Dopamine-Induced Changes in $\alpha_{\text{olf}}$ Protein Levels in Striatonigral and Striatopallidal Medium Spiny Neurons Underlie the Genesis of L-DOPA-Induced Dyskinesia in Parkinsonian Mice

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The dopamine precursor, L-3,4-dihydroxyphenylalanine (L-DOPA), exerts powerful therapeutic effects but eventually generates L-DOPA-induced dyskinesia (LID) in patients with Parkinson's disease (PD). LID has a close link with deregulation of striatal dopamine/cAMP signaling, which is integrated by medium spiny neurons (MSNs). Olfactory type G-protein $\alpha$ subunit ($\alpha_{\text{olf}}$), a stimulatory GTP-binding protein encoded by the $\text{GNAL}$ gene, is highly concentrated in the striatum, where it positively couples with dopamine $D_1$ ($D_1$R) receptor and adenosine $A_2A$ receptor ($A_2A$R) to increase intracellular cAMP levels in MSNs. In the striatum, $D_1$Rs are mainly expressed in the MSNs that form the striatonigral pathway, while $D_2$Rs and $A_2A$Rs are expressed in the MSNs that form the striatopallidal pathway. Here, we examined the association between striatal $\alpha_{\text{olf}}$ protein levels and the development of LID. We used a hemi-parkinsonian mouse model with nigrostriatal lesions induced by 6-hydroxydopamine (6-OHDA). Using quantitative immunohistochemistry (IHC) and a dual-antigen recognition in situ proximity ligation assay (PLA), we here found that in the dopamine-depleted striatum, there appeared increased and decreased levels of $\alpha_{\text{olf}}$ protein in striatonigral and striatopallidal MSNs, respectively, after a daily pulsatile administration of L-DOPA. This leads to increased responsiveness to dopamine stimulation in both striatonigral and striatopallidal MSNs. Because $\alpha_{\text{olf}}$ protein levels serve as a determinant of cAMP signal-dependent activity in striatal MSNs, we suggest that L-DOPA-induced changes in striatal $\alpha_{\text{olf}}$ levels in the dopamine-depleted striatum could be a key event in generating LID.

Keywords: olfactory type G-protein $\alpha$ subunit, dopamine, striatum, Parkinson's disease, L-DOPA-induced dyskinesia

INTRODUCTION

Human pathology has shown that Parkinson’s disease (PD) results from dopamine deficiency in the neostriatum, particularly in the putamen, due to degenerative loss of nigrostriatal dopaminergic cells (Kish et al., 1988; Goto et al., 1989). Treatments with the dopamine precursor, L-3,4-dihydroxyphenylalanine (L-DOPA), remain the gold standard of drug...
therapy for PD. However, after prolonged and pulsatile exposure to L-DOPA, PD patients eventually develop L-DOPA-induced dyskinesia (LID; Jenner, 2008; Calabresi et al., 2010; Huot et al., 2013). LID is an adverse event that occurs in more than 50% of patients after 5–10 years (Ahlskog and Munter, 2001; Rascol et al., 2006). Importantly, once LID has been established, its severity increases unless dopaminergic drug dosage is reduced (Brotchie, 2005). It is known that the severity of loss of nigral dopaminergic cells represents the most important factor that determines the severity of LID (Guridi et al., 2012; Bastide et al., 2015). However, the nature of the cellular and molecular key events that lead to a progressive increase in responsiveness to dopaminergic stimulation in LID remains unclear.

LID is closely linked with pathological changes in dopaminergic transmissions in the striatum (Bastide et al., 2015; Calabresi et al., 2016). Dopamine receptors are categorized into two subclasses, D₁- and D₂-type receptors, based on their functional properties to stimulate and inhibit the adenylyl cyclase and, thereby, increase intracellular cAMP levels in MSNs (Hervé, 2011). As GαolfRs and DolfRs are mainly expressed in striatonigral and striatopallidal MSNs (D2-cells). A prototypical Gs-coupled receptor, olfactory type G-protein (Golf), the rate-limiting factor for the D₁R- and A₂AR-dependent cAMP production (Kull et al., 2000; Corvol et al., 2001), Gαolf protein level serves as a determinant of cAMP signal-dependent activity in both D₁R-expressing striatonigral MSNs (D1-cells) and D₂R-expressing striatopallidal MSNs (D2-cells). D₁R/Golf-mediated increases in intracellular cAMP levels facilitate D1-cell activity (Hervé, 2011), while the elevation of intracellular cAMP levels via A₂AR/Golf activation functionally opposes the actions of D₁Rs on D2-cells (Schwartzschild et al., 2006; Fuxe et al., 2007). It is also known that Golf protein levels in striatal MSNs are regulated by posttranslational usage-dependent mechanism through the activation of D₁Rs (Hervé et al., 2001; Corvol et al., 2004, 2007; Alcacer et al., 2012; Ruiz-DeDiego et al., 2015) and A₂ARs (Hervé et al., 2001).

The aim of this study was to clarify the association of striatal Golf protein levels with LID development. For this purpose, we used a hemi-parkinsonian mouse model with nigrostriatal lesion induced by 6-hydroxydopamine (6-OHDA). Using quantitative immunohistochemistry (IHC) and a highly-sensitive in situ proximity ligation assay (PLA), we show that in the 6-OHDA-lesioned striatum, daily pulsatile injections of L-DOPA might cause changes in Golf levels in not only D1-cells but also D2-cells, and lead to elevated responsiveness to dopamine stimulation in both D1-cells and D2-cells. This novel finding suggests that L-DOPA-induced changes in striatal Golf levels in the dopamine-denervated striatum may serve as a principal cause for generating LID.

**MATERIALS AND METHODS**

**Experimental Animals**

All experimental procedures involving the use of animals and the analysis of brain anatomy were approved by the Institutional Care and Use Committees of Tokushima University, Japan. Adult male C57BL/6 mice aged 8–9 weeks were purchased from Nihon SLC Co. (Shizuoka, Japan). Mice were housed in a controlled environment (23 ± 1°C, 50 ± 5% of humidity) with 12 h light/dark cycle. Mice were allowed to take food and tap water ad libitum.

**Stereotaxic Injection of 6-OHDA**

Mice were anesthetized with isoflurane (Sigma-Aldrich, St. Louis, MO, USA) and were mounted on a stereotaxic frame (Narishige, Tokyo, Japan). Each mouse received a stereotaxic injection of 6-OHDA-HCl (8.2 µg) dissolved in 4 µl of saline containing 0.02% ascorbic acid. Two 2 µl injections were administered into the striatum at a rate of 1 µl/min. The needle was left in place for 5 min to allow diffusion away from the injection site. The stereotaxic coordinates according to the mouse brain atlas (Paxinos and Franklin, 2001) were anterior-posterior, +0.5; medial-lateral, +2.4; and dorsal-ventral, −4.0 and −3.0. Mice were allowed to recover for 3 weeks and then apomorphine (Sigma-Aldrich; 0.5 mg/kg)-induced rotation behavior was studied over the course of 60 min. Mice with contralateral rotations (>7 times/min) were chosen and used for further studies.

**L-DOPA Treatments**

Three weeks after the 6-OHDA-lesioning, mice received intraperitoneal injections of L-DOPA (Sigma-Aldrich; 20 mg/kg of free base) dissolved in 0.9% saline and intraperitoneal injections of benserazide-HCl (Sigma-Aldrich; 12 mg/kg) dissolved in 0.9% saline 20 min before daily administration of
L-DOPA over 10 days. On day 11, the mice underwent behavioral studies and were then sacrificed for histological studies.

Assessment of Abnormal Involuntary Movements (AIMs)
AIM scoring was performed according to previous reports (Cenci et al., 1998; Pavón et al., 2006; Santini et al., 2007). AIM scores were obtained after the last injection of L-DOPA for 1 min every 10 min over a period of 140 min. For the evaluation, each mouse was placed in a glass cylinder (diameter of 12 cm). Purposeless movements were classified on the basis of their topographic distribution. The following four subtypes of AIMs were present: locomotive (tight contralateral turns), axial (twisted posturing of the neck and upper body toward the contralateral side), forelimb (jerky movements of the contralateral forelimb, and/or grabbing movement of the contralateral paw), and orolingual (jaw movements and tongue protrusion toward the contralateral side). Each subtype was scored as follows: 0, absent; 1, occasional; 2, frequent; 3, continuous; 4, continuous, not interrupted by sensory stimuli.

Tissue Preparations
Immediately after the last AIM scoring, the mice were intraperitoneally administered a lethal dose of pentobarbital (Sigma-Aldrich). They were then transcardially perfused with 0.01 M phosphate-buffered saline (PBS) at pH 7.2, followed by cold 4% paraformaldehyde in 0.1 M phosphate buffer at pH 7.2. The brains were removed, post-fixed overnight in the same fixative at 4°C, and stored in a 10–30% sucrose gradient in 0.1 M phosphate buffer at 4°C for cryoprotection. Sixteen-micrometre-thick sections were cut on a cryostat and stored in PBS containing 0.05% NaN₃ until use.

IHC
Immunostaining was performed on free-floating sections using the tyramide signal amplification (TSA) method, as in our previous report (Okita et al., 2012). After blocking endogenous peroxidases activity, the sections were incubated in PBS containing 3% bovine serum albumin (BSA) for 60 min. They were then incubated with antibodies against one of the following (diluted in PBS-BSA): G_olf (rabbit polyclonal, 1:5000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), tyrosine hydroxylase (TH, rabbit polyclonal, 1:100,000) (Sato et al., 2008; Morigaki and Goto, 2016), D₁R (mouse monoclonal, 1:5000; Novus Biologicals, Littleton, CO, USA), A_2AR (mouse monoclonal, 1:5000; Santa Cruz Biotechnology), D₂R (rabbit polyclonal, 1:2000; Merck Millipore, Billerica, MA, USA) or c-Fos (rabbit polyclonal, 1:50,000; Oncogene Science, Cambridge, MA, USA) for 18 h. The bound antibodies were detected using the Histofine Simple Stain Kit (Nichirei, Tokyo, Japan) and the TSA-system with Cyanine3 or Fluorescein (Perkin Elmer) against the µ-opioid receptor (MOR; 1:20,000; Millipore, Billerica, MA, USA), a mouse monoclonal antibody against D₁R (1:5000; Novus Biologicals), or a mouse monoclonal antibody against A_2AR (1:5000; Santa Cruz Biotechnology). The bound antibodies were detected using the Histofine Simple Stain Kit (Nichirei) and the TSA-system with Fluorescein (Perkin Elmer).

Dual-Antigen Recognition In Situ PLA
Dual-antigen recognition PLA experiments were conducted using the Brightfield Duolink PLA kit reagents (Sigma-Aldrich) according to the manufacturer’s recommendations with some modifications. Briefly, after blocking endogenous peroxidases in PBS containing 0.1% H₂O₂ for 30 min, the free-floating sections were incubated in PBS containing 3% normal goat serum for 60 min. They were then incubated in PBS containing 3% normal goat serum and a rabbit polyclonal antibody against G_olf (1:500; Santa Cruz Biotechnology) in combination with a mouse monoclonal antibody against D₁R (1:500; Novus Biologicals) or a mouse monoclonal antibody against A_2AR (1:500; Santa Cruz Biotechnology) for 18 h at room temperature. After subsequent secondary labeling with rabbit PLA minus and mouse PLA plus probes, we used the Brightfield Duolink Detection reagents for ligation and amplification and label probe binding according to the manufacturer’s instructions. For final signal visualization, we used the TSA-system with Cyanine3 (Perkin Elmer). After mounting on slides, the stained sections were counterstained with hematoxylin and were cover-slipped using 10% glycerol in PBS.

Digital Imaging and Morphometry
Digital microscopy images were captured using an Olympus BX51 microscope (Olympus, Tokyo, Japan) equipped with a DP40 digital camera (Olympus). They were imported into Adobe Photoshop CS4 and processed digitally. We adjusted contrast, brightness, and color balance. Using an image analyzer (MetaMorph, Molecular Device, Tokyo, Japan), we measured the optical densities of immunoreactive products and PLA signals in the striatum, which were represented by gray levels on non-colored digital images (Sato et al., 2008; Goto et al., 2013; Morigaki and Goto, 2015). Using the same protocol described above, we also measured optical densities of G_olf immunoreactive products in the striosome and matrix subfields in the striatal sections double-stained for G_olf and MOR. We also counted the numbers of neuronal nuclei positive for c-Fos in a 0.5 mm × 0.5 mm field in the striatum and globus pallidus, as in our previous report (Tanabe et al., 2014). These morphometric analyses were carried out in a blind manner.

Statistical Analysis
All experimental values are expressed as means ± SEM. For two-group comparisons, we used a paired two-tailed t-test. Multiple comparisons were analyzed using one-way or two-way analysis of variance (ANOVA), followed by Bonferroni’s post hoc tests for pair wise comparisons. Statistical analyses were performed using Stat View 5.0 (SAS Institute, Cary, NC, USA).
software. P-values of less than 0.05 were considered statistically significant.

RESULTS

Generation of a Mouse Model with LID

To model the generation of AIMS in PD following repeated L-DOPA treatments, we employed a well-established PD mouse model in which mice first received unilateral injection of 6-OHDA into the striatum (Santini et al., 2007). After 3 weeks of recovery and an apomorphine test, the mice were subjected to L-DOPA treatment for 10 days according to standardized protocols (Figure 1A). In this study, 6-OHDA-lesioned mice administered daily injections of benzerazide-HCl (12 mg/kg) alone for 10 days were designated as “PD” models. Six-OHDA-lesioned mice that received daily injections of L-DOPA (20 mg/kg) and benzerazide-HCl (12 mg/kg) for 10 days and finally exhibited LIDs with total AIM scores of more than 20 were designated as “PD with Dyskinesia (PD-D)” models. Among 6-OHDA-lesioned mice that received daily injections of L-DOPA (n = 28), 25 mice (∼90%) were grouped into the PD-D model. Mice that received no drug treatment, except for anesthetic drugs, were used as “naïve controls”.

In both PD and PD-D mice, IHC with anti-TH antibody revealed a severe loss of nigral dopaminergic cells (Figure 1B) and striatal dopaminergic afferents (Figure 1C) on the side of the 6-OHDA injection. Quantitative measurements (Figure 1D) revealed a greater-than-90% reduction in TH labeling in the dorsolateral (DL) striatum on the lesioned side when compared to the non-lesioned side (lesion side, 3.9 ± 2.1; non-lesion side, 56.5 ± 8.9; means ± SEM; n = 10; two-tailed t-test, P < 0.01). Figure 1E shows the time course of changes in LIDs as determined by AIM scoring in PD-D mice. AIMS were maximal 40 min after L-DOPA administration, declined after 70 min, and almost disappeared after 120 min.

Regional and Cellular Localization of Gαolf in the Normal Mouse Striatum

Figure 2A depicts the known distributional patterns of Gαolf, D1R, and A2A R in a simplified basal ganglia circuit diagram. Note that Gαolf is mainly localized with D1R in the D1-cells that form the striatonigral pathway, while it is localized with A2AR in the D2-cells that form the striatopallidal pathway. Using IHC, we reappraised the localization profile of Gαolf immunoreactivity (IR) in the mouse striatum. Low-magnification microscopic images show strong Gαolf labeling in the striatum (Figure 2B), particularly in the DL region (Figure 2B’, arrows). As in our previous report (Sako et al., 2010), Gαolf IR was differentially concentrated in the different striatal compartments, with heightened Gαolf labeling in the striosomes relative to the matrix (Figures 2C,D). Optical density measurements (Figure 2E) also revealed that Gαolf IR in the striosomes was significantly higher than that in the matrix (striosomes, 39.8 ± 5.0; matrix, 17.2 ± 1.9; means ± SEM; n = 10 per group; paired two-tailed t-test, P < 0.01 vs. Non-lesion).
FIGURE 2 | Striatal localization of Gαolf protein in normal mice. (A) Localization pattern of Gαolf in a simplified basal ganglia circuit. Note that Gαolf is colocalized with D1R in strionigral medium spiny neurons (MSNs; D1-cells), but with adenosine A2A receptor (A2AR) in D2R-expressing striatopallidal MSNs (D2-cells). The strionigral and striatopallidal pathways arising from the striosome are omitted in this scheme. Abbreviations: SNc, substantia nigra pars compacta; VTA, ventral tegmental area. (B,B') Multiple frontal sections of the striatum stained for Gαolf from naïve control mice (B) and their graded color-converted images (B'). Note that Gαolf immunoreactivity (IR) is highly concentrated in the DL portion of the striatum (arrows). (C) Representative photomicrograph of the striatum stained for Gαolf. Asterisk indicates an example of the striosomes. (D,D') Representative photomicrographs of the DL striatum double-stained for Gαolf (D) and µ-opioid receptor (MOR) (D'). Corresponding striosomes are indicated by arrows. (E) Quantification of mean Gαolf staining intensity in the striosome and matrix compartments in the DL striatum. Data are means ± SEM (n = 10). Paired two-tailed Student's t test: **P < 0.01, Striosome vs. Matrix. (F) Representative photomicrographs of the DL striatum stained for Gαolf with DAPI (4,6-diamidino-2-phenylindole)-staining. Tiny dots positive for Gαolf (inset) are shown. (G–H) Representative photomicrographs of neurons double-stained for Gαolf and D1R (G) or A2AR (H) in the DL striatum. (I,J) Representative photomicrographs of the DL striatum stained with the dual recognition in situ proximity ligation assay (PLA) for Gαolf-D1R (I) or Gαolf-A2AR (J). Tiny dots showing the PLA signals for Gαolf-D1R (I) or Gαolf-A2AR (J) are abundant. Microscopic images at higher magnifications are shown in the insets (arrows) in (I,J). Scale bars: (B,C) = 1 mm; (D,D') = 200 µm; (F–J) = 10 µm; inset in (F) = 5 µm; insets in (I,J) = 2.5 µm.

Microscopic images with high magnification show numerous tiny dots of Gαolf IR densely distributed in the DL striatum (Figures 2F–H). In the double-labeling study, Gαolf-positive dots were frequently localized in MSNs labeled for D1R (Figure 2G) or A2AR (Figure 2H). Using dual-antigen recognition in situ PLA, which indicates that two proteins are in close proximity (Söderberg et al., 2006), we also found that dot signals indicating the presence of Gαolf protein in close proximity to D1R protein (D1R-Gαolf; Figure 2I) or A2AR protein (A2AR-Gαolf; Figure 2J) were abundantly distributed in the DL striatum.

Dopaminergic Regulation of Striatal Gαolf Protein Levels
To examine the dopaminergic regulation of Gαolf protein levels in the striatum, we performed a quantitative IHC using an anti-Gαolf antibody on striatal sections prepared from naïve control, PD, and PD-D mice. In low-magnification
microscopic images, PD mice (Figure 3A) showed dramatic increases in $\alpha$olf IR in the dorsal striatum on the 6-OHDA-lesioned side when compared to non-lesioned side. In contrast, PD-D mice (Figure 3B) only had a modest increase in striatal $\alpha$olf IR on the 6-OHDA-lesioned side relative to non-lesioned side. Higher-magnification microscopic images of the DL striatum also show that, when compared to naïve controls (Figure 3C), there is a marked, but, slight increase in $\alpha$olf IR in the 6-OHDA-lesioned striatal areas of PD (Figure 3D) and PD-D (Figure 3E) mice. As indicated in a previous report (Ruiz-DeDiego et al., 2015), it is likely that dopamine depletion increases $\alpha$olf IR mainly in the matrix of the 6-OHDA-lesioned striatum, leading to a loss of the striosome-predominant pattern of $\alpha$olf IR expression in PD mice. However, daily treatment with L-DOPA reverses the lesion-induced increase in $\alpha$olf IR primarily in the matrix, leading to reappearance of the striosome-predominant pattern of $\alpha$olf IR expression in PD-D mice. These visual impressions were confirmed by quantitative densitometry analyses of the DL striatum (Figures 3F–H), as follows. We found a significant and marked increase of 101% ($P < 0.001$, two-way ANOVA) in $\alpha$olf IR levels in the 6-OHDA-lesioned striatum of PD, when compared to naïve controls. There was a 67% decrease ($P < 0.01$, two-way ANOVA) in $\alpha$olf IR levels in the 6-OHDA-lesioned striatum of PD-D mice when compared to that of PD mice (Figure 3F; PD mice: non-lesion, 102 ± 17% and 6-OHDA-lesion, 201 ± 28%; PD-D mice: non-lesion, 103 ± 19% and 6-OHDA-lesion, 134 ± 16%; % of naïve control mice ± SEM; $n = 15$).

In the striatal compartments of the DL striatum, we found a significant increase of 38% ($P < 0.01$, two-way ANOVA) $\alpha$olf IR levels in the striosomes of 6-OHDA-lesioned striatum of PD, when compared to naïve controls. There was no apparent
difference ($P > 0.05$, two-way ANOVA) in striosomal levels of $G_{olf}$ IR in the 6-OHDA-lesioned striatum between PD and PD-D mice (Figure 3G; PD mice: non-lesion, 101 ± 22% and 6-OHDA-lesion, 138 ± 26%; PD-D mice: non-lesion, 102 ± 19% and 6-OHDA-lesion, 122 ± 20%; % of naïve control mice ± SEM; $n = 15$). We also found a significant increase of 96% ($P < 0.001$, two-way ANOVA) in matrix levels of $G_{olf}$ IR in the 6-OHDA-lesioned striatum of PD mice, when compared to those in naïve controls. There was a 66% decrease ($P < 0.01$, two-way ANOVA) in matrix levels of $G_{olf}$ IR in the 6-OHDA-lesioned striatum of PD-D mice when compared to those of PD mice (Figure 3H; PD mice: non-lesion, 99 ± 23% and 6-OHDA-lesion, 196 ± 24%; PD-D mice: non-lesion, 102 ± 12% and 6-OHDA-lesion, 130 ± 22%; % of naïve control mice ± SEM; $n = 15$). These findings indicate that dopamine depletion causes a dramatic increase in $G_{olf}$ levels in the DL striatum, particularly in the matrix. Daily exposure to L-DOPA induces a down-regulation of this lesion-induced increase in $G_{olf}$ expression.

**Dopaminergic Regulation of Striatal Expression of $D_1R$, $A_2AR$, and $D_2R$**

To examine dopaminergic regulation of $D_1R$, $A_2AR$ and $D_2R$ expression in the DL striatum, we performed quantitative IHC on sections prepared from 6-OHDA-lesioned striata of PD and PD-D mice (Figure 4). We observed no significant changes ($P > 0.05$, one-way ANOVA) in the expression levels of $D_1R$ IR in PD or PD-D mice when compared to naïve controls (Figures 4A,B; PD mice, 101 ± 21%; PD-D mice, 98 ± 17%; % of naïve control mice ± SEM; $n = 15$). The expression levels of $A_2AR$ IR in PD and PD-D mice were not significantly different ($P > 0.05$, one-way ANOVA) from those in naïve controls (Figures 4C,D; PD mice, 103 ± 15%; PD-D mice, 102 ± 19%; % of naïve control mice ± SEM; $n = 15$). The optical density quantification of $D_2R$ IR in PD and PD-D mice was expressed as percentage (Figures 4E,F; PD mice, 107 ± 13%; PD-D mice, 99 ± 15%; % of naïve control mice ± SEM; $n = 15$). The optical density quantification of $D_2R$ IR in the DL striatum from PD ($n = 15$) and PD-D ($n = 15$) mice. Data are expressed as percentage of levels in naïve control mice ($n = 15$) and are means ± SEM.

**FIGURE 4 | Dopaminergic regulation of striatal expression of $D_1R$, $A_2AR$, and $D_2R$.** (A) Representative photomicrographs of striatal expression of $D_1R$ in normal (naïve controls) and lesioned hemispheres from 6-OHDA-lesioned mice treated with daily injections of benzerazide alone for 10 days (PD model), and from 6-OHDA-lesioned mice that received daily injections of benzerazide and L-DOPA for 10 days and exhibited dyskinesia (PD-D model). (B) Optical density quantification of $D_1R$ IR in the DL striatum from PD ($n = 15$) and PD-D ($n = 15$) mice. Data are expressed as percentage of naïve control mice ($n = 15$) and are means ± SEM. No significant changes in striatal levels of $D_1R$ IR in PD and PD-D mice were observed when compared to naïve controls; one-way ANOVA ($F_{(2,42)} = 0.0$) followed by Bonferroni’s test. (C) Representative photomicrographs of striatal expression of $A_2AR$ from naïve control, PD and PD-D mice. (D) Optical density quantification of $A_2AR$ IR in the DL striatum from PD ($n = 15$) and PD-D ($n = 15$) mice. Data are expressed as percentage of levels in naïve control mice ($n = 15$) and are means ± SEM. No significant changes in striatal levels of $A_2AR$ IR in PD and PD-D mice were observed when compared to naïve controls; one-way ANOVA ($F_{(2,42)} = 1.2$) followed by Bonferroni’s test. (E) Representative photomicrographs of striatal expression of $D_2R$ from naïve control, PD and PD-D mice. (F) Optical density quantification of $D_2R$ IR in the DL striatum from PD ($n = 15$) and PD-D ($n = 15$) mice. Data are expressed as percentage of levels in naïve control mice ($n = 15$) and are means ± SEM. **$P < 0.01$ vs. naïve controls; one-way ANOVA ($F_{(2,42)} = 16.9$) followed by Bonferroni’s test. Scale bars: (A,C,E) = 1 mm.
118 ± 13%; % of naïve control mice ± SEM; n = 15). We found a significant increase in the expression of D2R in PD (P < 0.01, one-way ANOVA), but not PD-D (P > 0.05, one-way ANOVA), mice when compared to naïve controls (Figures 4E,F; PD mice, 123 ± 15%; PD-D mice, 112 ± 22%; % of naïve control mice ± SEM; n = 15). These findings indicate that dopamine depletion causes a significant increase in striatal D2R expression, which is reversed by daily treatment with L-DOPA. In addition, dopamine depletion and L-DOPA replacement cause no significant changes in striatal expression of D1R and A2AR in the dopamine-denervated striatum.

Dopaminergic Regulation of Striatal Levels of PLA Signals for D1R-Gαolf

To examine the dopaminergic regulation of striatal levels of Gαolf protein in close proximity to D1R protein, we used a sensitive in situ PLA in sections prepared from 6-OHDA-lesioned striata from PD and PD-D mice (Figure 5). In low-magnification microscopic images, a marked and moderate increase in D1R-Gαolf PLA signals was observed in the dorsal striatum in PD and PD-D mice when compared to naïve controls (Figures 5A,B). Higher-magnification microscopic images of the DL striatum also show that compared to naïve controls (Figure 5C), there is a marked and moderate increase in the D1R-Gαolf PLA signals in 6-OHDA-lesioned striatal areas in PD (Figure 5D) and PD-D (Figure 5E) mice. Quantitative densitometry analyses of the DL striatum revealed increases of 92% (P < 0.001, one-way ANOVA) and 50% (P < 0.001, one-way ANOVA) in the D1R-Gαolf PLA signal in PD and PD-D mice, respectively, when compared to naïve controls. There was a decrease of 42% (P < 0.001, one-way ANOVA) in the D1R-Gαolf PLA signal in PD-D mice when compared to PD mice (Figure 5F; PD mice, 192 ± 25%; PD-D mice, 150 ± 21%; % of naïve control mice ± SEM; n = 10). These findings indicate that dopamine depletion causes a marked increase in striatal D1R-Gαolf PLA signal, which is downregulated by daily treatment with L-DOPA. However, there is a significant increase of striatal D1R-Gαolf PLA signal in PD-D mice compared to naïve controls.

Dopaminergic Regulation of Striatal Levels of PLA Signals for A2AR-R-Gαolf

To examine the dopaminergic regulation of striatal levels of Gαolf protein in close proximity to A2AR protein, we used a sensitive in situ PLA in sections prepared from 6-OHDA-lesioned striata from PD and PD-D mice (Figure 6). Notably, low-magnification microscopic images show an apparent decrease in the A2AR-
αolf PLA signal in the DL striatum of PD-D mice when compared to both naïve control and PD mice (Figures 6A,B). Higher-magnification images also show the localization patterns of A2AR-αolf PLA signals in the DL striatum of naïve control (Figure 6C), PD (Figure 6D), and PD-D (Figure 6E) mice. Quantitative densitometry analyses of the DL striatum revealed decreases of 41% (P < 0.001, one-way ANOVA) and 45% (P < 0.001, one-way ANOVA) in A2AR-αolf PLA signal levels in PD-D mice, when compared to naïve controls and PD mice, respectively (Figure 6F; PD mice, 104 ± 24%; PD-D mice, 59 ± 21%; % of naïve control mice ± SEM; n = 10). These findings indicate that L-DOPA replacement, but not dopamine depletion, causes a significant decrease in the striatal A2AR-αolf PLA signal in the dopamine-denervated striatum.

**Differences in Striatal Responsiveness to Dopamine Stimulation between PD and PD-D Mice**

To assess changes in striatal responsiveness to dopamine stimulation in PD and PD-D mice, we performed IHC using an antibody against c-Fos, which is known to be induced in the striatum and globus pallidus following the stimulation of D1Rs and D2Rs (Marshall et al., 1993; LaHoste and Marshall, 1994). We prepared striatal sections from PD and PD-D mice that received injections of L-DOPA (20 mg/kg) and benserazide-HCl (12 mg/kg) 2 h before sacrifice on day 11 (see Figure 1A). Microscopic images of the DL striatum stained for c-Fos from naïve control, PD and PD-D mice are shown in Figure 7A. Compared to naïve controls that also received the injections of L-DOPA (20 mg/kg) and benserazide-HCl (12 mg/kg) 2 h before sacrifice, we found a marked increase in the densities of c-Fos-positive (c-Fos+) nuclei in the 6-OHDA-lesioned striatum in both PD and PD-D mice. Quantitative densitometry analyses also showed a marked increase (P < 0.001, two-way ANOVA) in the density of c-Fos+ nuclei in the 6-OHDA-lesioned striatum in both PD and PD-D mice when compared to naïve controls. However, there was a decrease of ~40% (P < 0.001, two-way ANOVA) in the density of c-Fos+ nuclei in the 6-OHDA-lesioned striatum of PD-D mice when compared to PD mice (Figure 7B; naïve controls: 20 ± 12; PD mice: non-lesion, 31 ± 10 and 6-OHDA-lesion, 475 ± 55; PD-D mice: non-lesion,

**FIGURE 6 | Dopaminergic regulation of striatal levels of Gαolf proteins in close proximity to A2AR proteins.** Dual-antigen recognition *in situ* PLA used to detect Gαolf proteins in proximity to A2AR proteins (A2AR-Gαolf) was carried out on normal hemispheres of naïve controls and on lesioned hemispheres from 6-OHDA-lesioned mice treated with daily injections of benserazide alone for 10 days (PD model) and from 6-OHDA-lesioned mice that received daily injections of benserazide and L-DOPA for 10 days and exhibited dyskinesia (PD-D model). (A,B) Representative photomicrographs of striatal expression of A2AR-Gαolf PLA signals in normal and lesioned hemispheres from PD and PD-D mice (A), and their graded color-converted images (B). (C–E) Representative photomicrographs of the DL striatum stained with the *in situ* PLA for A2AR-Gαolf from naïve control (C), PD (D) and PD-D (E) mice. Their higher-magnification images are also shown in the insets in (C–E). (F) Optical density quantification of A2AR-Gαolf PLA signals in the DL striatum from PD (n = 10) and PD-D (n = 10) mice. Data are expressed as percentage of naïve control mice (n = 10) and are means ± SEM. ***P < 0.001 vs. naive controls; # # # P < 0.001 vs. PD; one-way ANOVA (F2,27) = 72.6) followed by Bonferroni’s test. Scale bars: (A,B) = 2 mm; (C–E) = 25 µm; insets in (C–E) = 10 µm.
FIGURE 7 | Effects of dopamine stimulation on striatal and pallidal c-Fos expression in 6-OHDA-lesioned mice. An immunohistochemical study with anti-c-Fos antibody was carried out on the striatal sections from PD and PD-D mice, which received injections of L-DOPA (20 mg/kg) and benserazide-HCl (12 mg/kg) 2 h before sacrifice on day 11 (see Figure 1A), and those from naïve controls that also received the injections of L-DOPA (20 mg/kg) and benserazide-HCl (12 mg/kg) 2 h before sacrifice. The density measurements were performed by counting the numbers of c-Fos-positive (c-Fos+) nuclei in a 0.5 mm × 0.5 mm field in the striatum and globus pallidus from each animal. (A) Representative photomicrographs of the DL striatum stained for c-Fos in naïve control, PD and PD-D mice. The inset (red open box) in the naïve control is the corresponding figure from the atlas of Paxinos and Franklin (2001) to show the striatal area that were analyzed in all naïve control, PD and PD-D mice. (B) Density quantification of c-Fos+ nuclei in the DL striatum from naïve controls (n = 10), and of those on non-lesion and lesion sides in PD (n = 10) and PD-D (n = 10) mice. Data are expressed as means ± SEM. ***P < 0.001 vs. naïve controls; ###P < 0.001 vs. PD; two-way ANOVA (F(1,36) = 140.9) followed by Bonferroni’s test. (C) Representative photomicrographs of the globus pallidus stained for c-Fos in naïve control, PD and PD-D mice. The inset (red open box) in the naïve control is the corresponding figure from the atlas of Paxinos and Franklin (2001) to show the pallidal area that were analyzed in all naïve control, PD and PD-D mice. (D) Density quantification of c-Fos+ nuclei in the globus pallidus from naïve controls (n = 10), and of those on non-lesion and lesion sides in PD (n = 10) and PD-D (n = 10) mice. Data are expressed as means ± SEM. ***P < 0.001 vs. naïve controls; ##P < 0.01 vs. PD; two-way ANOVA (F(1,36) = 80.8) followed by Bonferroni’s test. Scale bars: (A) = 200 µm; (C) = 100 µm.

29 ± 12 and 6-OHDA-lesion, 292 ± 49; means ± SEM; n = 10). Microscopic images of the globus pallidus stained for c-Fos obtained from naïve control, PD, and PD-D mice are shown in Figure 7C. Compared to naïve controls, we found increased densities of c-Fos+ nuclei in the globus pallidus on the lesioned sides in both PD and PD-D mice. Quantitative densitometry analyses also indicated a significant increase (P < 0.001, two-way ANOVA) in the density of c-Fos+ nuclei in the globus pallidus on the lesioned sides in both PD and PD-D mice when compared to naïve controls. Importantly, we found that there was an increase of ∼140% (P < 0.01, two-way ANOVA) in the density of c-Fos+ nuclei in the globus pallidus on the lesioned side in PD-D mice when compared to PD mice (Figure 7D; naïve controls: 4 ± 2; PD mice: non-lesion, 5 ± 3 and 6-OHDA-lesion, 38 ± 8; PD-D mice: non-lesion, 8 ± 7 and 6-OHDA-lesion, 92 ± 11; means ± SEM; n = 10). These findings indicate that dopamine depletion causes a marked increase in the responsiveness of striatal D1-cells to dopamine stimulation, which is downregulated by daily treatments with L-DOPA. Given the changes in striatal D2-R expression in PD and PD-D mice (see above), it is likely that in the dopamine-denervated striatum, dopamine depletion may cause increased striatal D2-R expression, which then enhances the responsiveness of D2-cells to dopamine stimulation (Cai et al., 2002). Notably, L-DOPA replacement could induce a further increase in the responsiveness of D2-cells to dopamine stimulation despite no obvious increase in striatal D2-R expression in the dopamine-denervated striatum.

DISCUSSION

Here we used IHC and in situ PLA to determine the region- and cell-type-specific distributions of Gaolf proteins in the mouse striatum. Using a mouse model of hemiparkinsonism induced by 6-OHDA, we also found that daily pulsatile
administration of L-DOPA might induce usage-dependent changes in G_{olf} expression not only in D1-cells, but also in D2-cells in the dopamine-depleted striatum (see Figure 8). This raises the possibility that LID might result from reduced A2A R/G_{olf}/cAMP signal levels in D2-cells, which may be caused by intermittent and pulsatile activation of postsynaptic D1 Rs in the striatum. Our results support and provide new insights into the hypothesis that LID is associated with a decrease in activity of “indirect” striatopallidal pathway (Crossman, 1990; DeLong, 1990; Brotchie, 2005; Guridi et al., 2012).

**Strategic Localization of G_{olf} Proteins in the Striatum**

We used IHC to demonstrate that G_{olf} IR is highly concentrated in the DL striatum, which corresponds to the motor-sensory territory in rodents and is analogous to the putamen in primates (Graybiel, 2008). This implies that G_{olf} may have a unique position in regulating the activities of the cortico-thalamo-basal ganglia circuit involved in motor functions, i.e., the motor loop (Alexander and Crutcher, 1990), at the striatal level. Although a previous study revealed no obvious compartmental difference in G_{olf} mRNA expression throughout striatal development in rats (Sakagami et al., 1995), we observed differential concentrations of G_{olf} IR in the striosome and matrix compartments, with higher densities of G_{olf} IR in the striosomes relative to the matrix. This finding suggests that G_{olf} may be a key molecule for controlling differential responses of striosome-matrix systems to D1R activation in adult mice. There is evidence that in experimental animal models with 6-OHDA-lesions (Hervé et al., 1993; Corvol et al., 2004; Alcacer et al., 2012; Ruiz-DeDiego et al., 2015) or in those with a total absence of D_{1} Rs due to D_{1}R gene targeting (Hervé et al., 2001), the upregulation of G_{olf} levels in the striatum is not accompanied by a parallel increase in G_{olf} mRNA expression. Thus, homeostatic regulation of striatal G_{olf} protein levels is thought to occur via post-translational mechanisms, wherein the altered expression of G_{olf} protein depends directly on its rate of usage (Hervé, 2011). We suggest that when compared to the matrix, the striosomes might have the lower levels of D1 Rs in D2-cells due to A2AR/G_{olf}/cAMP signal levels, which might result in an enhanced responsiveness to D1R activation. Abbreviations: PD, Parkinson’s disease; PD-D, PD with dyskinesia; ACh, acetylcholine; D1-cell, dopamine D1 receptor-expressing striatonigral medium spiny neuron; D2-cell, dopamine D2 receptor-expressing striatopallidal medium spiny neuron; D1R, dopamine D1 receptor; D2R, dopamine D2 receptor; G_{olf}, olfactory type G-protein α subunit.

**FIGURE 8 | Proposed diagram for dopaminergic regulation of G_{olf} levels that determine responsiveness to dopamine stimulation in striatal D1- and D2-cells.** The heights of the red and blue columns indicate the abundance of G_{olf} proteins in D1-cells and D2-cells, respectively. In PD mice, D1-cells might exhibit the dopamine D1R hypersensitivity caused by a dramatic increase in their G_{olf} levels, while D2-cells might show no apparent changes in their G_{olf} levels. In PD-D mice, D1-cells might show an increase in their G_{olf} levels, while D2-cells might show a decrease in their G_{olf} levels, which might result in an enhanced responsiveness to D1R activation. Abbreviations: PD, Parkinson’s disease; PD-D, PD with dyskinesia; ACh, acetylcholine; D1-cell, dopamine D1 receptor-expressing striatonigral medium spiny neuron; D2-cell, dopamine D2 receptor-expressing striatopallidal medium spiny neuron; D1R, dopamine D1 receptor; D2R, dopamine D2 receptor; G_{olf}, olfactory type G-protein α subunit.
unclear, it was also noted that in striatal membrane, the content in Gαolf protein would be almost one to two orders of magnitude higher than that in D1R or A2AR (Hervé, 2011).

**Striatal Gαolf as a Determinant of the Increased Responsiveness of D1-cells to Dopamine Stimulation in LID**

As shown in previous studies (Alcacer et al., 2012; Ruiz-DeDiego et al., 2015), we found a marked increase in striatal Gαolf protein levels in PD mice with 6-OHDA lesions. This is in line with evidence that dopamine depletions may lead to up-regulation of Gαolf protein expression in the rat striatum (Hervé et al., 1993; Marcotte et al., 1994; Penit-Soria et al., 1997; Corvol et al., 2004; Rangel-Barajas et al., 2011) and in the putamen in patients with PD (Corvol et al., 2004). Given the evidence that striatal levels of D1R (Shinotoh et al., 1993; Turjanski et al., 1997; Hurley et al., 2001) and other major mediators of D1R signaling (Girault et al., 1989; Nishino et al., 1993) are unchanged in PD patients, the dramatic increase in striatal Gαolf protein level may be a key event in the D1R hypersensitivity that develops in PD (Alcacer et al., 2012). In support of this notion, we detected no obvious changes in striatal D1R expression in PD mice.

Previous data have suggested that the up-regulation of Gαolf protein levels in the dopamine-depleted striatum is post-translational (Hervé et al., 1993; Ruiz-DeDiego et al., 2015) and results from the disuse of the D1Rs (Hervé et al., 2001). Indeed, daily administration of L-DOPA for 10 days resulted in a down-regulation of the increased Gαolf protein levels in the 6-OHDA-lesioned striatum in PD-D mice. However, we also found a significant increase in striatal Gαolf levels in PD-D mice when compared to naïve controls. In agreement with the changes in striatal Gαolf levels in PD and PD-D mice, in situ PLA also revealed that striatal D1R-Gαolf PLA signals were dramatically increased in PD mice and moderately increased in PD-D mice. These findings imply an increased responsiveness of D1-cells to D1R activation in PD-D mice, although this responsiveness is lower than that found in PD mice. Our assumption is also supported by the fact that, compared to naïve controls, a significant increase in the number of striatal c-Fos+ nuclei consequent to L-DOPA administration was evident in PD-D mice, although this increase was more pronounced in PD mice. Since Gαolf represents the rate-limiting factor in D1R-mediated cAMP production in D1-cells, these findings suggest that striatal Gαolf level acts as a determinant for the increased responsiveness of D1-cells to dopamine stimulation in LID (see Figure 8).

**Striatal Gαolf as a Determinant of the Increased Responsiveness of D2-cells to Dopamine Stimulation in LID**

It has been postulated that repeated exposure to dopaminergic agents leads to increased sensitivity of D2-cells to D2R activation in the dopamine-depleted striatum in experimental animals (Engber et al., 1989; Asin et al., 1995; Kashihara et al., 2000). However, no obvious increase in striatal D2R expression has been observed in PD patients treated with dopaminergic drugs (Rinne et al., 1981; Gutman and Seeman, 1985; Antonini et al., 1997; Thobois et al., 2004). In agreement with this notion, we found that pallidal c-Fos induction consequent to L-DOPA administration was more marked in PD-D mice compared to PD mice. On the other hand, there was increased expression of striatal D2Rs in PD mice, but not in PD-D mice. This indicates that the repeated administration of L-DOPA results in an increased responsiveness of D2-cells to striatal D2R activation in the dopamine-denervated striatum, and suggests that this phenomenon might underlie LID. Using an in situ PLA, we found that A2AR-Gαolf PL signals were markedly reduced along with Gαolf protein levels in the 6-OHDA-lesioned striatum of PD-D mice. This novel finding indicates that as in D1-cells, repeated exposure to L-DOPA causes down-regulation of Gαolf protein levels in D2-cells in the dopamine-depleted striatum. This then leads to the facilitation of the effects of dopamine on D2-cells by reducing A2AR/Gαolf signaling-mediated cAMP production (see Figure 8). This may be the reason that PD-D mice display an increased responsiveness of D2-cells to dopamine stimulation. However, the mechanism by which repeated and pulsatile injections of L-DOPA causes a decrease in A2AR/Gαolf PLA signals in PD-D remains a matter of speculation, as follows.

A2AR usage by endogenous adenosine results in a basal rate of Gαolf degradation (Hervé et al., 2001). It has been shown that in experimental animals with 6-OHDA-lesions, chronic (or persistent) dopamine depletions caused no significant changes (Ballarin et al., 1987; Herrera-Marschitz et al., 1994; Nomoto et al., 2000) or slight decrease (Pinna et al., 2002) in the extracellular adenosine levels in the striatum. In accordance with these findings, our present results also showed no significant changes in striatal levels of A2AR-Gαolf PLA signals in PD mice. Thus, we suggest that chronic dopamine depletions per se might cause no obvious changes in A2AR-Gαolf signaling activities that depend on the endogenous adenosine levels in striatal D2-cells. However, it is known that endogenous levels of adenosine are increased in response to the activation of N-methyl-D-aspartate (NMDA) receptors (Delaney and Geiger, 1998; Delaney et al., 1998), which can be facilitated by D1R stimulation (Cepeda and Levine, 2012; Morikagi and Goto, 2015), in the striatum. A landmark report has shown that in the rat striatum, transient (pulsatile) stimulation of D1Rs facilitates the NMDA receptor-dependent increase in extracellular adenosine levels (Harvey and Lacey, 1997). These findings suggest that in 6-OHDA-lesioned mice with D1R hypersensitivity, repeated exposure to L-DOPA may lead to a transient activation of D1Rs, which then enhances the NMDA receptor-dependent increase in adenosine release in the dopamine-denervated striatum. Moreover, Nash and Brotchie (2000) have shown that in striatal slices prepared from rats with 6-OHDA lesions, NMDA receptor activation could cause a marked increase in adenosine release and, thereby, indirectly stimulate A2ARs. Taken together, we speculate that in the
6-OHDA-lesioned striatum of PD-D mice, decreased Go_{olf} levels in D2-cells might be due to increased extracellular adenosine levels caused by the daily pulsatile activation of striatal D_{1}Rs. If our assumption is correct, striatal D_{1}R signals might contribute to regulation of the Go_{olf} protein levels in not only D1-cells but also D2-cells in the dopamine-depleted striatum.

Because adenosine/A_{2A}R signaling functionally opposes the actions of D_{2}Rs on D2-cells by its ability to increase the A_{2A}R/Go_{olf}-dependent cAMP production, it has so far been suggested that A_{2A}R antagonism may boost the anti-parkinsonian action of D_{2}R agonists in treating PD symptoms (Jenner, 2003; Schwarzschild et al., 2006; Fuxe et al., 2007; Huot et al., 2013). In addition, based on the evidence that striatal A_{2A}R expression might be increased in PD patients with dyskinesia (Calon et al., 2004; Ramachandran et al., 2011) and in dyskinetic animal models of PD (Jenner et al., 2009), it has also been suggested that adenosine A_{2A} sites might be a potential pharmacologic target for reducing LIDs (Jenner et al., 2009; Ramachandran et al., 2011; Huot et al., 2013; Kanda and Uchida, 2014). In Japan, istradefylline, an A_{2A}R antagonist, is currently used in clinics for treating PD patients (Kondo and Mizuno, 2015). The drug has shown to improve “off” time in patients with advanced PD, but has not shown anti-LID effects in the absence of a reduction in dopaminergic drug dosage. Adjunct use of istradefylline often causes dyskinetic effects in the absence of a reduction in dopaminergic drug dosage. Adjunct use of istradefylline often causes dyskinetic symptoms as a major adverse effect (Kondo and Mizuno, 2015). Considering usage-dependent Go_{olf} degradation through adenosine/A_{2A}R, we assume that in PD patients treated with L-DOPA, adenosine/A_{2A}R antagonism might be effective in reducing the “priming” of LID. However, once LID is established, adenosine/A_{2A}R antagonism might exacerbate dyskinetic symptoms. Our assumption may corroborate the notion that A_{2A}R activation might be required for dyskinesia “priming” mechanism (Brotchie, 2005).

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**CONCLUSION**

Because Go_{olf} protein level serves as a determinant of cAMP signal-dependent activity in both D1-cells and D2-cells in the striatum, Go_{olf} may represent an ideal target for the modulation of striatal functions under physiological and pathological conditions. Dysregulation of Go_{olf} expression has been associated with the pathophysiology of several brain disorders (Hervé, 2011). Of our particular interest is that the GNAL gene, which encodes Go_{olf}, is a causative gene in primary (toryon) dystonia (Fuchs et al., 2013). This is direct evidence that Go_{olf} plays a pivotal role in the “motor loop” of the cortico-basal ganglia circuits. Under parkinsonian conditions, dopamine depletion results in a crucial D_{1}R hypersensitivity in the striatum, which leads to the beneficial effects of L-DOPA in PD patients, but also generates LID. In this study, we found that in the 6-OHDA-lesioned striatum of PD mice, daily pulsatile administrations of L-DOPA may cause usage-induced changes in striatal Go_{olf} levels, leading to increased responsiveness to dopamine stimulation in both D1-cells and D2-cells. Thus we suggest that L-DOPA-induced changes in Go_{olf} levels in the dopamine-depleted striatum may be a key event in LID development.

**AUTHOR CONTRIBUTIONS**

SG conceived and designed the experiments. RM, SO and SG performed the experiments; analyzed the data; contributed reagents/materials/analysis tools. SG wrote the manuscript.

**ACKNOWLEDGMENTS**

This work was supported in part by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan (grants-in-aid for Scientific Research no. 24390223, 26461272, 26430054 and 16K10788) and Japan Agency for Medical Research and Development (AMED; no. 16ek0109182h0001).
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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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