored. After pretesting, mice were separated into 4 groups with AIMs scores counterbalanced between experimental groups. After five days' rest, in the post-test phase, mice were administered saline, ranitidine (20 mg/kg), DOPA (5 mg/kg), or DOPA + ranitidine formulation (DOPA-5 mg/kg; ranitidine 20 mg/kg) daily for 15 days; AIMs were again assessed every third day. The AIMs were represented as total AIM score/session.

2.4. Sample preparation and Western blotting

After behavioral testing, mice received their assigned treatment injection, sacrificed by decapitation 45 minutes later under anesthesia. Tissue samples were processed and the Western blots were run as previously described (Ahmed et al., 2008a, 2015a, b; Bychkov et al., 2007; Bychkov, E.R. et al., 2011). Sample loading was counterbalanced across experimental groups. We used the list of antibodies as shown in Supplemental Table 1 to quantify the levels of different proteins by Western blotting.

2.5. Tissue immunohistochemistry and imaging

Immunohistochemical analysis was performed as previously described (Ahmed et al., 2008a, 2010, 2015a, 2015b; Bychkov et al., 2007; 2011a, b, 2012). For tyrosine hydroxylase (TH) immunostaining, 30 μm coronal sections were taken around both caudate putamen and substantia nigra, to examine dopaminergic denervation and neuronal cell body loss (Ahmed et al., 2008a, 2015a). For phosphorylated extracellular signal regulated kinase (pERK), choline acetyl transferase (ChAT), FoxP, and Δ FosB expression, only coronal sections at the level of striatum were used. The z stack images were taken using Zeiss laser scanning confocal microscope LSM 800 at Stanford Cell Sciences Imaging facility. The fluorescence intensity (FI) of the immunoreactivity was quantified by reported methods using NIH version of Image J software (Gangarossa et al., 2016). To quantify the signal intensity, the pictures were processed in the following way: all pictures were converted to gray scale and the background was subtracted to reduce the noise level. The cells of interest were defined by adjusting the threshold, and also by size and circularity features. The FI was quantified only in the particles with a pixel size $\geq$400 and circularity of 0.3–1. This type of analysis assures that the signal was cell specific.

2.6. Data analysis

Statistical analysis was performed using StatView statistical software (SAS Institute, Cary, NC). The AIM scores for each session were compared with the nonparametric Mann-Whitney test. The data for the cylinder test were analyzed using the Wilcoxon signed-rank test or Mann-Whitney test to evaluate the effect of L-DOPA or for group comparison, respectively. Western blots were quantified using band densitometry in Image J software (NIH version). A calibration curve was constructed for each blot from samples of known concentration and fitted using linear equations in Prism 7.0 (Graphpad Software, La Jolla, CA) as previously described, to avoid variability among different blots (Ahmed et al., 2008a, 2008b, 2010, 2011, 2015a, 2015b; Bychkov et al., 2007, 2011a, 2011b, 2012; Hanson et al., 2007). The immunohistochemical images were quantified using Image J software. The Western blot and immunohistochemical quantitation data were analyzed by repeated measures analysis of variance (ANOVA) with group/treatment as main factor and with hemisphere (intact vs. lesion) as a within-group factor. Post hoc analysis with Bonferroni/Dunn test was performed when there was a significant difference observed between the treatment groups. The value of $p < 0.05$ was considered statistically significant.

3. Results

Dysregulation of HA in PD has been suggested to contribute to pathophysiology leading to LID (Ferrada et al., 2008; Garcia-Ramirez et al., 2004; Johnston et al., 2010; Lim et al., 2015). We hypothesized that LID could be reduced with HA-H2R antagonism by GRK-mediated regulation of the signaling cues. We tested the amelioration of LID using ranitidine by behavioral and biochemical studies.

3.1. Behavioral testing to study relief of akinesia and dyskinesia

Akinesia in 6-OHDA lesion model was tested by cylinder test using DOPA, ranitidine, and DOPA + ranitidine combinations. The number of paw touches of the normal and impacted paw to support the full body weight was counted in a blinded manner. The contralateral paw usage with ranitidine alone was similar to the saline treatment, whereas both DOPA and DOPA + ranitidine combination treatment produced significantly increased contralateral paw usage (saline vs. DOPA, $p < 0.001$ in DOPA and DOPA + ranitidine groups, respectively, as measured by Wilcoxon rank nonparametric test), compared to saline or ranitidine alone treatments. The cylinder test suggests that DOPA treatments either alone or in combination with ranitidine was able to relieve the akinesia seen in hemiparkinsonian mice (Fig. 1B) and ranitidine by itself did not affect the relief of akinesia without DOPA. DOPA and DOPA + ranitidine formulations did not differ in their ability to increase contralateral paw usage.

Dyskinetic behavior was quantified using AIMs every third day after drug treatments in pretesting and post-testing sessions. Total AIMs are represented as cumulative scores per session (Fig. 1C) for all the 4 groups tested. In the pretesting session, the mice belonging to all the 4 groups were treated with DOPA over the course of 9 days to establish the baseline AIMs for group separation. After 9 days of DOPA/benserazide treatments, mice were separated into four groups, balanced for AIMs as previously described (Ahmed et al., 2010, 2015b). In the post-testing sessions, mice received daily subcutaneous injections of saline, ranitidine alone, DOPA alone, and DOPA + ranitidine as a combined formulation dissolved in saline. The saline and ranitidine alone groups did not show any AIMs without the DOPA in the post-testing sessions while daily DOPA injections produced increasing AIMs and coadministration of ranitidine with DOPA significantly attenuated AIMs across all the sessions tested except post-testing session 3 (Fig. 1C) ($p = 0.0061$, 0.0238, 0.0517, 0.0205, and 0.0086, respectively, for post-testing sessions 1 through 5 per Mann-Whitney rank nonparametric test). The total AIMs were further analyzed into ALO-AIMs (Fig. 1D) and locomotor AIMs (Fig. 1E). The ALO-AIMs showed that the DOPA + ranitidine combination treatment had significantly reduced ALO-AIM scores than the animals that received DOPA alone ($p = 0.0098$, 0.0272, 0.0454, 0.0134, and 0.0086, respectively, for post-testing sessions 1 through 5 per Mann-Whitney rank nonparametric test). The trend of locomotor AIMs mirrored the effect seen with the axial limb orofacial abnormal involuntary movement (ALO AIM) behavior with DOPA + ranitidine combination treatment having decreased AIM behavior than the DOPA alone treatment across the testing sessions ($p = 0.0286$, 0.011, 0.0439, 0.1128, and 0.026, respectively, for post-testing sessions 1 through 5 per Mann-Whitney rank nonparametric test). We were then interested in the dynamics of the AIMs during the testing period and plotted the ALO-AIMs over 20 minutes bin for post-testing sessions 1 and 5 to see the dyskinetic behavior. As anticipated, the DOPA-alone group without ranitidine addition had higher ALO-AIMs in both the testing sessions and the DOPA + ranitidine combination treatment having lesser AIM scores and earlier attenuation times during post-testing session 1 with significant changes seen at 60, 80, and 120 minutes bin intervals ($p = 0.0069$, 0.0355, and 0.0055, respectively) (Fig. 1F–G). In the post-testing session 5, the attenuation times for the DOPA + ranitidine combination treatment shifts to the right as we could see significant reductions in ALO-AIM scores only between 100 ($p = 0.0127$) and 120 minutes ($p =$

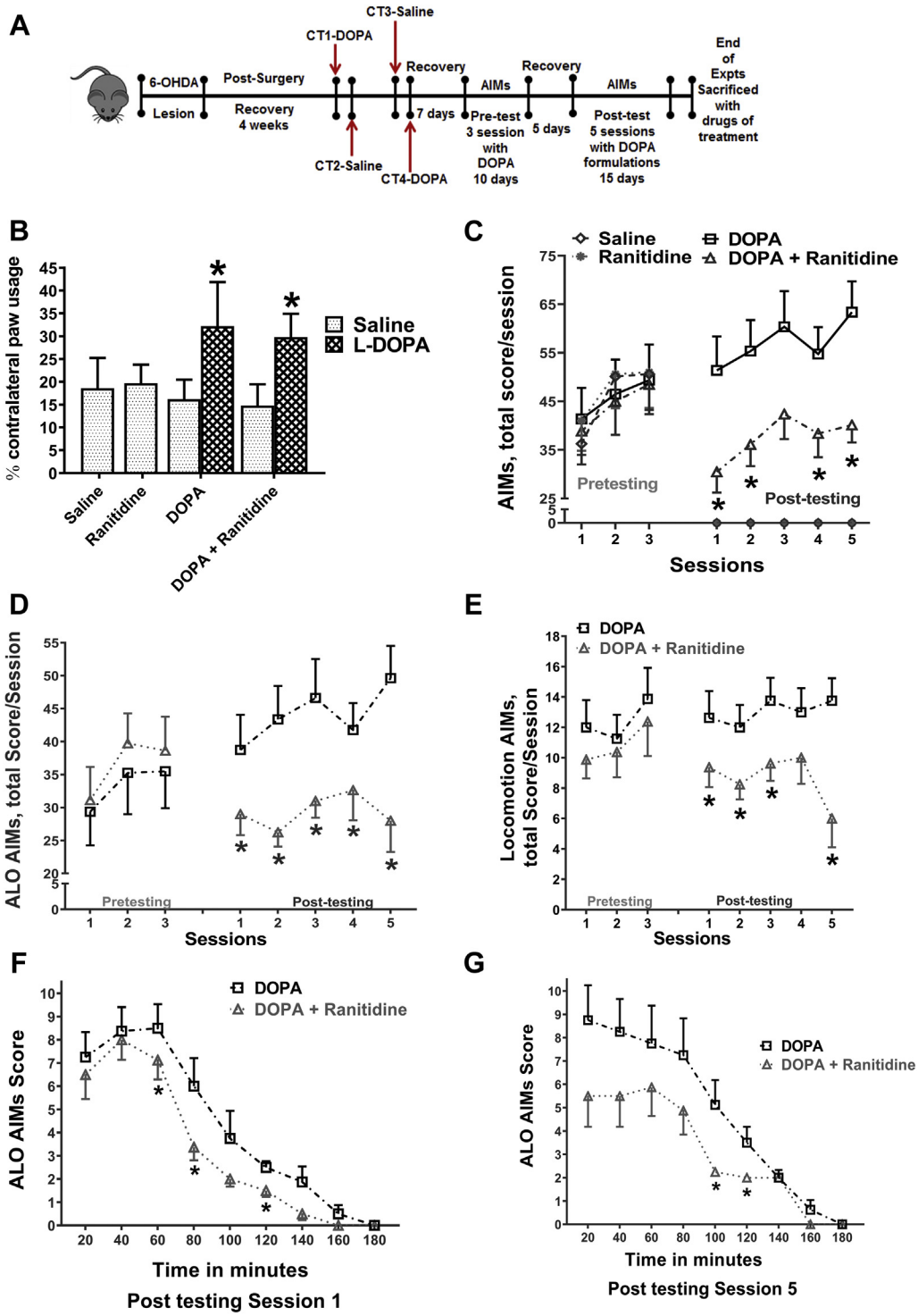

Fig. 1. (A) Sequence of experimental setup for the study paradigm in mice. CT denotes cylinder test, AIMs denote abnormal involuntary movements. (B) Usage of the paw contralateral to the 6-OHDA lesion in hemiparkinsonian mice with saline, ranitidine, DOPA, and DOPA + ranitidine combination treatments. DOPA and DOPA + ranitidine combination treatments showed significantly increased contralateral paw usage relative to saline and ranitidine alone treatments showing relief from akinesia rendered due to Parkinsonian defect. The difference in the improvement of akinesia between saline or ranitidine and DOPA, DOPA + ranitidine combination treatments was statistically significant ($^*p < 0.0001$) as measured by Wilcoxon rank nonparametric testing. (C) Abnormal involuntary movement studies (AIMs) in mice after DOPA pretesting and post-testing with DOPA + ranitidine combination treatments. AIMs are the measurement of total score comprising the orolingual-forelimb dyskinesia (involuntary contralateral movements of the tongue and orofacial muscles), axial dystonia (twisting movements of the body), and locomotor/rotational behavior. The animals were given a score based on the duration of the dyskinetic behavior for a period of 3 hours with 20-minute interval between the scoring. During pretesting, mice were administered with DOPA/benzaseride (5 mg/kg) for 9 days; AIMs were quantified every third day (total 3 sessions) and were significantly increased over this period. Mice were then divided into 4 groups (n = 8 per group) exhibiting equivalent AIMs at the end of the pretesting period and were rested for one week. Then, in the post-testing sessions, mice were injected daily with saline, ranitidine, DOPA, or DOPA + ranitidine combination treatments for 15 days (totaling 5 sessions with 3-day interval between each session for AIM scoring). In the post-testing session, treatment with DOPA + ranitidine combination significantly reduced the total AIMs score than the DOPA-alone treatment group as analyzed by Mann-Whitney rank nonparametric test ($^*p = 0.0061$, 0.0238, 0.0517, 0.0205, and 0.0086, respectively, for sessions 1 through 5. Note that the AIMs were lower on session 3, but the significance was lost for this session).

0.0204) (Fig. 1G) after which the dyskinetic behavior was similar in both the groups.

3.2. Effect of ranitidine and HA deficiency on the levels of GRKs

The confirmation of DA depletion in hemiparkinsonian mice was determined by tyrosine hydroxylase analysis by Western blotting and immunohistochemistry in the intact and the lesioned hemispheres (Supplemental Fig. 1A, B, respectively). Previous studies have reported minimal to no damage with the vehicle injection compared to the 6-OHDA–injected mice (Zurkovsky et al., 2013) and in the unilateral rat models where the unilateral lesion does not affect the biochemical pathways on the contralateral intact side (Ahmed et al., 2008a; Bychkov et al., 2007). For GRK quantification, intact and lesioned hemisphere samples were run on the same blot for the 3 groups, ranitidine and DOPA-alone groups, and DOPA + ranitidine–treated animals (Fig. 2A). We represented the expression of GRK protein according to a previously described methodology (Ahmed et al., 2008a). The levels of GRK expression between the intact and lesioned hemispheres were normalized to the expression levels of actin and the differences were plotted as a percentage expression of the intact side (Fig. 2A). The graphical representation for the quantitation of GRK2, GRK3, GRK5, and GRK6 is shown in Supplemental Fig. 1C–F. No changes were seen in GRK2 expression between the intact and the lesioned hemispheres; however, the effect of treatment could be seen with DOPA + ranitidine combination treatment having generally lower expression of GRK2 in both the intact and the lesioned hemisphere than ranitidine or DOPA-alone treatment groups (Supplemental Fig. 1C). Fig. 2A shows that GRK3 was significantly reduced in the lesioned hemisphere in the DOPA-alone group, as previously reported; but the expression was normalized after treatment with ranitidine or DOPA + ranitidine combination treatment ($F(2,21) = 4.895$, $p = 0.018$ by one-way ANOVA with treatment as the between-group factor). The post hoc Bonferroni/Dunn analysis of the percent decrease in GRK3 in the lesioned hemisphere was significantly attenuated in the ranitidine ($p < 0.0145$) and DOPA + ranitidine group ($p < 0.0133$) relative to the L-DOPA alone–treated group. The lesioned side had slightly higher expression of GRK5 in all the experimental groups (Fig. 2A) without any statistical difference between the groups. The expression of GRK6 has been variable (Ahmed et al., 2008a, 2010, 2015a, Bezard et al., 2005). We observed only a marginal degree of downregulation of GRK6 in the DOPA-alone and DOPA + ranitidine combination group in the lesioned hemisphere than the intact side (Fig. 2A) unlike the differences seen with rat hemiparkinsonian studies. Ranitidine treatment alone increased the expression of GRK6 in the lesioned side ($F(2,21) = 3.925$, $p = 0.0349$), although the difference between the groups were not statistically significant and didn't survive the Bonferroni/Dunn post hoc analysis. The representative Western blots for the expression of GRKs and β-actin loading control in the drug-treated groups are shown in Fig. 2B.

We then were interested in studying the effect of histamine depletion as in the Hdc-Ko mice along with dual loss of dopamine (6-OHDA injection into median forebrain bundle) on the expression profiles of GRKs. We found that GRK3 and GRK6 were decreased in the lesioned side of wild-type (WT) littermates in line with the previous rat hemiparkinsonian studies, and this loss was not reflected in the Hdc-Ko mice in the lesioned hemisphere. Similar to the ranitidine- and DOPA-treated cohorts, we looked at the % expression in the lesioned side from that of the intact side and found that both GRK3 and GRK6 were significantly increased in the lesioned hemispheres of the Hdc-Ko mice compared to the WT littermates according to one-way ANOVA with Bonferroni/Dunn post hoc analysis with genotype as the between-group factor ($p < 0.0127$ and 0.0156 for GRK3 and GRK6, respectively, Fig. 2C). GRK2 was decreased on the lesioned side than the intact hemisphere in both Hdc-Ko and their WT littermates, whereas GRK5 was increased slightly mirroring the effect seen with ranitidine or DOPA alone and with DOPA + ranitidine treatments. The expression profile of GRKs and β-actin loading control for treatment groups, Hdc-Ko mice, and WT littermates is shown in Fig. 2D. The graphical representation for the quantitation of GRK2, GRK3, GRK5, and GRK6 in the Hdc-Ko and their WT littermates are shown in Supplemental Fig. 2A–D.

3.3. Signaling changes after HA-H2R antagonism in LID

The percentage expression of the activated mitogen-activated protein kinase pathway proteins ERK, p38, and stress activated protein kinase (SAPK)/Jun amino terminal kinase (JNK) is shown in Fig. 3A, and the respective immunoblots along with β-actin loading controls are shown in Fig. 3B. The quantitation of the mitogen-activated protein kinase pathway proteins for both activated and total proteins is shown in Supplemental Fig. 3A–F. The levels of expression of the activated Akt proteins as a percentage expression to the intact hemisphere are shown in Fig. 3C, and the respective immunoblots for activated Akt along with β-actin loading controls are shown in Fig. 3D. Acute DOPA treatment results in supersensitive ERK responsivity; this is deactivated by chronic DOPA treatment (Ahmed et al., 2015a; Ding et al., 2011; Gerfen, 2003; Lim et al., 2015; Moreno et al., 2011). ERK was activated by chronic treatments with ranitidine or DOPA alone and with DOPA + ranitidine combination in the lesioned hemisphere than the intact side (Fig. 3A). One-way ANOVA with treatments as between-group factor did not yield statistical significance ($F(2,21) = 2.54$, $p = 0.1024$), but when we analyzed the data by two-way ANOVA using treatment as the between-group factor and hemisphere (intact vs. lesioned) as with in group factors we found statistical significance ($F(2,21) = 44.928$, $p < 0.0001$ for hemisphere and $F = 3.8$, $p = 0.039$ for treatment vs. hemisphere). The Bonferroni/Dunn post hoc analysis showed significant difference between the DOPA alone and DOPA + ranitidine treatment groups ($p = 0.0093$). There was no difference between ranitidine-alone and DOPA-alone groups or

(D) Shows the ALO AIMs scores for the pretesting and post-testing sessions for DOPA and DOPA + ranitidine groups. In the post-testing session, DOPA-alone group had significantly higher ALO AIMs score than the DOPA + ranitidine combination treatment group (*$p = 0.0098$, 0.0272, 0.0454, 0.0134, and 0.0086, respectively, for sessions 1 through 5 as per Mann-Whitney nonparametric analysis). The data for the ALO AIMs containing (orolingual, forelimb, and axial dystonia) were separated from the locomotion or rotational AIMs to have a better understanding. (E) Locomotion AIMs in the pretesting and post-testing session for all the experimental groups are represented here. The locomotion behavior mirrored the changes seen with the ALO AIMs between DOPA alone and DOPA + ranitidine combination treatment group. The locomotion behavior more or less mirrored the ALO-AIMs behavior except for post-testing session 4 which was not significant between the 2 groups tested (*$p = 0.0286$, 0.011, 0.0439, 0.1128, and 0.026, respectively, for sessions 1 through 5 as per Mann-Whitney nonparametric analysis). (F) Representation of ALO AIMs in 20 minutes bin during the post-testing session 1 for the 2 groups tested. The 20 minutes bin showed significant difference during the 60, 80, and 120-minute time points between the DOPA alone and DOPA + ranitidine combination treatment groups (*$p = 0.0069$, 0.0355, and 0.0055, respectively, for the denoted time points per Mann-Whitney rank nonparametric testing). (G) Representation of ALO AIMs in 20 minute bin during the post-testing session 5 for the 2 groups tested. In the post-testing session 5, the significance in the ALO AIMs between the DOPA alone and the DOPA + ranitidine combination treatment group shifted to the right. DOPA + ranitidine combination treatment group had significantly less ALO AIMs scores in the 100- and 120-minute time points than DOPA-alone group, *$p = 0.0127$ and $p = 0.0204$, respectively. Abbreviations: 6-OHDA, 6-hydroxydopamine; DOPA, dihydroxyphenylalanine.

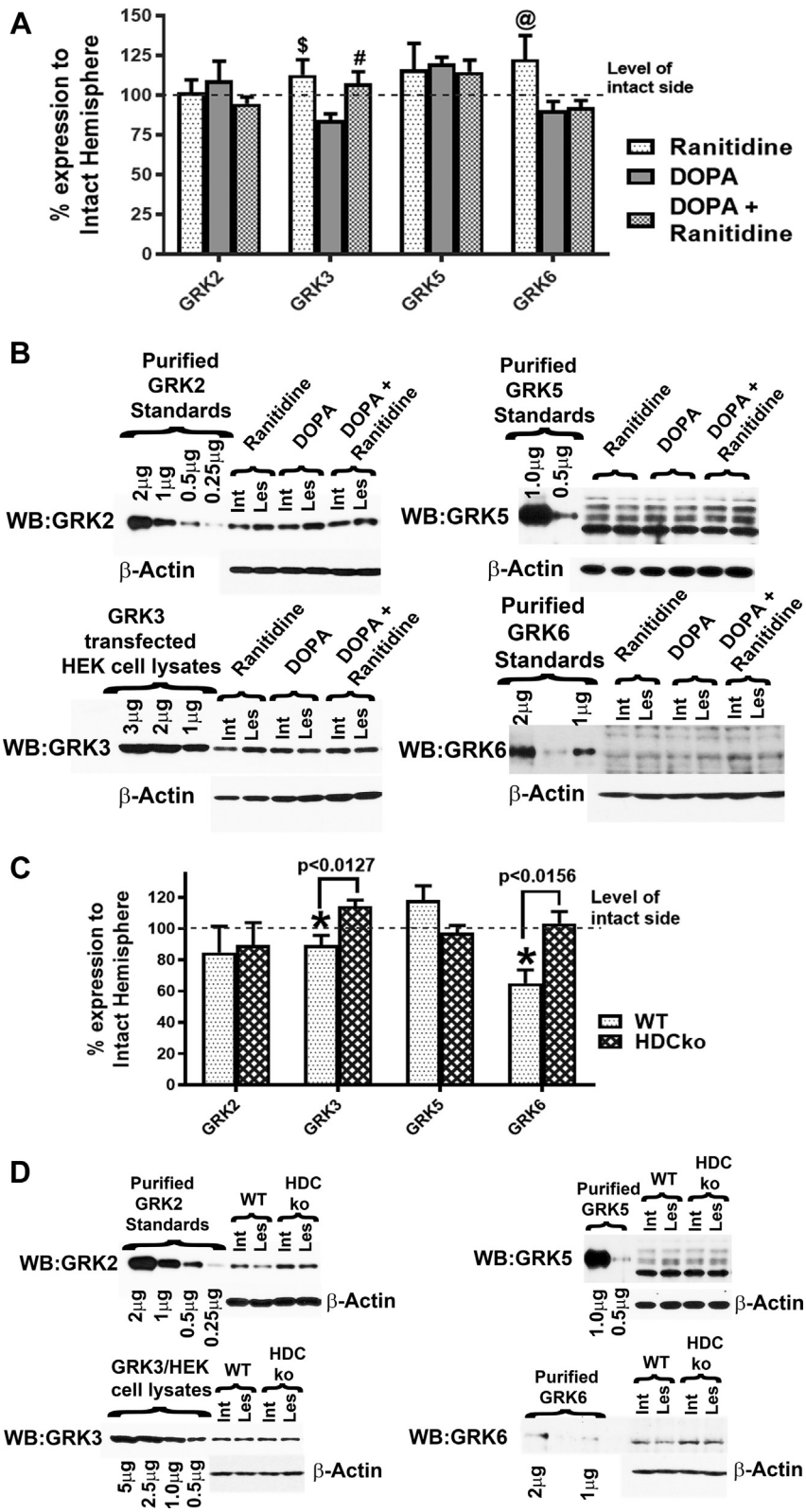

Fig. 2. (A) Shows the % expression of GRK levels in the lesioned hemisphere, relative to the intact hemisphere which is represented at 100% (dotted line). All the GRK proteins were quantified by previously reported methods after normalization of the proteins to β-actin levels. GRK3 expression was significantly reduced in the lesioned hemisphere in 6-OHDA–treated mice treated with DOPA; this effect was reversed in the groups treated with ranitidine or with DOPA + ranitidine combination treatments ($F(2,21) = 4.895$, $p = 0.018$ by one-way ANOVA with treatment as between-group factor) ($ represents statistical difference between ranitidine vs. DOPA, $p = 0.0145$ and # represents statistical difference between DOPA and DOPA + ranitidine $p = 0.0133$ by Bonferroni/Dunn post hoc analysis). Ranitidine alone increased the expression of GRK6 in the lesioned hemisphere (@ represents statistical difference between ranitidine versus DOPA, $p < 0.0173$ [NS], ranitidine versus DOPA + ranitidine, $p < 0.0199$ [NS]). (B) Western blotting images of GRKs along with

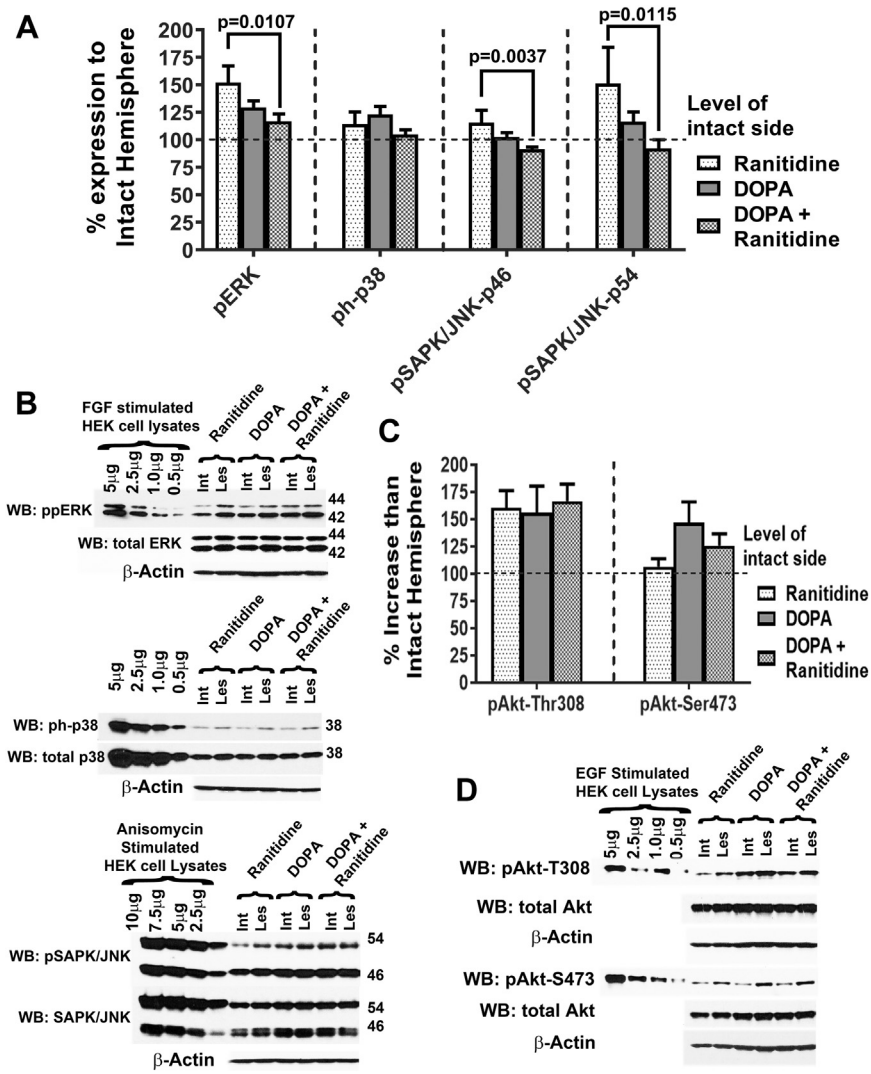

Fig. 3. (A) Shows the quantitation of the activated MAPK proteins such as ERK, p38, SAPK/JNK (p46, p54) in the lesioned hemispheres of the experimental groups. Data are shown as % expression to intact hemisphere (dotted line). All the MAPK proteins were quantified by previously reported methods after normalization of the proteins to their respective total proteins and β-actin levels. All MAPK pathway proteins were found to be increased in the lesioned hemisphere than the intact side with DOPA + ranitidine combination treatments normalizing most of the MAPK pathway proteins. The percentage increase in the expression of the MAPK pathway proteins was analyzed by one-way ANOVA with treatment as between-group factor and were subjected to Bonferroni/Dunn post hoc analysis when we observed a significant difference with a $p < 0.05$. (B) Western blotting images of MAPK pathway proteins in the treatment groups along with β-actin loading controls. (C) Akt signaling in the experimental groups at the major (T308) and minor (S473) phosphorylation sites in the experimental groups. Data are shown as % increase than intact hemisphere (dotted line) after normalization to β-actin levels. Akt phosphorylation at Thr308 was elevated in the lesioned striatum after DOPA treatment, with or without ranitidine. Lesion-induced increases in the levels of Akt-Ser473 were not seen with ranitidine treatment suggesting that only DOPA was able to induce changes in Akt-Ser473 activation and ranitidine tends to diminish this effect. (D) Western blotting images of Akt phosphorylation at Thr308 and Ser473 along with total Akt and β-actin loading controls are shown. Abbreviations: ANOVA, analysis of variance; MAPK, mitogen-activated protein kinase; DOPA, dihydroxyphenylalanine; ERK, extracellular signal regulated kinase; JNK, Jun amino terminal kinase; SAPK, stress activated protein kinase; Akt, protein kinase B; Ser473, serine 473 site of Akt/protein kinase B.

between ranitidine-alone and DOPA + ranitidine combination treatment groups suggesting that ranitidine when given along with DOPA reduced the activation of ERK, whereas ranitidine or L-DOPA alone could not.

Two-way ANOVA with treatment as between-group factor and hemisphere as within-group factors yielded difference only for the hemisphere and not for the treatment for p38 ($F(2,21) = 2.216$, $p = 0.1339$ for treatment and $F = 9.394$, $p = 0.0059$ for the hemisphere) suggesting that there was increased activation of p38 in the lesioned hemisphere than the intact side. The activation of p38 was slightly upregulated in the lesioned hemisphere of the DOPA-alone group but no difference was seen when we compared the treatment versus hemisphere for the groups ($F(2,21) = 2.692$, $p = 0.091$) (Fig. 3A).

The % expression of pSAPK/JNK is shown in Fig. 3A. The levels of p46 and p54 expression were significantly lower in the ranitidine

β-actin loading controls. (C) GRK protein levels in hemiparkinsonian WT and Hdc-Ko mice showing the % change in GRK protein, relative to the intact hemisphere; GRK3 and GRK6 were reduced in the lesioned hemisphere of WT mice but not of *Hdc*-Ko mice (WT vs. *Hdc*-KO: $F = 12.33$, $N = 4$, $^*p = 0.0127$ and 0.0156 for GRK3 and $F = 11.15$, $N = 4$, $p = 0.0156$ for GRK6, respectively, by one-way ANOVA with genotype as between-group factor). (D) Western blotting images of GRKs along with β-actin loading controls in WT littermates and *Hdc*-Ko mice. Abbreviations: 6-OHDA, 6-hydroxydopamine; DOPA, dihydroxyphenylalanine; ANOVA, analysis of variance; GRK, G-protein-coupled receptor kinase; Hdc-Ko, histidine decarboxylase deficient mice; WT, wild-type.

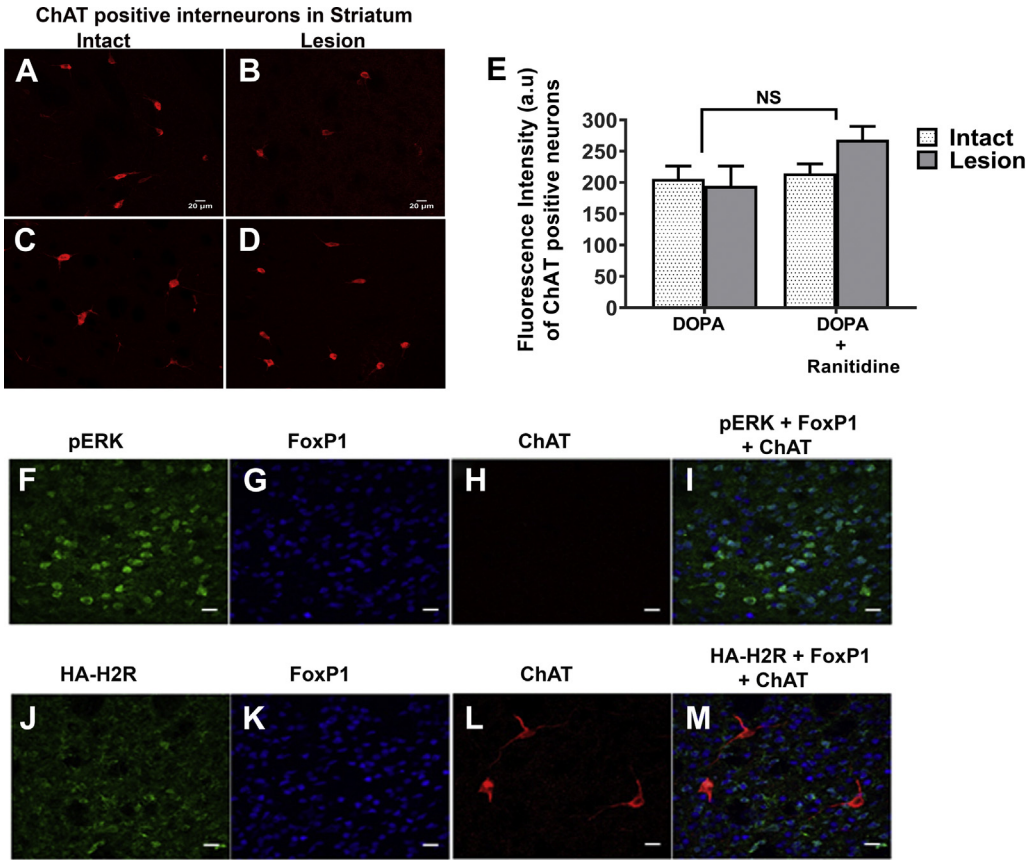

Fig. 4. (A–D) Immunohistochemical staining for ChAT-positive neurons in the striatum of DOPA-treated animals. Photomicrographs in A and B indicate the ChAT-positive neurons in the intact and the lesioned hemispheres of DOPA treatment group and the photomicrographs in the panels C and D indicate ChAT-positive neurons in the intact and the lesioned hemispheres of DOPA + ranitidine treatment groups, respectively. (E) Graphical representation of the quantification of the fluorescence intensity (FI) in the intact and lesioned sides of the experimental groups. The FI of ChAT-positive neurons were more or less similar in both the experimental groups. (F–I) Immunohistochemical staining for pERK, ChAT, and FoxP1 in the lesioned striatum to show the expression of activated ERK signal in FoxP1-positive MSNs and ChAT-positive interneurons in DOPA-treated hemiparkinsonian mice. FoxP1 is a neuronal marker for the MSNs. pERK (green—F), FoxP1 (blue—G), and ChAT (red—H) panel I shows the triple staining. (J–M) Immunohistochemical staining for HAH2R, ChAT, and FoxP1 in the lesioned striatum to show the expression of HAH2R in FoxP1-positive MSNs and ChAT-positive interneurons in DOPA-treated hemiparkinsonian mice. HAH2R (green—J), FoxP1 (blue—K), and ChAT (red—L) panel M shows the triple staining. pERK and H2R was colocalized in FoxP1-positive MSNs (panel I, M); however, FoxP1 staining was not seen in ChAT-positive large cholinergic interneurons (panel M). Abbreviations: ChAT, choline acetyl transferase; DOPA, dihydroxyphenylalanine; ERK, extracellular signal regulated kinase; pERK, phosphorylated extracellular signal regulated kinase; MSNs, medium spiny neurons. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

alone group than the DOPA alone or DOPA + ranitidine treatment groups ($F = 5.257$, $p = 0.0141$; $F = 4.531$, $p = 0.0231$) by one-way ANOVA with treatment as the factor for p46 and p54, respectively. There was no difference between the hemispheres between the groups as far as the p46 and p54 expression was concerned. The expression was generally lower with ranitidine alone and only DOPA activated SAPK/JNK proteins and ranitidine had minimal effect in activating the SAPK/JNK proteins. The SAPK/JNK activation of both p46 and p54 were slightly higher in the ranitidine group which could be due to the effect of lesioning and DOPA counteracted this effect.

Supersensitive Akt response has been implicated in dyskinesia in animal models of PD (Ahmed et al., 2015a; Bychkov et al., 2007) in particular at the phosphorylation site threonine 308 site of Akt/protein kinase B. The difference in the activation of Akt at threonine 308 site of Akt/protein kinase B between the intact and the lesioned side was significant in all the treatment groups ($F(2,21) = 18.516$, $p = 0.003$); however, no between-group differences were observed ($F(2,21) = 2.929$, $p = 0.0755$) by two-way ANOVA (Fig. 3C, Supplemental Fig. 4A). There was no difference seen with the activation of Akt serine 473 site of Akt/protein kinase B between the experimental groups ($F = 2.241$, $p = 0.1312$), although the lesion-induced changes ($F = 7.725$, $p = 0.0112$) were preserved in the DOPA-treated groups but not with the ranitidine-alone group. The quantitation of activated and total Akt is shown in Supplemental Fig. 4A–B.

3.4. Activation of ERK and upregulation of ΔFosB in the lesioned striatum

Activation of ERK was lower with DOPA + ranitidine combination than DOPA or ranitidine alone treatments as shown by Western blots. ERK is expressed in both the MSNs and the large ChAT-positive cholinergic interneurons (CINs) and we were interested in studying which cell type contributed to the increased ERK activation in the lesioned striatum with the drug treatments. We used the immunohistochemical approach to study the pattern of activated ERK expression. Previous studies have suggested that the development of dyskinesia corresponds to a switch in the activation of ERK from MSNs to CINs (Ding et al., 2011). First, we quantified the ChAT immunoreactivity of both the intact and lesioned striatum, which were similar in the intact and lesioned side in the DOPA-treated and DOPA + ranitidine–treated animals (Fig. 4A–E). In the DOPA + ranitidine combination treatment, the FI was higher in the lesioned side than the intact side but was not statistically significant and thus the ChAT expression was more or less similar with

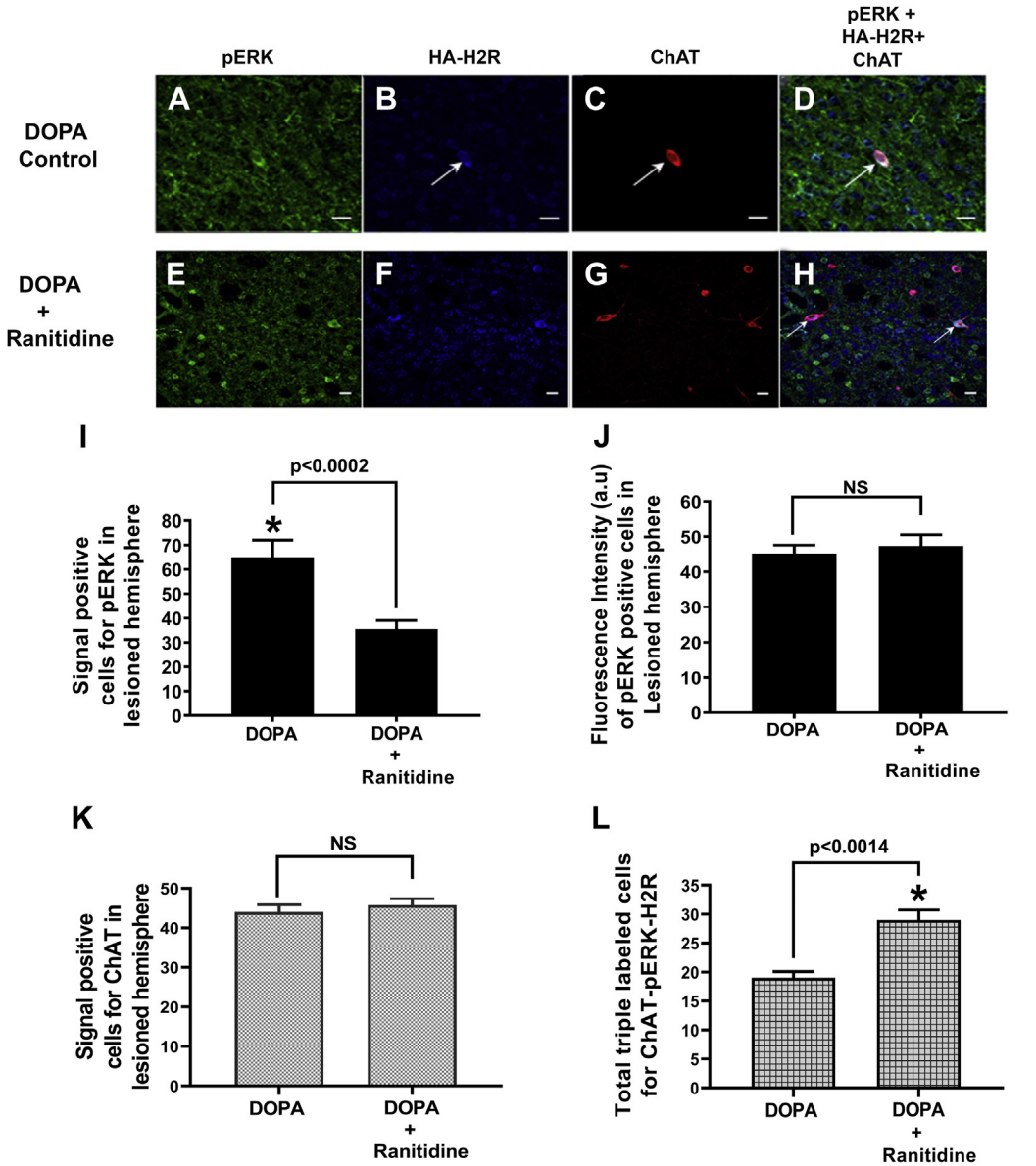

Fig. 5. (A–H) Immunohistochemical colocalization of pERK, HAH2R in ChAT-positive interneurons with DOPA and DOPA + ranitidine combination treatment groups of hemiparkinsonian mice. pERK (green A and E), H2R (blue B and F), and ChAT (red C and G) signal in the striatum of the lesioned side. More pERK colocalization in HAH2R-positive ChAT (panel H) cholinergic interneurons could be seen in the DOPA + ranitidine combination treatment group. Arrow indicates colocalization. Scale bar = 20 μm. (I) Graphical representation of activated ERK (pERK)–positive cells in the lesioned side in the experimental groups. The number of activated ERK-positive cells increased statistically in the lesioned side of the DOPA-treated animals than DOPA + ranitidine combination treatment group ($p < 0.0002$). (J) Graphical representation of the fluorescent intensity of pERK signal–positive cells in the lesioned side in the experimental groups is shown. The individual cell fluorescent intensity for pERK signal did not differ between DOPA alone treatment or with DOPA + ranitidine combination treatments. (K) Graphical representation of ChAT-positive cells in the lesioned side in the experimental groups. The number of ChAT-positive cells was similar in the lesioned side of the DOPA and DOPA + ranitidine–treated animals. The total ChAT-positive cells were quantified to study the coexpression of activated ERK (pERK), HA-H2R in CINs. (L) Graphical representation of the cells showing triple labeling for pERK, HA-H2R, and ChAT in the lesioned side of the experimental groups. The number of CINs showing HA-H2R and activated ERK increased statistically in the lesioned side of the DOPA + ranitidine–treated animals than DOPA treatment group ($p < 0.0014$). Abbreviations: ChAT, choline acetyl transferase; CINs, cholinergic interneurons; DOPA, dihydroxyphenylalanine; pERK, phosphorylated extracellular signal regulated kinase; ERK, extracellular signal regulated kinase. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

DOPA-alone treatment or in combination with ranitidine. We used neuronal markers such as FoxP1 to study the coexpression of activated ERK (pERK) and HA-H2R in MSNs (Fig. 4F–M). Fig. 4F–I shows the localization of pERK in MSNs. HA-H2R (Fig. 4J–M) was also seen colocalized in FoxP1-positive cells indicating that both pERK and HA-H2R are expressed in the MSNs after dopaminergic stimulation of the lesioned striatum. We then studied the colocalization of pERK and HA-H2R signaling in the neurons and analyzed the degree of coexpression of both ERK and HA-H2R (Fig. 5A–J). We did see occasional triple labeling of pERK, HA-H2R, and ChAT in the DOPA alone–treated group (Fig. 5D); this was more prominent in mice treated with DOPA + ranitidine combination treatment (Fig. 5H). There was striking increase in the number of pERK-positive cells in the lesioned hemisphere of the DOPA alone–treated animals than the DOPA + ranitidine combination–treated groups mimicking the results of the Western blot analysis ($F = 16.819$, $p < 0.0002$ by one-way ANOVA with treatment as the between-group factor) (Fig. 5I). The individual cell fluorescent intensity of pERK signal–positive cells in the lesioned side was unchanged between DOPA alone and DOPA + ranitidine

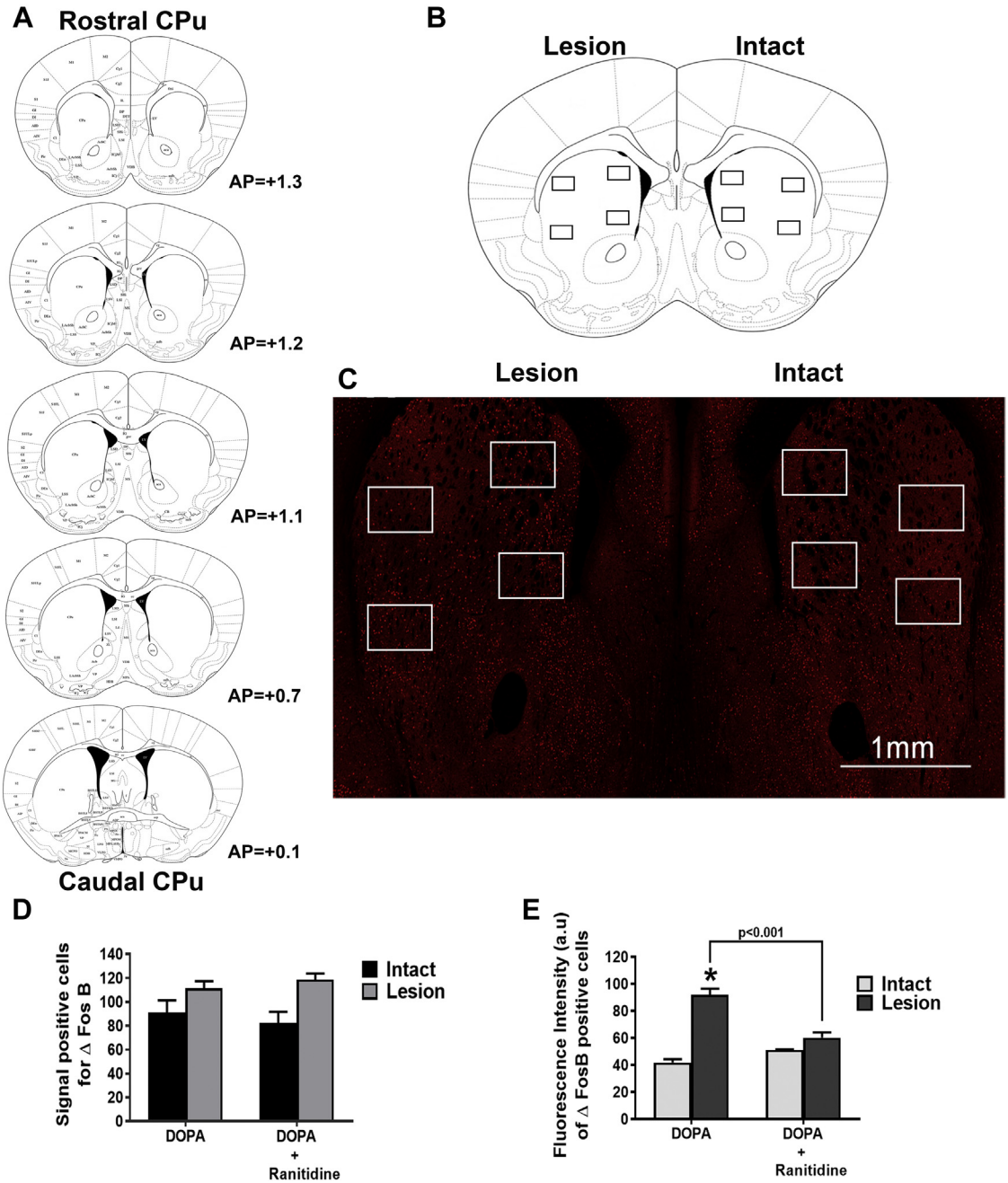

Fig. 6. (A–E) ΔFosB expression in the intact and lesioned striatum in the experimental groups. The expression of ΔFosB tends to increase in the rostrocaudal direction with increased expression seen in the caudate putamen. (A) Shows the coronal sections of the striatum in the rostrocaudal direction at different levels of the bregma. (B) Illustration of ΔFosB quantification in the coronal section. The 4 rectangular boxes in the intact and the lesioned sides indicate the quantification of the number of ΔFosB-positive cells and the fluorescent intensity of the ΔFosB expressing cells. (C) Coronal section at the level of striatum (bregma AP = +0.7) in the DOPA-treated animals. The ΔFosB stained immunohistochemical sections (n = 4 animals and n = 4 sections from each animal) from the experimental groups were selected at this level and the images were captured at the designated magnification and quantified using Image J software as described in the Materials and Methods section. (D) Graphical representation of the ΔFosB signal–positive cells in the intact and the lesioned side in the experimental groups. The number of ΔFosB-positive cells increased in the lesioned side than the intact side, but the difference was not statistically significant in the experimental groups and hence the individual cell fluorescent intensity was measured. (E) Graphical representation of the fluorescent intensity of ΔFosB signal–positive cells in the intact and the lesioned side in the experimental groups. The individual cell fluorescent intensity for ΔFosB significantly increased on the lesioned side in the DOPA alone–treated group, which was significantly reduced when ranitidine was coadministered with DOPA. Abbreviation: DOPA, dihydroxyphenylalanine.

combination therapy groups. (Fig. 5J). The quantification of triple staining of pERK, HA-H2R, and ChAT in CINs showed increased labeling in DOPA + ranitidine treatment groups than DOPA-alone treatment group ($F = 25.926$, $p = 0.0014$ by one-way ANOVA with treatment as the between-group factor) (Fig. 5K–L).

ΔFosB has been shown to accumulate in the DA-depleted brain with repeated L-DOPA administration (Andersson et al., 1999; Cenci et al., 1999, Engeln et al., 2016; Tekumalla et al., 2001). ΔFosB expression profile increases in the caudate putamen in a rostrocaudal direction as shown in the coronal diagrams of the striatum (Fig. 6A) from bregma at the level AP = +1.3 to AP = +0.1 with the highest levels at level 0.1 from bregma per the mouse atlas (Paxinos and Franklin, 2013). The immunohistochemical expression profile of ΔFosB in DOPA alone–treated animals is shown in

Supplemental Fig. 5. For the quantification of ΔFosB, we selected sections representing the section shown in Fig. 6B (AP = +1.2 from bregma) and captured the image at a constant magnification and exposure times. We selected 4 sections from each animal (n = 4 per group) as shown in Fig. 6B and C. We quantified ΔFosB expression by image analysis (Fig. 6C). The number of signal-positive cells for ΔFosB expression was increased in the lesioned striatum of the DOPA alone or DOPA + ranitidine combination treatment group than the intact side, with no difference between animals treated with either DOPA alone or with DOPA + ranitidine combination (Fig. 6D). There was no effect of ranitidine alone on ΔFosB levels (data not shown). We then looked at the individual cell fluorescent intensity for ΔFosB expression (Fig. 6E). The FI of the cells for ΔFosB in the lesioned side of the DOPA alone–treated animals were significantly higher than DOPA + ranitidine combination treatment group ($F = 5.696$, $p = 0.0249$ for treatment and $F = 27.757$, $p < 0.0001$ for ΔFosB FI for hemisphere [intact vs. lesioned] by two-way ANOVA) suggesting that ranitidine could suppress the ΔFosB expression when given along with DOPA. The pERK and ΔFosB expression data suggest that ranitidine suppresses dyskinesia by mitigating the activation of ERK and suppressing ΔFosB expression in the MSNs and CINs.

4. Discussion

We studied the signaling mechanism by which ranitidine suppressed dyskinesia while preserving the beneficial effects of DOPA in relieving akinesia. HA-H2R antagonism to suppress dyskinetic behavior in L-DOPA therapy has been primarily through inhibiting the firing of the ChAT-positive CINs (Lim et al., 2015). Recent studies support the idea that HA excites the postsynaptic excitatory effect on both dopamine (DA) D1 receptor (D1R) and dopamine D2 receptor (D2R) MSNs (Zhuang et al., 2018). The HA-H1R and H2R mRNA has also been found to be coexpressed in the DA-D1R and D2R MSNs. Previous studies showed blocking the effect of histaminergic system with antagonists such as famotidine and ranitidine had beneficial outcomes on LID with L-DOPA (Cui et al., 2014; Johnston et al., 2010; Lim et al., 2015; Shi et al., 2015). In the histidine decarboxylase knockout (Hdc-Ko.) mice, GRK3 and GRK6 which are downregulated in DA-depleted striatum in PD remained unchanged or slightly higher in the striatum of the histidine decarboxylase knockout mice lesioned with 6-OHDA. The levels of GRK3 and GRK6 are not reversed by DOPA treatment in hemiparkinsonian animal models (Ahmed et al., 2008a, 2010, 2015a, 2015b; Bychkov et al., 2007). Unlike the hemiparkinsonian rodent models, MPTP-treated monkey model of PD had higher levels of GRK6 which were normalized by L-DOPA treatment (Bezard et al., 2005). Because the loss of GRK3 and GRK6 in the PD patient brains is permanent, attempts have been made to develop ideal drug candidates that could enhance the expression of these 2 rate-limiting kinases in the brain. The interaction of dual loss of DA and HA has not been previously reported before as far as the expression of GRKs is concerned. One would expect loss of HA along with DA would further reduce the expression of GRKs, but what we found was quite the opposite effect in Hdc-Ko mouse lines. The exact mechanism by which the loss of HA results in the upregulation of GRKs is not known at this point and needs further investigation. It has been suggested that H2R is selectively downregulated by GRK-mediated phosphorylation especially by GRK2 and GRK3. GRK5 and GRK6 seem to play little or no role in the desensitization of the H2R (Shayo et al., 2001).

GRK-mediated regulation and desensitization of both DA and HA receptors could be pivotal in GPCR signaling in the striatum. Suppressing the histaminergic excitation in the striatum especially in the DA-D1R and D2R MSNs and cholinergic system could provide tonic inhibition and reduce neurotransmitter release thereby impeding the firing potential (Ding et al., 2011). Previous studies have suggested enhanced HA-H2R excitation of the striatal CINs as a possible mechanism for LID in animal models of PD (Lim et al., 2015). Others have reported that the behavioral expression of LID has been associated with increased activation of ERK in CINs. In another mouse model, the ablation of striatal CINs has resulted in reduced dyskinesia with L-DOPA therapy (Won et al., 2014). These previous studies have indicated that the modulation of striatal cholinergic tone could be a potential target for treating LID. We focused more on the signaling pathway especially of ERK and ΔFosB to counteract the effect of LID and checked whether there were changes in the levels of GRKs with ranitidine treatment along with DOPA. Ranitidine treatment enhanced the expression of GRK3 subtly in the lesioned striatum than the DOPA alone treatment group. Addition of ranitidine had a profound effect on the LID in the mouse model tested and we believe that it is through the normalization of GRK3. We quantitated the ERK activation in DOPA alone and with DOPA + ranitidine combination treatments and found that DOPA alone treatment had significantly higher pERK signal-positive cells than the animals treated with DOPA + ranitidine combination treatment. There was no difference in the per cell intensity between the DOPA alone and DOPA + ranitidine combination treatment groups. HA-H2R antagonism selectively suppresses the activation of ERK over prolonged periods of time. The immunohistochemical studies showed coexpression of pERK, HA-H2R in ChAT-positive neurons in the experimental groups only at a specific time point of 45 minutes after drug treatment. The reduction in LID was measured over time and we found that the DOPA + ranitidine combination treatment group had better outcomes past this time period as per the ALO-AIMs bin analysis at post-testing sessions 1 and 5. The ERK and the ΔFosB are the best studied pathways in the context of DA depletion and subsequent L-DOPA therapy. Enhanced ERK response to dopaminergic stimulation in the DA-depleted striatum has been linked to the D1R supersensitivity (Gerfen et al., 2002; Santini et al., 2007, 2009, 2010, 2012; Westin et al., 2007) and directly influences the transcription factor ΔFosB which tends to accumulate in the DA-depleted striatum with subsequent DOPA treatment and has been shown to contribute to the development of LID (Berton et al., 2009; Cao et al., 2010; Engeln et al., 2016). We studied the expression of activated ERK and ΔFosB accumulation with DOPA therapy and with combinational therapy of DOPA + ranitidine. We found some degree of colocalization of active ERK and ΔFosB in the striatum with the localization segregated in the striatum. ΔFosB expression was significantly higher in DOPA alone treatment than the DOPA + ranitidine combination therapy in the lesioned side supporting the role of ΔFosB accumulation in the progression of LID in the DOPA alone group. Previous studies have shown that the lentivirus-mediated expression of GRK3 and GRK3–regulator of G-protein signaling domain had been successful in dampening down LID by suppressing the accumulation of ΔFosB in hemiparkinsonian rats (Ahmed et al., 2015b). The role of ranitidine in reducing dyskinesia has been previously reported (Cui et al., 2014; Shi et al., 2015); however, the exact signaling mechanism by which ranitidine counteracts dyskinesia has never been studied before. Even marginal cell-specific increases in the levels of GRK3 could be beneficial in counteracting the signaling deficits due to DOPA therapy and ranitidine provides it in the DA-depleted striatum. Taken together, these results suggest the idea that H2R antagonism might be a beneficial therapeutic option in DA replacement therapy with DOPA in alleviating the debilitating side effects of DOPA in PD via counteracting the activation of ERK and the excessive accumulation of ΔFosB.

5. Conclusions

The present study demonstrated the mechanistic pathway by which the H2R antagonist ranitidine inhibited the LID via normalizing the levels of ERK, ΔFosB in the DA-depleted striatum by normalizing GRK3 in the striatum.

Disclosure

The authors declare no conflicts of interest.

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No human subjects were involved in the present study and the animal studies were carried out following National Institute of Health guidelines and were approved by the Institutional Administrative Panel on Laboratory Animal Care (APLAC).

The authors duly acknowledge that there are no human case studies involved representing any individual personal data.

The data sets used and/or analyzed during the present study are available from the corresponding author on reasonable request. All data generated or analyzed during this study are included in the published article.

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Authors' contributions: MRA and JR conceived the idea for the study. MRA did the animal surgeries, behavioral analysis, biochemical studies, and statistical analyses. MJ, MSA, AZ, EHL, and JT performed the biochemical and microscopic analyses under the supervision of MRA and JR. HO and CP provided the HDC-Ko mouse line. JJ made the spray-dried formulations of the drugs. MRA, CP, and JR analyzed the data and wrote the manuscript. All authors read and approved the final manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neurobiolaging.2019.06.004.

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