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Rho GTPase regulation of α-synuclein and VMAT2: Implications for pathogenesis of Parkinson's disease

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ABSTRACT

Accumulation of α-synuclein (Asyn) in neuronal perikarya and dystrophic neurites is characteristic of idiopathic and familial Parkinson's disease. In this study, we investigated the relationship between α-synuclein expression and neurite outgrowth-maturation using MN9D dopaminergic cells and demonstrated key features of Asyn regulation in hippocampal neurons. Neurite elongation elicited by inhibition of Rho GTPase activity with C3 transferase or by db-cAMP treatment was associated with marked reduction of α-synuclein mRNA and protein expression. Rho inhibition resulted in reduction of transcription factor SRF in the nuclear fraction and retention of MKL-1 – the SRF co-transactivator of SRE – in cytosol, indicating that these effects of Rho inhibition may be mediated though reduction of SRF-SRE transcription. Inhibition of Rho GTPase activity led to decreased nuclear localization of GATA2, a key regulator of α-synuclein promoter activity. Rho inhibition-induced neurite extension was associated with increased VMAT2 and SNARE proteins synaptophysin and synapsin I. These results indicate that in the MN9D dopaminergic cell line, α-synuclein transcription and levels of synaptic vesicle associated proteins are inversely correlated with neurite growth. We confirm that in mature hippocampal neurons inhibition of RhoA and knock down of SRF by siRNA also lead to decrease GATA2 and Asyn. The results suggest that RhoA signaling may be potential therapeutic target for the treatment of synucleinopathies.

Introduction

Synucleinopathies are a group of degenerative diseases characterized by the accumulation of α-synuclein (Asyn) in dystrophic neurites and in neuronal perikarya that include idiopathic and familial Parkinson's disease (PD) and dementia with Lewy bodies (DLB) (Marti et al., 2003). The discovery that missense mutations in the *SCNA* (α-synuclein) gene result in rare dominant forms of PD (Polymeropoulos et al., 1997) was followed by observations that duplication/triplication of the normal *SCNA* gene results also in autosomal dominant PD-DLB. Increased levels of α-synuclein correlate with disease onset and severity (Bandopadhyay and de Belleroche, 2009; Farrer et al., 2004; Singleton et al., 2003). The importance of α-tsynuclein levels in PD-DLB pathogenesis is supported further by observations that promoter polymorphisms causing increased transcription levels of α-synuclein are associated with the sporadic form of the disease (Maraganore et al., 2006), and observations from animal studies that overexpression of wild type or mutant Asyn in the substantia nigra of rodents causes dopaminergic denervation of the striatum and neurite pathology with accumulation of synaptic vesicles in varicosities (Lo Bianco et al., 2002). Asyn regulates activity of tyrosine hydroxylase (TH) (Lou et al., 2010), plays a key role in synaptic vesicle clustering and regulates neurotransmitter release (Nemani et al., 2010). In pathological states, Asyn accumulation in neurites is associated with dystrophic changes, neurite retraction and loss of synaptic connectivity. In PD, innervation of target fields is extensively lost early in the disease process (Albin et al., 2008; Bohnen et al., 2006), and it has been proposed that the vulnerability of select neuronal populations is in part related to the extent of axonal branching and the size of the synaptic fields supported by those neurons (Matsuda et al., 2009).

To explore the relationship between the development of dystrophic neurites and α-synuclein expression, we undertook a series of studies of neurite dynamics and α-synuclein expression. Rho GTPases function as molecular switches that translocate to the plasma membrane to transduce signals regulating growth cone morphology, and axon extension and branching. We used MN9D cells, a dopaminergic cell line that expresses the plasmalemmal dopamine transporter (DAT), TH, the vesicular monoamine transporter (VMAT), and release dopamine (DA); and we confirmed key findings in mature hippocampal neurons *in vitro*.

We found that inhibition of Rho-GTPase signaling by C3 transferase or by PKA activation resulted in neurite extension and was accompanied by a substantial reduction in expression of Asyn mRNA and protein.

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These effects were mediated through the reduction in nuclear transcription factors SRF and MKL-1, and in GATA-2, a transcriptional regulator of Asyn.

Results

Link between neurite growth and α-synuclein expression uncovered by Rho inhibition

RhoA plays a central role in regulating growth cone collapse and for inhibiting neurite extension (Wang et al., 2002). C3 transferase is an enzyme produced by *Clostridium botulinum* that inhibits Rho activity through ADP-ribosylation of the Rho effector domain (Rubin et al., 1988). Targeted inhibition of RhoA by C3 transferase promotes neuronal regeneration and axonal extension after injury and during development (Dergham et al., 2002; Jin and Strittmatter, 1997; Niederost et al., 2002). To study the relationship between neurite growth and α-synuclein expression we used a cell permeable C3 transferase to induce neurite growth, C3 transferase treatment for 48 h resulted in robust extension of neurites from what were otherwise rounded MN9D cells (Fig. 1A). Morphometric analysis of neurites stained with an antibody against β-III tubulin demonstrated an increased number of processes, increased length of processes and an increase in the number of branches per process (Fig. 1B). Neurite extension induced by Rho inhibition in these dopaminergic cells was associated with decreased Asyn mRNA and protein expression (Figs. 2A and B). There was no change in phosphorylated or total TH (Fig. 2B).

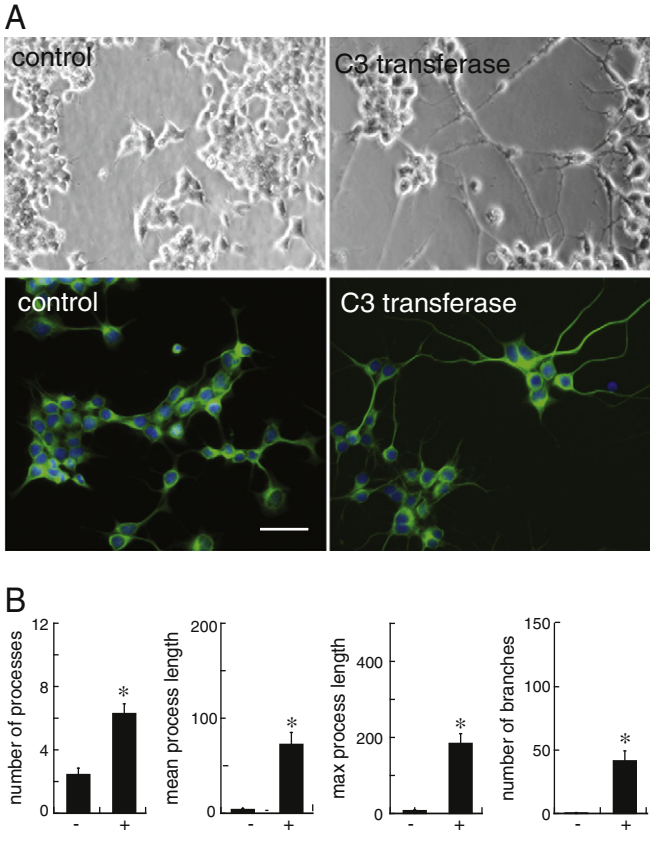

Fig. 1. A. MN9D cells treated with C3 transferase (2.5 μg/ml for 48 h) show extension of neurites (top: phase contrast; bottom: immunostained) with an antibody against β-III tubulin (Tuj-1). Bar = 40 μm. B. Quantitative analysis of the number of processes per cell, the mean length of processes per cell in μm, the maximum length of processes from individual cells in μm, and the number of branches per neuritic process. Mean ± SEM; * $P<0.05$.

Rho inhibition reduces nuclear SRF-MKL-1 and GATA-2

The effects of Rho-GTPases are mediated by downstream signaling through the serum response factor (SRF) transcription factor binding to the serum response element (SRE) in the promoter region of target genes (Liu et al., 2003). SRF acts as sensor of actin monomer (G-actin) levels in the cytoplasm. The G-actin level transducer of SRF is identified as the megakaryoblastic leukemia 1 (MKL-1) factor. MKL-1 is bound to G-actin in the cytoplasm; on activation of Rho and polymerization to F-actin, MKL-1 translocates to the nucleus to form a MKL-SRF complex that binds to SRE sites to coordinate the transcription of immediate early genes, including SRF (Knoll and Nordheim, 2009; Miralles et al., 2003; Sunavala-Dossabhoy et al., 2004). Treatment of MN9D cells with C3 transferase for 24 h resulted in a marked decrease in SRF in the nuclear fraction and retention of MKL-1 in the cytosolic fraction (Figs. 3Aand B). SRF forms multiprotein complexes with the GATA family of transcription co-regulators. GATA-2 is abundantly expressed in dopaminergic neurons in brain regions affected by PD and binds to intron-1 of *SCNA* to regulate expression of endogenous neuronal Asyn (Scherzer et al., 2008). There was a marked decrease in GATA-2 protein in the nuclear fraction of C3 transferase treated compared to control MN9D cells suggesting that Rho signaling may alter Asyn transcription through regulation of GATA-2 (Fig. 3B).

cAMP-PKA activation results in neurite extension and a reduction in α synuclein mRNA and protein

Exposure of MN9D cells to dibutyryl cyclic AMP (db-cAMP) results in the formation of neurites similar to those seen in mature neurons. Treatment of MN9D cells with 2 mM db-cAMP resulted in a marked increase in the number and length of neuritic processes visualized by immunostaining with an antibody against β-III tubulin (Fig. 4A). The amount of phosphorylated and total TH was unchanged by the db-cAMP treatment, but the amount of α synuclein protein decreased as early as 24 h after exposure to db-cAMP, and remained reduced through 4 days of treatment (Fig. 4B). The reduction in α-synuclein protein was probably a result of decreased expression, manifest by a reduction in Asyn mRNA (Fig. 4C).

PKA activation by cyclic AMP (cAMP) results in phosphorylation of Ser188 of RhoA leading to inactivation of RhoA (Lang et al., 1996). In order to understand the mechanism by which cAMP down-regulates α-synuclein expression during neurite outgrowth, we examined the effect of db-cAMP on Ser188 RhoA phosphorylation. We observed an increase in Ser188 phosphorylation of RhoA with unchanged levels of total RhoA (Fig. 5A), suggesting that PKA activation inhibits Rho activity through Ser188 phosphorylation. This was confirmed by the effect of 1 μM of the PKA activator forskolin, which reduced the amount of SRF in the nuclear fraction of treated MN9D cells (Fig. 5B). These results point to the convergence of cAMP-PKA and Rho-GTPase signaling pathways in promoting neurite growth and regulating Asyn transcription.

Upregulation of VMAT2 and synaptic vesicle protein expression by Rho inhibition

The vesicular monoamine transporter VMAT2, responsible for vesicle loading of neurotransmitter amines, is important in protecting neurons against MPTP toxicity (Takahashi et al., 1997; Uhl et al., 2000). We found that treatment with C3 transferase to inhibit Rho signaling resulted in a marked increase in VMAT2 protein levels as well as increased synaptic vesicle proteins synaptophysin and synapsin I (Fig. 6A), suggesting a coordinated relationship between Asyn expression and synaptic vesicle