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RGS proteins as critical regulators of motor function and their implications in Parkinson's disease[#]

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Running Title: RGS protein modulation of movement and Parkinson's disease

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List of non-standard abbreviations: ACh, acetylcholine; CNS central nervous system, DA, dopamine; DEP, disheveled, Egl-10, Pleckstrin homology; DHEX, DEP helical extension; DR, dopamine receptor; D_1R, dopamine 1 receptor; D_2R, dopamine 2 receptor; GAP, GTPase-activating protein; GGL, G gamma subunit-like; GPCR, G protein-coupled receptor; LPS, lipopolysaccharide; MPTP, methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MSNs, medium spiny neurons; PD, Parkinson's disease; RGS, regulator of G protein signaling; SNc, substantia nigra pars compacta; 6-OHDA, 6-hydroxydopamine; α-syn, α-synuclein.

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Abstract

Parkinson's disease (PD) is a devastating, largely non-familial, age-related disorder caused by the progressive loss of dopamine (DA) neurons in the SNc. Release of DA from these neurons into the dorsal striatum is crucial for regulating movement and their loss causes PD. Unfortunately, the mechanisms underlying SNc neurodegeneration remain unclear, and currently there is no cure for PD, only symptomatic treatments. Recently, several regulator of G protein signaling (RGS) proteins have emerged as critical modulators of PD pathogenesis and/or motor dysfunction and dyskinesia: RGSs 4, 6, 9 and 10. Striatal RGS4 has been shown to exacerbate motor symptoms of DA loss by suppressing M_4-autoreceptor-$G\alpha_{i/o}$ signaling in striatal cholinergic interneurons. RGS6 and RGS9 are key regulators of D_2R-$G\alpha_{i/o}$ signaling in SNc DA neurons and striatal medium spiny neurons (MSNs), respectively. RGS6, expressed in human and mouse SNc DA neurons, suppresses characteristic PD hallmarks in aged mice, including SNc DA neuron loss, motor deficits, and α-synuclein accumulation. Following DA depletion, RGS9, through its inhibition of MSN D_2R signaling, suppresses motor dysfunction induced by L-DOPA or D_2R-selective agonists. RGS10 is highly expressed in microglia, the brain's resident immune cells. Within the SNc, RGS10 may promote DA neuron survival through the upregulation of pro-survival genes and inhibition of microglial inflammatory factor expression. Thus, RGSs 4, 6, 9, and 10 are critical modulators of cell signaling pathways that promote SNc DA neuron survival and/or proper motor control. Accordingly, these RGS proteins represent novel therapeutic targets for the treatment of PD pathology.

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Significance statement

Parkinson's (PD), the most common movement disorder, is a progressive neurodegenerative disease characterized by SNc dopamine (DA) neuron loss and subsequent motor deficits. Current PD therapies only target disease motor symptomology and are fraught with side effects. Therefore, researchers have begun to explore alternative therapeutic options. Regulator of G protein signaling (RGS) proteins, whether primarily expressed in SNc DA neurons (RGS6), striatal neurons (RGSs 4 and 9), or microglia (RGS10), modulate key signaling pathways important for SNc dopamine neuron survival and/or proper motor control. As such, RGS proteins represent novel therapeutic targets in PD.

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Introduction

Parkinson's disease (PD) is a largely non-familial, progressive, neurodegenerative disorder characterized by the loss of dopamine (DA) neurons in the substantia nigra pars compacta (SNc) (Fahn, 2008, Meissner et al., 2011, Shulman et al., 2011, Mhyre et al., 2012). Under normal conditions, these DA neurons project to the striatum where they release DA onto postsynaptic targets, allowing for proper control of motor behavior. Therefore, loss of SNc DA neurons results in the dysregulation of DA signaling within the nigrostriatal pathway (**Figure 1**) and the hallmark motor deficits associated with PD, including bradykinesia, muscle rigidity, and resting tremors. PD is widely considered an idiopathic disorder (Fearnley and Lees, 1991) with identifiable genetic mutations accounting for only 5-9% of clinical cases (Nussbaum and Ellis, 2003). Currently, the primary risk factor associated with PD is aging (Fearnley and Lees, 1991), with the idiopathic form of the disorder affecting 2% of the population over 60 and 15% of those individuals over the age of 85. Despite decades of research, the molecular characteristics/mechanisms that predispose SNc DA neurons to age-related degeneration remain unknown. As a result, there is currently no cure for PD, only symptomatic treatments.

The prevalence of PD is vast, with nearly 1 million people in the United States suffering from the disease, and continues to rise as the proportion of elders in our society expands. With 60,000 new PD diagnoses annually, there has been considerable interest in developing novel and improved therapeutic alternatives. Currently, primary therapeutic strategies used to treat PD-related motor symptoms work to correct the dysregulated DA signaling, and include drugs such as levodopa (L-DOPA), a blood brain barrier-permeable DA precursor, and DA receptor (DR) agonists. However, while these drugs are effective in the short-term at correcting PD-related motor deficits, they are fraught with problems of their own, including *wearing-off* phenomena (Pahwa and Lyons, 2009, Jenner, 2013), dyskinesias (Schrag and Quinn, 2000, Thanvi et al., 2007), and various non-motor complications (Chaudhuri et al., 2006, Poewe,

2008) . Due to these issues, researchers have begun to explore whether drugs that modulate non-dopaminergic neurotransmission systems could be of use in PD. G protein-coupled receptors (GPCRs), the largest family of cell-surface receptors encoded by the genome, have provided promising avenues for alternative PD therapies. In particular, animal models of PD have implicated modulation of noradrenergic, cholinergic, adenosinergic, glutamatergic and serotonergic neurotransmission as possible adjunctive therapies to current dopaminergic treatments (reviewed by Lemos et al., 2018). Unfortunately, high levels of conservation in the orthosteric binding site within GPCR subfamilies has limited the development of receptor-subtype specific drugs and thus, like the dopaminergic therapies currently employed in PD, these treatment options would likely have numerous side effects. As such, it is of the utmost importance to identify alternative methods of modulating GPCR signaling within a subfamily. Regulator of G protein signaling (RGS) proteins may hold the key.

RGS proteins modulate the magnitude and duration of GPCR signaling by facilitating heterotrimeric G protein inactivation through their GTPase-activating (GAP) activity toward Gα subunits, a function bestowed by their RGS domain. The RGS protein family includes 20 canonical RGS proteins, four of which have been implicated in PD pathogenesis, RGSs 4, 6, 9, and 10 (**Figure 2**). Whether they are expressed primarily in SNc DA neurons (RGS6), striatal neurons (RGSs 4 and 9), or the brain's resident immune cells (microglia, RGS10), these RGS proteins have been shown to modulate key signaling pathways that are important for SNc DA neuron survival and/or proper motor control. As such, these RGS proteins represent novel therapeutic targets for the treatment of Parkinson's pathology.

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RGS4

RGS4 is a small 23 kDa member of the R4 family (**Figure 2**) selectively expressed in the central nervous system (CNS) and heart of both humans and rodents (Bansal et al., 2007, Erdely et al., 2004, Zhang et al., 1998). As a member of the R4 family, RGS4 functions as a GAP for $G\alpha_q$ and $G\alpha_{i/o}$. Within the CNS, RGS4 mRNA is predominantly expressed in the amygdala and striatum but is also present in most cortical neuronal layers (Ebert et al., 2006).

Various studies have implicated striatal RGS4 in the regulation of CNS cholinergic and dopaminergic signaling, as well as endocannabinoid-mediated long-term depression (Lerner and Kreitzer, 2012, Ding et al., 2006, Geurts et al., 2003). In PD, degeneration of SNc DA neurons is accompanied by an increase in striatal acetylcholine (ACh) release, resulting in exacerbated motor symptoms. It was first hypothesized that this increase in cholinergic neurotransmission resulted from reduced D_2R-mediated inhibition of synaptic Ca_v2 channels in striatal cholinergic interneurons (**Figure 1**). However, Ding *et al.* (2006) demonstrated that, while Ca_v2 activity remained unchanged following DA depletion, its activity was attenuated by the M_4 muscarinic autoreceptor (M_4R) expressed on these interneurons.

Given that M_4 autoreceptors are $G\alpha_{i/o}$-coupled, their signaling may be regulated by RGS4 (**Figure 3**). Several studies, some conflicting, have examined the expression and function of striatal RGS4 in the context of DA depleted rodent PD models. Geurts *et al.* (2003) initially described a significant *reduction* in striatal mRNA expression of both RGS4 and RGS9 following unilateral 6-hydroxydopamine (6-OHDA) lesion or reserpine (adrenergic blocker)-mediated DA depletion. However, Ding *et al.* (2006) demonstrated the opposite effect of these treatments on striatal RGS4 mRNA expression. In their study, *increased* RGS4 mRNA expression following either of these treatments was accompanied by a marked attenuation in M_4R signaling and increased striatal ACh release. Using intracellular dialysis, they discovered that RGS4 inhibits M_4 autoreceptor-mediated Ca_v2 activity in striatal cholinergic interneurons (**Figure 3**). In agreement with these findings, Ko *et al.* (2014) reported not only elevated RGS4

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expression in L-DOPA treated 6-OHDA-lesioned rats, but also that RGS4 mRNA suppression during L-DOPA priming attenuated the development of drug-induced dyskinesia. Similarly, RGS4$^{-/-}$ mice exhibited fewer motor behavioral deficits following 6-OHDA lesion (Lerner and Kreitzer, 2012). However, another study reported that RGS4$^{-/-}$ mice are not protected from 6-OHDA-induced injury and motor dysfunction (Ashrafi et al., 2017).

Collectively, these studies (Ding et al., 2006, Ko et al., 2014) suggest RGS4 inhibition may be useful in treating drug-induced dyskinesias in PD. In light of this, Blazer *et al.* (2015) described the selectivity of a thiadiazolidinone inhibitor (CCG-203769) they discovered for RGS4 over other RGS proteins that might represent a novel therapeutic option for PD. However, RGS4 has not been implicated in modulating SNc DA neuron loss and it remains unclear whether it promotes motor deficits following DA depletion through its striatal actions.

RGS6

RGS6 is a member of the R7 RGS subfamily, which modulates $G\alpha_{i/o}$ signaling (Hooks et al., 2003) and shares two unique domains in addition to the RGS domain: the disheveled, Egl-10, Pleckstrin homology (DEP)/DEP helical extension (DHEX) domain and the G gamma subunit-like (GGL) domain. The DEP/DHEX domain allows R7 family members to associate with the membrane anchor proteins R7BP and R9AP (Drenan et al., 2006, Martemyanov et al., 2005), while the GGL domain promotes interaction with the atypical Gβ subunit, $G\beta_5$, which is required for stabilization of all R7 family members (**Figure 2**) (Witherow et al., 2000, Snow et al., 1999, Posner et al., 1999, Chen et al., 2003, Porter et al., 2010, Narayanan et al., 2007, Cheever et al., 2008).

RGS6 is expressed in a wide variety of tissues throughout the body (Gold et al., 1997, Bifsha et al., 2014, Stewart et al., 2015, Maity et al., 2012, Maity et al., 2011, Yang et al., 2010, Stewart et al., 2014) with highest mRNA and protein levels expressed in the brain . When Chatterjee *et al.* first cloned RGS6 (2003) using a Marathon-ready human brain cDNA library,

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they described multiple RGS6 splice variants predicted to produce 36 distinct RGS6 protein isoforms containing either long (RGS6L, ~49-56kDa) or short (RGS6S, ~32-40kDa) N-terminal domains, an incomplete or intact GGL domain, and 9 alternative C-terminal sequences. While sequence similarities have complicated the study of individual RGS6 protein isoforms, Bifsha *et al.* (2014) and Luo *et al.* (2019) have demonstrated that RGS6L isoforms may be key survival factors for SNc DA neurons.

RGS6 was first implicated in PD when it was discovered that *RGS6* was the most differentially lost gene in ventral SNc DA neurons in a developmental PD model, Pituitary homeobox 3 (PitX3)-deficient mice (Bifsha et al., 2014). Subsequent immunohistochemical analyses revealed that RGS6 was exclusively expressed in DA neurons within the SNc of both mice and humans that are lost with PD (Luo et al., 2019, Bifsha et al., 2014). Comparative phenotyping of $RGS6^{+/+}$ and $RGS6^{-/-}$ mice revealed that RGS6 acts as a critical survival factor for SNc DA neurons that, when lost, results in their late-age degeneration (Bifsha et al., 2014), as well as PD-like motor deficits, including reduced mobility (open field test and rotarod) and abnormal gait (DigiGait analysis) (Luo et al., 2019). In aged $RGS6^{-/-}$ mice, SNc DA neuron degeneration is associated with markers of pathological change (Fluoro-Jade C and Nissl staining) as well as reduced levels of the DA precursor synthesizing enzyme, tyrosine hydroxylase (TH), and the vesicular DA transporter, Vmat2. In addition, SNc DA neuron degeneration is accompanied by enhanced D_2-autoreceptor signaling, increased expression of the DA transporter (DAT) (Bifsha et al., 2014), and increased sensitivity of $RGS6^{-/-}$ mice to quinpirole (D_2R agonist) suppression of locomotion (Luo et al., 2019), known to be mediated by the nigrostriatal D_2-autoreceptor (Bello et al., 2011, Lindgren et al., 2003, Usiello et al., 2000, Wang et al., 2000). All of these molecular changes likely contribute to the dysregulated production and release/re-uptake of DA in the nigrostriatal circuit of aged $RGS6^{-/-}$ mice, cytotoxic DA byproduct (DOPAL) accumulation, and the observed PD-like motor deficits (**Figure 4**) (Luo et al., 2019).

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The expression of several genes that had previously been associated with Parkinson's, such as: *DJ-1* (*PARK7*), *PINK1* (*PARK6*), *LRRK2* (*PARK8*) and *SNCA* (α-synuclein, α-syn) were also altered in RGS6$^{-/-}$ mice (Bifsha et al., 2014). Of particular interest, immunohistochemical analysis of aged RGS6$^{-/-}$ mice revealed that they exhibited abnormally high levels of the α-syn protein (Luo et al., 2019), a hallmark of PD which is believed to contribute to neurodegeneration (Spillantini et al., 1998, Spillantini et al., 1997, Chartier-Harlin et al., 2004, Kim, 2013, Singleton et al., 2003, Stefanis, 2012, Chu and Kordower, 2007, Li et al., 2004, Giasson et al., 2002, Masliah et al., 2000). The α-syn protein observed in aged RGS6$^{-/-}$ mice, unlike that observed in young or wild type animals, was primarily extracellular and, as revealed by western analysis, highly oligomeric (Luo et al., 2019).

RGS6 likely suppresses late-age-onset SNc DA neuron death and α-syn accumulation through its negative regulation of the SNc D_2-autoreceptor-$G\alpha_{i/o}$-cAMP/PKA signaling axis (**Figure 4**) (Luo et al., 2019). Neuronal cAMP/PKA levels are controlled by GPCRs coupled to either $G\alpha_s$ or $G\alpha_{i/o}$, which function to increase or decrease cAMP, respectively. Mittal *et al.* (2017) discovered that β-agonists, which signal through $G\alpha_s$-linked β-adrenergic receptors, dramatically reduce both α-syn expression and human PD incidence while also inhibiting 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced SNc DA neuron loss in mice. RGS6$^{-/-}$ mice exhibit hyperactive SNc D_2 autoreceptor-$G\alpha_{i/o}$ signaling and reduced SNc DA neuron PKA signaling (Luo et al., 2019), suggesting that RGS6 likely regulates SNc DA neuron survival and α-syn expression through cAMP-mediated mechanisms as well.

In summary, the findings of Bifsha *et al.* (2014) and Luo *et al.* (2019) are significant, as they reveal that the loss of a single gene, *RGS6*, phenocopies late-age-onset PD in mice (**Figure 4**).

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RGS9

RGS9 is a member of the R7 RGS protein subfamily, and therefore, like RGS6, modulates $G\alpha_{i/o}$ signaling (Hooks et al., 2003), contains the DEP/DHEX and GGL domains, and is stabilized by $G\beta_5$ (**Figure 2**) (Witherow et al., 2000, Chen et al., 2003, Porter et al., 2010, Cheever et al., 2008). RGS9 exists as two isoforms: RGS9-1, a ~56kDa protein expressed in the retina (He et al., 1998), and RGS9-2, a ~77kDa protein largely expressed in the striatum (Zhang et al., 1999, Rahman et al., 1999, Gold et al., 1997, Thomas et al., 1998, Liou et al., 2009).

RGS9 was first implicated in PD when Tekumalla *et al.* (2001) reported elevated RGS9 protein expression in the striatum of PD patients. One complication of this study was that patients had received L-DOPA, which made it difficult to determine whether the elevation in RGS9 expression was the result of the disease or its treatment. This issue remained unclarified until Geurts *et al.* (2003) reported that rat striatal RGS9 mRNA expression was reduced following 6-OHDA nigrostriatal lesioning, a finding that was further corroborated by Kovoor *et al.* (2005) who reported that $RGS9^{-/-}$ mice were more susceptible to 6-OHDA-induced PD than $RGS9^{+/+}$ mice.

In the striatum, RGS9-2 inhibits D_2R-$G\alpha_{i/o}$ signaling to modulate motor function (**Figure 5**), as demonstrated through investigation of the impact of D_1R- and D_2R-selective agonists on circling behavior of rats overexpressing RGS9-2 in the ventral striatum (nucleus accumbens) (Rahman et al., 2003). Unilateral striatal overexpression of RGS9-2 and treatment with apomorphine or quinpirole (D_2R-selective agonists) induced a strong directional rotation bias towards the side of RGS9-2 overexpression. In contrast, treatment with the D_1R-selective agonist (SKR81297) did not induce a directional rotation bias. Since the authors had demonstrated that RGS9-2 can regulate DA signaling in the basal ganglia, they subsequently wanted to determine the impact of RGS9-2 loss on locomotion and DA receptor expression. Therefore, they performed locomotor analyses on $RGS9^{-/-}$ mice. These analyses revealed that

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RGS9 loss is associated with an enhanced locomotor response to amphetamine despite D_1R and D_2R expression levels remaining unaltered in the ventral striatum (Rahman et al., 2003).

Kovoor *et al.* (2005) bolstered these findings by revealing that, while $RGS9^{-/-}$ mice display normal locomotory behavior, they exhibit severe abnormal involuntary movements following treatment with reserpine (adrenergic blocker) in combination with quinpirole or apomorphine. In contrast, reserpine in combination with the D_1R-selective agonist did not induce abnormal movement behaviors. Similarly, RGS9-2 overexpression in striatum of monkeys with a MPTP lesion reduced the incidence of L-DOPA induced dyskinesia, without minimizing L-DOPA's antiparkinsonian effects (Gold et al., 2007).

In summary, these findings indicate that RGS9 plays a critical role in modulating motor movement through its ability to inhibit D_2R-$G\alpha_{i/o}$ signaling in the striatum. These findings implicate a post-synaptic role of RGS9 (**Figure 5**) versus the pre-synaptic role of RGS6 (**Figure 4**) in controlling nigrostriatal movement.

RGS10

RGS10 is a small 20 kDa member of the R12 RGS subfamily (**Figure 2**) that functions as a GAP for $G\alpha_{i/o/q/z}$ (Hunt et al., 1996) and is highly expressed in brain regions associated with higher brain function, including the hippocampus, striatum, and dorsal raphe (Gold et al., 1997). RGS10 is unique among the RGS proteins we have discussed thus far in that it is highly expressed in the brain's resident immune cells, microglia, in addition to its low neuronal expression (**Figure 6**) (Waugh et al., 2005).

Microglia and neuroinflammation have been widely implicated in PD pathogenesis (reviewed by Subhramanyam et al., 2019; Joers et al., 2017). Not only are reactive/activated microglia and the inflammatory mediators they produce observed in the brains of PD patients, but various PD-associated chemicals and neurotoxins, such as 6-OHDA, MPTP, and bacterial

lipopolysaccharides (LPS), are known to cause microglial activation and may at least partially induce neuronal cell death through this activation. Furthermore, α-syn may sensitize DA neurons to inflammation-induced cell death as well as activate microglia and be modified by microglia, subsequently promoting DA neuron death. Finally, it has been shown that non-steroidal anti-inflammatory drugs may lower the incidence of PD and inhibition of tumor necrosis factor α (TNFα) or the LPS receptor (toll-like receptor 4, TLR4) may reduce DA neuron death.

In addition to contributing to PD progression, there is evidence suggesting that inflammation may be one initiating factor in PD (reviewed by Tansey and Goldberg, 2010). Aging, the greatest and least understood risk factor associated with idiopathic PD, is known to prime microglia toward activation, resulting in exacerbated inflammation. This priming process and the resulting increased inflammation are predicted to be particularly detrimental in areas of the brain that contain a higher density of microglia, such as the midbrain, and to neuronal populations undergoing a high degree of oxidative processes, such as DA neurons. Supporting this prediction that midbrain DA neurons may be particularly susceptible to microglial priming/activation and inflammation, viruses/conditions associated with inflammation, such as influenza (influenza pandemic 1914-1918), Japanese encephalitis virus (JEV) exposure, and Crohn's disease, have all been associated with increased PD risk.

As RGS10 is enriched in microglia and a mutation in the *RGS10* gene had already been linked to neurodegenerative age-related maculopathy (Jakobsdottir et al., 2005, Schmidt et al., 2006), Lee *et al.* (2008) hypothesized that RGS10 loss could predispose an organism to PD, possibly through exaggerated microglial activation. In this initial investigation, Lee *et al*. (2008) demonstrated that RGS10$^{-/-}$ mice suffer from increased CNS microglial burden (seen on mixed 129/C57/BL6 background but not on pure C57/BL6 background (Kannarkat et al., 2015)) and activation. Furthermore, RGS10$^{-/-}$ mice were particularly susceptible to LPS-mediated SNc DA neuron degeneration. Consistent with this finding and the known role of LPS in microglia inflammatory induction, primary microglia isolated from RGS10$^{-/-}$ mice had dysregulated

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inflammatory gene expression profiles under basal conditions and following LPS stimulation. Similar results were obtained from the BV2 murine microglia cell line after RGS10 knockdown. Interestingly, Lee *et al.* also demonstrated that culture media taken from LPS-treated BV2 cells induced MN9D (mesencephalon DA neuroblastoma cell line) cell death which could be prevented by the TNF receptor decoy etanercept. Together, these results suggest that RGS10 functions as a pro-survival factor in PD by inhibiting microglia activation/inflammatory factor production and subsequent DA neuron cell death. However, Lee *et al.* argued that RGS10 not only promoted DA neuron survival indirectly, but also directly as RGS10 ablation in MN9D cells sensitized them to the toxic effects of LPS-treated BV2 cell media, an effect that was once again prevented by etanercept (**Figure 6**).

In subsequent publications, Lee *et al.* (2011, 2012) provided further mechanistic insight into the findings described above. In their 2008 publication, Lee *et al.* demonstrated that RGS10 translocates from the cytoplasm into the nucleus of primary microglia following LPS exposure. Therefore, they hypothesized that, in addition to regulating GPCR-$G_{i/o}$ signaling, RGS10 may also limit pro-inflammatory factor expression by inhibiting NF-κB (Lee et al., 2011). In support of this hypothesis, NF-κB subunit (p65 and p50) expression and transcriptional activity (NF-κB-luciferase reporter plasmid) were significantly increased in $RGS10^{-/-}$ primary microglia following TNFα and/or LPS exposure. Furthermore, re-expression of RGS10 in $RGS10^{-/-}$ primary microglia reduced LPS-stimulated inflammatory factor expression (*i.e.* TNFα), and media toxicity toward MN9D cells. Since TNFα is required for SNc DA neuron degeneration following 6-OHDA administration (McCoy et al., 2006, McCoy et al., 2008) and RGS10 appears to modulate microglia TNFα production, Lee *et al.* (2011) performed viral expression experiments to determine whether RGS10 overexpression could protect against 6-OHDA lesioning. Compared to 6-OHDA/lenti-GFP-injected rats, rats injected with 6-OHDA/lenti-RGS10 virus displayed significantly decreased microglial activation and DA neuron degeneration (Lee et al., 2011). Together, these results indicate that RGS10 promotes DA

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neuron survival indirectly by inhibiting microglial NF-κB-mediated expression of pro-inflammatory factors, particularly TNFα (**Figure 6**). In a later publication, Lee *et al.* (2012) described the direct pro-survival role of RGS10 in DA neurons. They reported that TNFα reduces MN9D RGS10 protein expression and that stable overexpression of wild type RGS10, but not the RGS10-S168A (RGS10SA, resistant to PKA phosphorylation) mutant, in the MN9D cells reduced TNFα-toxicity. This reduction in TNFα-toxicity may relate to RGS10's ability to potentiate PKA-CREB-mediated pro-survival gene (Bcl-2) expression. Together, these results indicate that PKA-mediated RGS10 phosphorylation and RGS10's subsequent promotion of PKA-CREB signaling may underly DA neuron survival (**Figure 6**).

In summary, the work by Lee *et al.* (2012, 2011, 2008) suggests that RGS10 may directly promote DA neuron survival by potentiating PKA-mediated CREB phosphorylation and pro-survival gene expression, as well as indirectly by inhibiting NF-κB-mediated inflammatory factor expression (**Figure 6**). Finally, this group has now published evidence suggesting that RGS10 expression may be reduced with age, causing dysregulation of immune/inflammatory pathways that could possibly contribute to PD initiation (Kannarkat et al., 2015).

Conclusion

The studies described here suggest that RGSs 4, 6, 9, and 10 are critical modulators of both G protein-dependent and -independent cell signaling pathways that promote SNc DA neuron survival and/or proper motor control. Together, these findings implicate RGSs 4, 6, 9, and 10 as novel therapeutic targets for the treatment of PD pathology, not just symptomology. Though the role of these RGS proteins in PD has clearly been established through genetic animal models, at present, they cannot be tested pharmacologically. Indeed, currently identified RGS protein inhibitors are not selective (Hayes et al., 2018, O'Brien et al., 2019). In addition, these inhibitors lack required tissue and neuronal specificity and likely will affect multiple tissues where these RGS proteins are expressed. Finally, no drugs have been identified that increase the activity of

any member of the RGS protein family as would be needed to target RGSs 6, 9 and 10 for PD treatment.

Currently, the majority of pharmaceuticals targeting GPCR signaling disrupt the pathway at the extracellular ligand-GPCR interface (reviewed by Neubig and Siderovski, 2002). While these pharmacological therapies successfully inhibit GPCR signaling, they often lack tissue specificity. Similarly, the search for selective RGS inhibitors and/or activators has proven difficult (Hayes et al., 2018, Neubig and Siderovski, 2002, O'Brien et al., 2019). In evidence of this, Hayes *et al.* (2018) recently demonstrated that each of 13 identified RGS4 inhibitors inhibited other members of the RGS protein family, sometimes with equal or greater potency than for RGS4. The polypharmacology of known RGS inhibitors may reflect their cysteine-dependent inhibition mechanism, and for this reason, none of these identified inhibitors affect the R7 members RGS6 and RGS7, which lack reactive Cys in their RGS domains. In addition, targeting of intracellular RGS protein-protein interactions has proven extremely challenging (reviewed by Neubig and Siderovski, 2002).

In recent years, limitations in both pharmacological compound selectivity and therapeutic benefits in PD have prompted the search for and development of novel non-pharmacological therapies. One of the most recent non-pharmacological approaches to emerge in PD treatment is gene therapy. Gene therapy entails viral delivery of genetic material to a patient either to modify (*i.e.* activate or suppress) endogenous gene expression or to introduce exogenous genes. Both AAV and lentiviral approaches, which are attractive due to their long-term expression efficacy and lack of immunogenicity, have been investigated in clinical trials for PD treatment in humans (reviewed by Hitti et al., 2019). Currently, clinical trials utilizing gene therapy for PD treatment are aimed at enhancing DA synthesis (AAV-AADC), promoting neuronal survival via enhanced neurotrophic factor expression (AAV-NTN), or promoting proper motor function through modification of basal ganglia signaling (AAV-GAD) (Hitti et al., 2019, Elkouzi et al., 2019, Muramatsu, 2010) . Finally, the use of CRISPR is also now under

investigation to modulate gene function in the mammalian brain (Zhou et al., 2018, Swiech et al., 2015, Heidenreich and Zhang, 2016) that may prove useful in PD therapeutics.

As discussed above, while RGSs 4, 6, 9, and 10 have been shown to be critical modulators or SNc DA neuron survival and/or motor function, the difficulty in creating selective activating (RGS6, 9, and 10) and inhibitory (RGS4) compounds limits their pharmacological usefulness. Therefore, future studies should focus on developing novel gene therapy approaches to selectively enhance (RGS6, RGS9, and RGS10) or diminish (RGS4) RGS protein signaling in the SNc (RGS6 and RGS10) or striatum (RGS9 and RGS4) of PD patients. Such strategies may provide new PD therapies that not only work to correct symptomology, but that also prevent pathology.

Authorship Contributions

Wrote or contributed to the writing of the manuscript: Ahlers-Dannen, K.E., Spicer, M.M., and Fisher, R.A.

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Legends for Figures:

Figure 1: Model of motor control by the nigrostriatal circuit. DA neurons (blue) originating in the substantia nigra project to the striatum, where they release DA onto D_1R- and D_2R-containing GABAergic (red) medium spiny neurons and D_2R-containing cholinergic interneurons (orange). DA, through its interaction with the $G\alpha_s$-coupled D_1R, promotes neuronal signaling. In contrast, D_2R-$G\alpha_{i/o}$ activation ultimately inhibits neuronal signaling. DA release in the striatum silences GABAergic neurons of the globus pallidus (GP) by enhancing the activity of D_1R-containing GABAergic MSNs of the direct pathway, increasing GABA release into the GP, and by silencing D_2R-containing GABAergic MSNs that begin the indirect pathway, ultimately decreasing glutamate release into the GP. Silencing of GP GABAergic neurons promotes thalamic glutamatergic (green) signaling to the cortex and proper motor control. Solid axons denote pathways that are "on" and dashed axons represent pathways that are "silent".

Figure 2: Comparison of the protein structure and interaction partners for RGS proteins implicated in PD and proper motor function. RGSs 4, 6, 9, and 10 have been implicated in PD pathogenesis and proper motor function. RGS4 is member of the R4 RGS subfamily that, through its RGS domain, functions as a GAP for $G\alpha_{i/o/q}$. RGSs 6 and 9 are members of the R7 RGS subfamily that, through their RGS domains, function as GAPs for $G\alpha_{i/o}$. Members of the R7 subfamily are characterized by two unique domains outside of their RGS domain, the DEP/DHEX domain and the GGL domain. The DEP/DHEX domain allows R7 family members bind to the membrane anchor proteins R7BP or R9AP, while the GGL domain promotes interaction with $G\beta_5$, which is required for stabilization of all R7 family members. RGS10 is the smallest RGS protein (~20kDa) and is a member of the R12 RGS subfamily, thus functioning as a GAP for $G\alpha_{i/o/q/z}$. Disheveled, Egl-10, Pleckstrin homology (DEP), DEP helical extension (DHEX), G gamma subunit-like (GGL)

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Figure 3: Model of RGS4's role in regulating striatal ACh release from cholinergic interneurons. RGS4 in striatal cholinergic interneurons inhibits M_4 autoreceptor signaling to promote calcium influx through $Ca_v2.2$. By preventing striatal cholinergic M_4-autoreceptor-$G\alpha_{i/o}$-mediated inhibition of $Ca_v2.2$, RGS4 promotes ACh packaging/release. This figure depicts striatal cholinergic interneurons (orange) synapsing on GABAergic D_1R- and D_2R-containing MSNs (red). These MSNs also likely express M_1 and M_2 ACh receptors. $G\alpha_{i/o}$-coupled receptors are red, $G\alpha_s$-coupled receptors are green, and $G\alpha_q$-coupled receptors are blue.

Figure 4: Model of RGS6's role in regulation of SNc D_2 autoreceptor signaling. RGS6 in SNc DA neurons inhibits D_2-autoreceptor signaling to promote proper DA homeostasis and neurotransmission as well as prevent aberrant α-synuclein accumulation. By inhibiting SNc D_2-autoreceptor-$G\alpha_{i/o}$ signaling, RGS6 promotes DA packaging/release by preventing Vmat2 downregulation and DA transporter (DAT) upregulation/activation. In addition, RGS6 inhibition of D_2-autoreceptor-$G\alpha_{i/o}$ signaling promotes cAMP/PKA signaling, increasing DA synthesis (TH phosphorylation) and suppressing α-synuclein expression. β-agonists, which have been shown to reduce PD incidence work in a similar fashion. In contrast, RGS6 loss, as seen in the $RGS6^{-/-}$ mouse model, disinhibits SNc D_2-autoreceptor-$G\alpha_{i/o}$ signaling, reducing cAMP–mediated DA synthesis and increasing α-synuclein accumulation. Furthermore, RGS6 loss is associated with cytotoxic DA (DOPAL) accumulation due to SNc D_2-autoreceptor-$G\alpha_{i/o}$-mediated Vmat2 downregulation and DAT upregulation. This figure depicts SNc DA neurons (blue) synapsing on GABAergic D_1R- and D_2R-containing MSNs (red). $G\alpha_{i/o}$-coupled receptors are red and $G\alpha_s$-coupled receptors are green.

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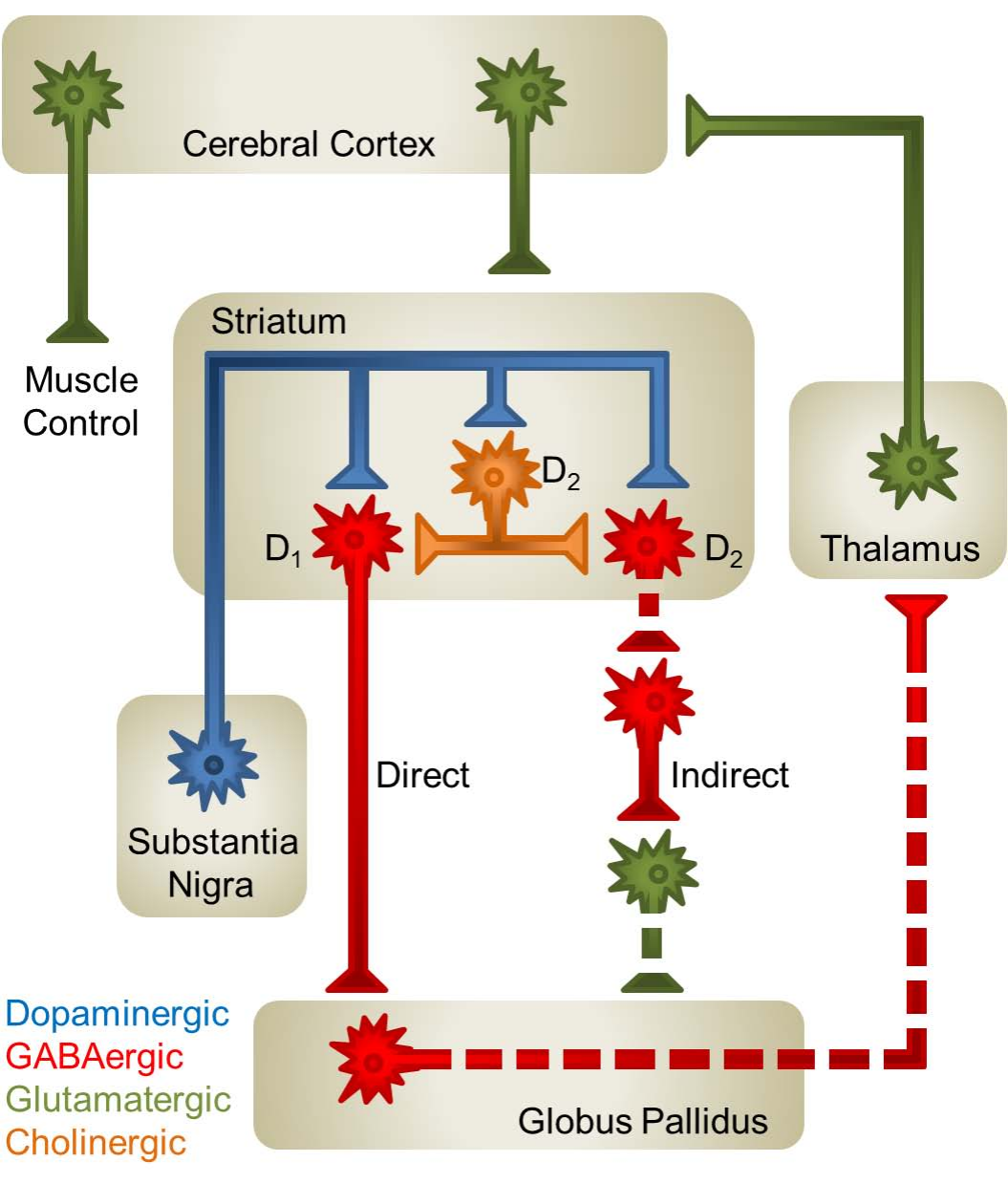
Figure 5: Model of RGS9's role in regulation of striatal D_2Rs to promote proper motor control. RGS9 inhibits D_2R signaling in striatal indirect MSNs (iMSNs) promoting neuronal activity/excitability to regulate motor function. By inhibiting striatal iMSN D_2R-G$\alpha_{i/o}$ signaling, RGS9 suppresses βγ-mediated GIRK channel activation, promoting depolarization and neuronal firing. This figure depicts SNc DA neurons (blue) synapsing on GABAergic D_2R-containing iMSNs (red). G$\alpha_{i/o}$-coupled receptors are red.

Figure 6: Model of RGS10's role in modulating SNc DA neuron survival. TNFα, an inflammatory factor, can induce neuronal cell death through activation of the TNFR-Fas-associated protein with death domain (FADD)-caspase pathway. Cell culture studies suggest that PKA phosphorylated RGS10 directly promotes MN9D DA cell survival by potentiating PKA-mediated CREB activation and pro-survival gene (Bcl-2) expression. However, RGS10 may also promote cell survival indirectly by inhibiting TNFR/TLR4 (LPS receptor)-NF-κB-mediated inflammatory factor (*i.e.* TNFα) expression by microglia, the brain's resident immune cells. Whether acting directly or indirectly to promote DA cell survival, TNFα works to counteract RGS10's positive effects by reducing its expression. This diagram depicts a microglial cell (tan) in close association with a DA neuron (blue) in the SNc. However, the role of RGS10 SNc DA neurons *in vivo* has yet to be directly examined. Tumor necrosis factor α (TNFα), Fas-associated protein with death domain (FADD), Toll-like receptor 4 (TLR4), bacterial lipopolysaccharides (LPS).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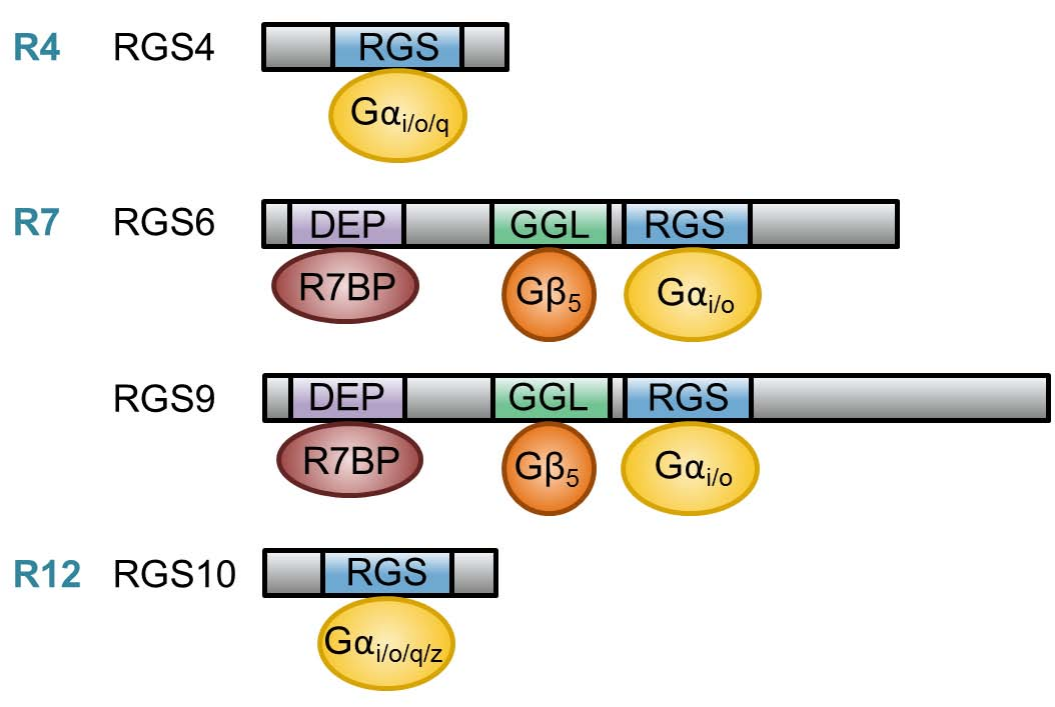
Striatum
Muscle Control
D2
D1
D2
Thalamus
Substantia Nigra
Direct
Indirect
Dopaminergic
GABAergic
Glutamatergic
Cholinergic
Globus Pallidus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RGS
Gαi/o/q
R7
RGS6
DEP
GGL
RGS
R7BP
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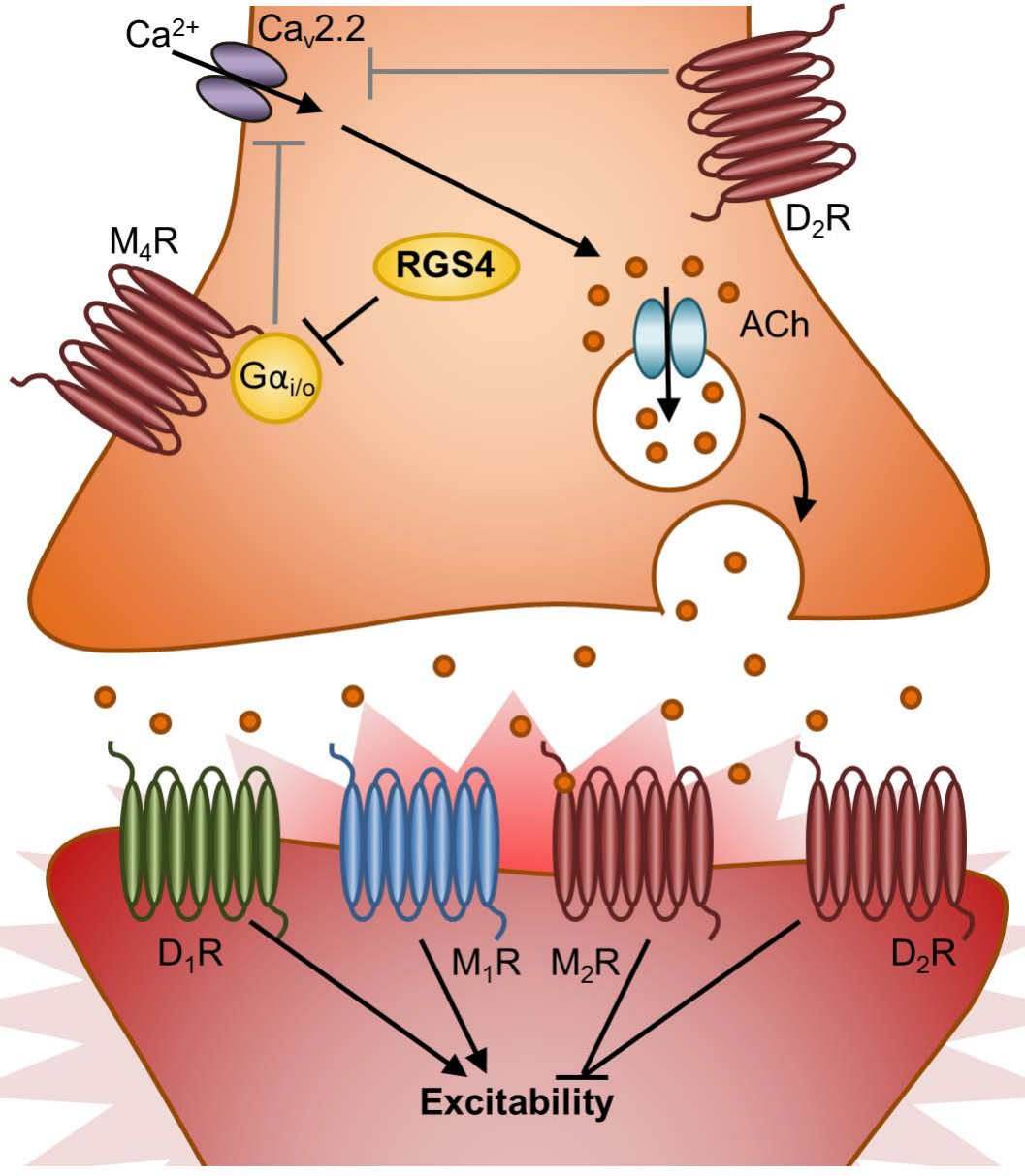
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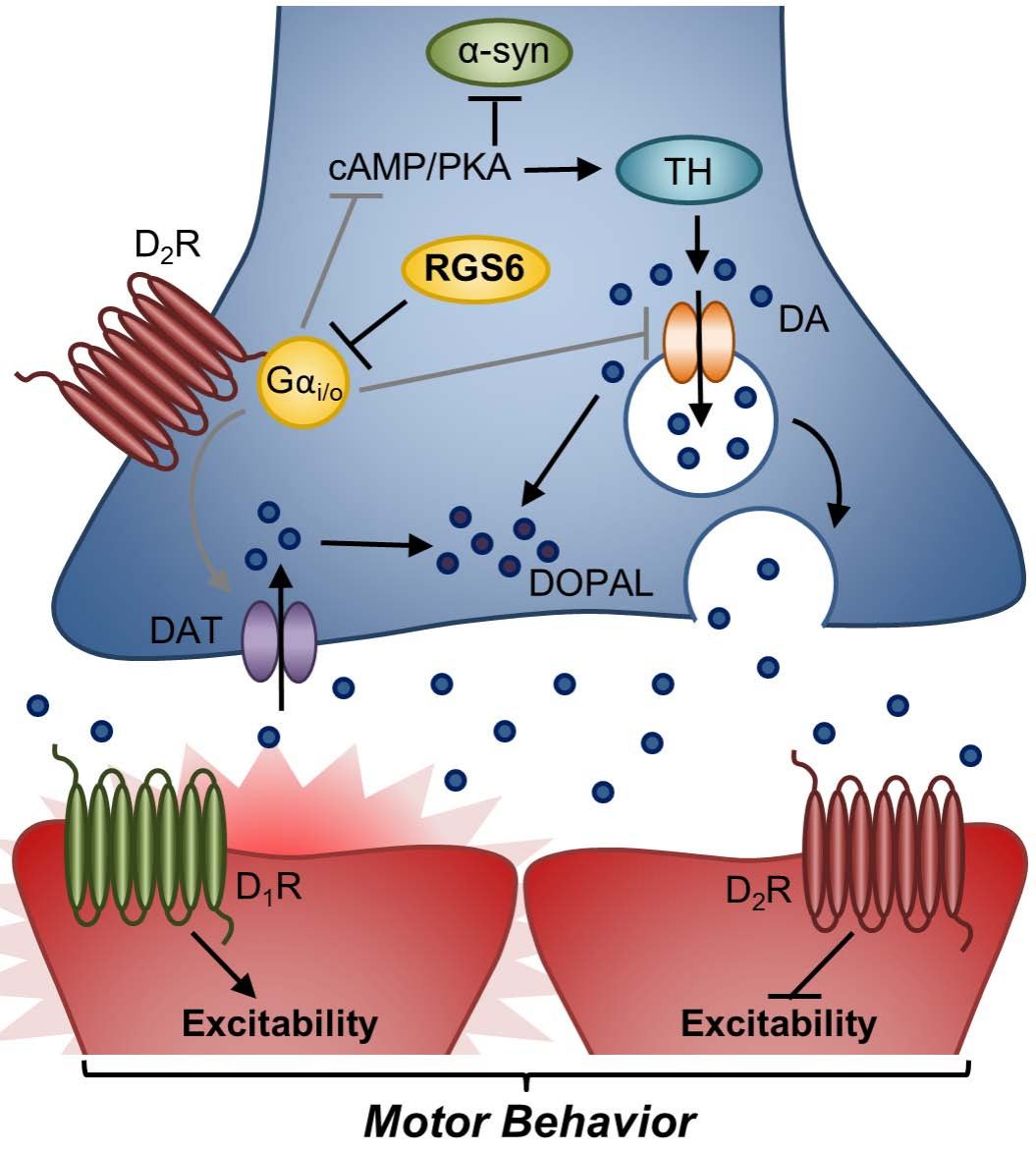
M4R
RGS4
Gαi/o
ACh
D1R
M1R
M2R
D2R
Excitability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cAMP/PKA
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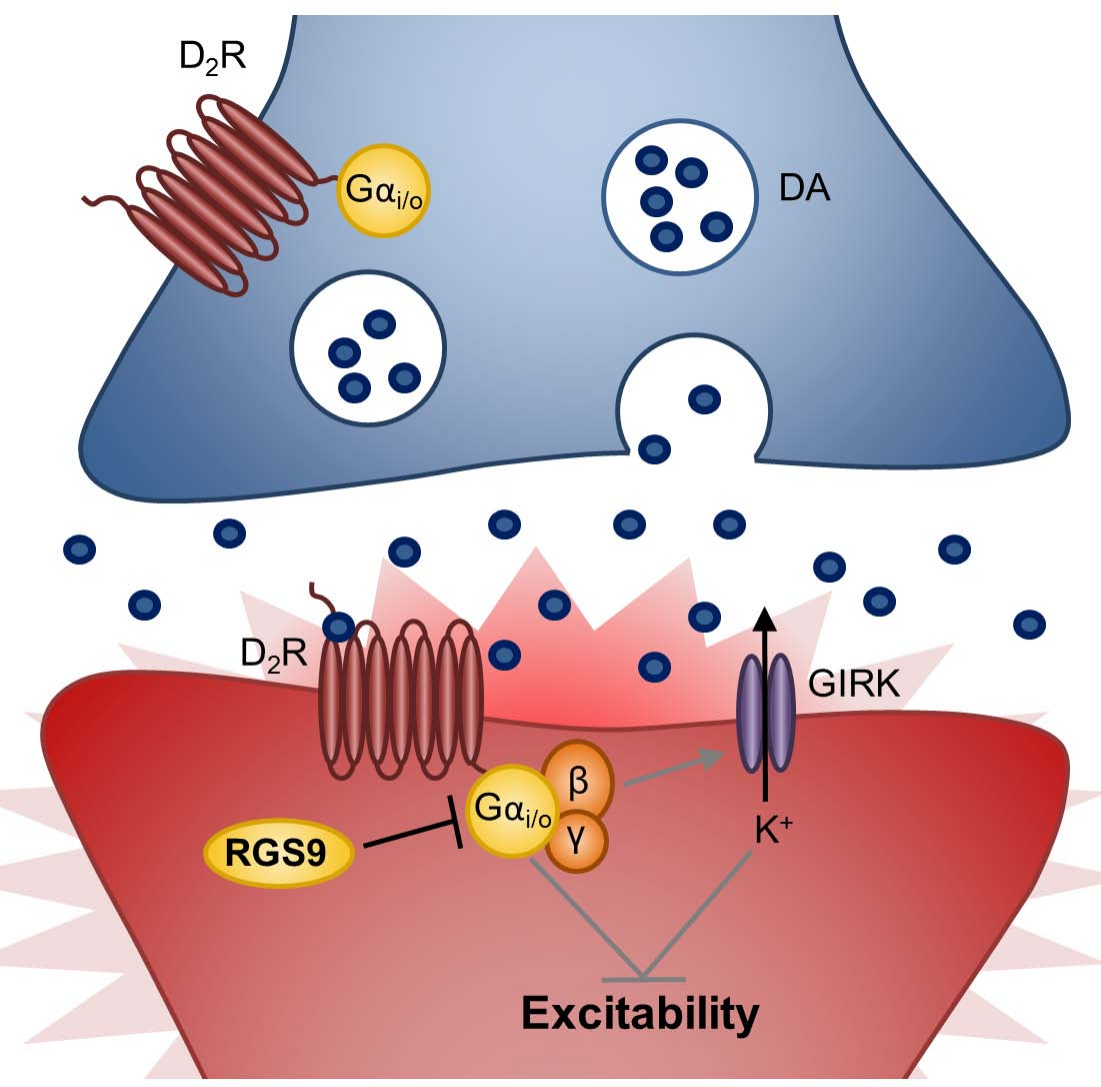
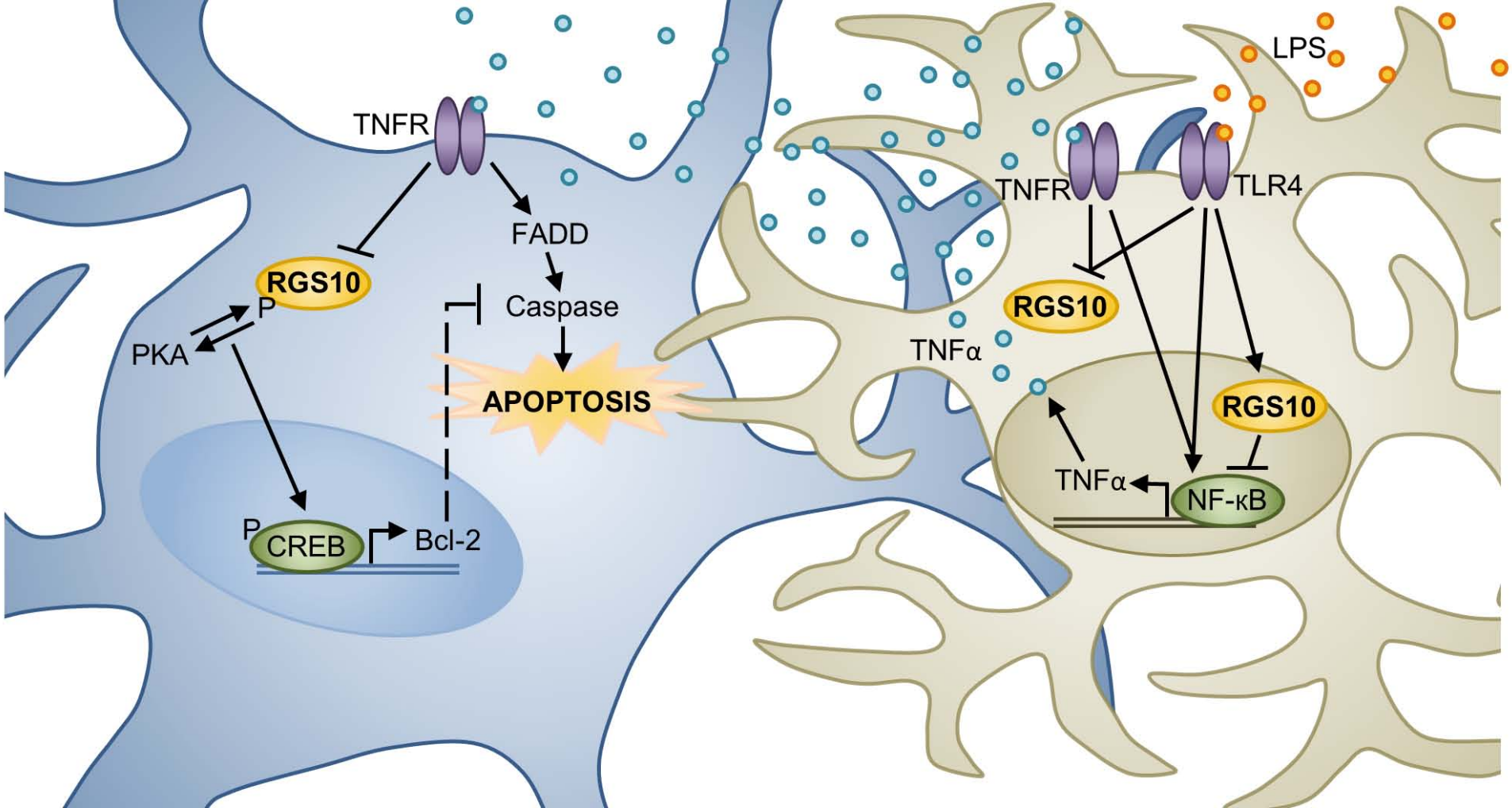
RGS6
DA
Gαi/o
DOPAL
DAT
D1R
D2R
Excitability
Excitability
Motor Behavior


D2R
Gαi/o
DA
D2R
GIRK
β
γ
Gαi/o
RGS9
K+
Excitability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RGS10
P
PKA
Caspase
APOPTOSIS
P
CREB
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RGS10
TNFα
NF-κB