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ORIGINAL PAPER

Lack of Alpha-Synuclein Modulates Microglial Phenotype In Vitro

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Abstract Alpha (α)-synuclein neuronal effects are continually being defined although its role in regulating glial phenotypes remains unclear. An ability to regulate microglial activation was investigated using primary cultures from wild type and α-synuclein deficient mice (*Snca*$^{-/-}$). *Snca*$^{-/-}$ microglia demonstrated increased secretion of the cytokine tumor necrosis factor-alpha (TNF-α), impaired phagocytic ability, elevated prostaglandin levels, and increased protein levels of key enzymes in lipid-mediated signaling events, cytosolic phospholipase (cPLA$_2$), cyclooxygenase-2 (Cox-2) and phospholipase D2 (PLD2) when compared to wild type cells. Increased cytokine secretion and cPLA$_2$ and Cox-2 levels in *Snca*$^{-/-}$ microglia were partially attenuated by inhibiting PLD-dependent signaling with n-butanol treatment.

Keywords Microglia · Phagocytosis · Cytokine · Parkinson · α-Synuclein · Phospholipase D

Introduction

Research interest in α-synuclein is partly based on data demonstrating that overexpression and mutations in α-synuclein are associated with early onset Parkinson's disease [1, 2]. It is highly expressed in brain within presynaptic terminals [3, 4]. α-Synuclein also binds a variety of proteins [5–7], lipid vesicles [8] and regulates fatty acid metabolism [9–12]. These data, along with the genetic and histological evidence of α-synuclein aggregates in neuronal Lewy bodies in diseased brains, suggest a role for α-synuclein in neuronal vesicular trafficking [13]. However, α-synuclein is also expressed in astrocytes, microglia, and oligodendroglia suggesting that it has yet to be defined roles in non-neuronal cells [9, 14–18].

Based upon its ability to regulate lipid metabolism and vesicle transport, α-synuclein could also have a role in regulating microglial physiology. Lipid-mediated signaling pathways are vitally important in achieving an activated state in microglia. For example, phospholipase D (PLD) is required for the activation of macrophage in response to lipopolysaccharide (LPS) and tumor necrosis factor (TNF)-α secretion [19–21]. PLD also mediates an integral enzymatic pathway involved in cytoskeletal restructuring and phagocytosis in these cells [22]. Since α-synuclein overexpression and interaction inhibits PLD activity it is likely that α-synuclein expression is involved in regulating PLD-dependent changes in microglial phenotype [5, 23–25].

We previously demonstrated that α-synuclein expression modulates microglial activation state [17]. Specifically, α-synuclein deficient (*Snca*$^{-/-}$) microglia have a basally reactive, secretory phenotype compared to wild type cells and an exacerbated reactive phenotype upon LPS stimulation. However, in spite of this reactive state *Snca*$^{-/-}$ cells have impaired phagocytic ability.

To determine whether the changes in microglial phenotype are due to loss of α-synuclein dependent PLD inhibition, we have continued to use postnatal brain derived primary microglia cultures from *Snca*$^{-/-}$ and wild type

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mice. *Snca*$^{-/-}$ mice displayed elevated protein levels of PLD2, cytosolic phospholipase ($cPLA_2$), and of cyclooxygenase (Cox)-2, consistent with an observed increase in basal prostaglandin levels compared to wild type microglia. Butanol treatment, used to attenuate PLD-mediated generation of phosphatidic acid, reduced *Snca*$^{-/-}$ cell cytokine secretion but was unable to lower it to levels secreted from comparably treated wild type cells. Butanol treatment partially attenuated increased *Snca*$^{-/-}$ protein levels of $cPLA_2$ and Cox-2 with no effect on PLD2 levels or neurotoxin secretion. These results suggest that α-synuclein-dependent regulation of PLD expression or activity is partially involved in regulating microglial secretory behavior and expression of a subset of lipid-signaling associated enzymes. These data demonstrate a broader role for α-synuclein in brain physiology besides previously characterized effects on neuronal function. More importantly, these findings offer the possibility that the role of α-synuclein-dependent regulation of lipid-associated signaling responses in disease are multi-factorial involving more than just neurons.

Experimental Procedures

Materials

The anti-Cox-2 and ERK2 antibodies were purchased from Santa Cruz Biotechnology. Butyl alcohol (n-butanol) was purchased from EM Science (Lawrence, KS). Anti-PLD1 and PLD2 were purchased from Abcam (Cambridge, MA). The anti-Cox-1 antibody was purchased from Cayman (Ann Arbor, MI). The anti-MAP2 antibody was purchased from Sigma (St Louis, MO). CD68 antibody was obtained from Serotec (Raleigh, NC). FITC-labeled Escherichia coli (K-12 strain) Bioparticles were purchased from Molecular Probes (Eugene, OR).

Tissue Culture

Synuclein gene ablated mice (*Snca*$^{-/-}$) were generated and characterized as previously described [26]. Microglia were derived from postnatal day 1–2 (P1-P2) wild type and *Snca*$^{-/-}$ (129/SvEv) mouse brains as previously described [17]. Briefly, meninges-free cortices were isolated, trypsinized, and plated onto tissue culture flasks with feeding every fifth day. At 14 days in vitro microglia were harvested from the mixed culture by rapid shaking (120 rpm, 30 min) and plated for use. Microglial purity was routinely verified at >98% purity by CD68 immunoreactivity. Primary cortical neuron cultures were also generated as previously described from cortices of embryonic day 16 C57Bl/6J mice [27]. Meninges-free cortices were isolated, trypsinized and plated onto poly-L-lysine-coated (0.05 mg/ml) tissue culture wells (260 cell/mm^2) for 7 days. Neuronal growth media was Neurobasal media with B27 supplements and glutamine (Life Technologies, Rockville, MD, USA) which consistently provide neuronal cultures greater than 95% pure and able to survive for at least 1 month in vitro. Culture purity was routinely evaluated by cell counting after immunostaining, to identify the neuronal cytoskeletal protein, microtubule-associated protein 2 (MAP2).

To assess the microglia-mediated neurotoxicity, neurons were coincubated either alone or with wild type or *Snca*$^{-/-}$ microglia for 72 h. Neurons were plated onto 24 well plates (40,000 cells/well) and at 7 days in vitro were co-cultured with microglia (4,000 cells/insert) that were plated onto cell culture inserts (0.4 μm Millicell, Millipore) in Neurobasal medium with B27 supplements (Gibco) with or without 0.01, 0.1 or 1.0% butanol (v/v) for 72 h. After the 72 h incubation, neurons were fixed in 4% paraformaldehyde and immunostained with antibody recognizing the neuronal cytoskeletal protein, microtubule-associated protein 2 (MAP2). A counting grid placed on the bottom of the wells was used to determine the number of viable neurons. Neurons were counted as viable if they were MAP2 positive, had a visible nuclei and immunostained processes which were at least two times the length of the cell body. Data is represented as mean number of neurons (±SD). Experiments were performed with 8 replicates per condition and repeated a minimum of three times.

Quantitation of Secreted TNF-α

Wild type and *Snca*$^{-/-}$ microglia were plated for 24 h in serum free DMEM/F12 to assess unstimulated cytokine secretion values. Medium was removed from the microglial cultures and the concentrations of secreted TNF-α were determined using commercially available mouse TNF-α colorimetric sandwich ELISA reagents purchased from R & D Systems (Minneapolis, MN). Experiments were performed with 8 replicates per condition and repeated a minimum of three times.

Phagocytosis Assay

Phagocytosis was quantified by measuring the uptake of a FITC-labeled bioparticle. Briefly, microglia, in 96 well plates, were incubated with or without FITC-labeled bioparticle (0.25 mg/ml) for 3 h. To quench the signal from extracellular or outer plasma membrane associated bioparticle, medium was removed and the cells were rinsed with 0.25 mg/ml trypan blue in PBS. Bioparticles without cells were added to wells and rinsed with trypan blue to provide negative controls for residual extracellular

fluorescence values. Intracellular fluorescence was read via fluorescent plate reader (Bio-Tek, Winooski, Vermont) at 480 nm excitation and 520 nm emission. Experiments were performed with eight replicates per condition and repeated a minimum of three times. Relative fluorescence units of wild type cells were compared to those from *Snca*$^{-/-}$ cells.

Western Blot

To perform Western blot analyses, microglia were plated onto 35 mm tissue culture plates in DMEM/F12 serum free media for 24 h. In butanol treatment experiments, microglia were treated with or without butanol (0, 0.01, 0.1 or 1.0% v/v) for 24 h. After incubation, cells were immediately collected and lysed. Protein lysates were quantified, resolved by 10% SDS–PAGE, transferred to PVDF and Western blotted using anti-cPLA$_2$, Cox-1, Cox-2, PLD1, PLD2 or ERK2 (loading control) antibodies. Due to differences in basal microglial morphology between *Snca*$^{-/-}$ and wild type microglia, typical cytoskeletal markers such as actin or tubulin are ineffective as loading controls for these cells and a cytosolic protein like ERK2 serves as a more appropriate reference [9, 17]. Indeed, our experience indicates that ERK2 expression level is unaltered in a variety of immune cell related stimulations [28, 29]. Affinity purified horseradish peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnologies. Antibody binding was visualized by chemiluminescence (Amersham). Quantitation of Western blot results was performed as previously described [30]. Optical densities of bands of interest were normalized to their respective loading control protein and the ratio averaged from five independent experiments using Adobe Photoshop Software (Adobe Systems).

Extraction of Prostaglandins

Prostaglandins (PG) were extracted from 2 ml of medium from microglial cultures under basal (unstimulated) conditions with 4 ml of acetone containing 0.005% butylated hydroxytoluene (BHT) [31]. PGE$_2$d$_4$ was used as internal standards. After extraction, the sample volume was reduced under a stream of nitrogen to complete dryness and the samples were then redissolved in 30 µl of acetonitrile:water (1:2 by volume).

Analysis of Prostaglandins

Reverse-phase LC electrospray ionization mass spectrometry was used for PG analysis. The PG were separated on a Luna C-18(2) (3 µm column, 100 A pore diameter, 150 × 2.0 mm) (Phenomenex, Torrance, CA, USA) with a stainless steel frit filter (0.5 µm) and security guard cartridge system (C-18) (Phenomenex, Torrance, CA, USA). The LC system consisted of an Agilent 1100 series LC pump with a wellplate autosampler (Agilent Technologies, Santa Clara, CA). The solvent system was composed of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). The flow rate was 0.2 ml/min. The separation program started with 10% of solvent B. At 2 min, the percentage of B was increased to 65% over 8 min, at 15 min the percentage of B was increased to 90% over 5 min, and at 35 min it was reduced to 10% over 2 min. Equilibration time between runs was 13 min.

MS analysis was performed using a quadrapole mass spectrometer (API3000, Applied Biosystem, Foster City, CA, USA) equipped with a TurboIonSpray ionization source. Analyst software version 1.4.2 (Applied Biosystem) was used for instrument control, data acquisition, and data analysis. The mass spectrometer was optimized in the multiple reaction-monitoring mode. The source was operated in negative ion electrospray mode at 450°C, electrospray voltage was −4,250 V, nebulizer gas was 8 l/min and curtain gas was 11 l/min. Declustering potential, focusing potential, and entrance potential were optimized individually for each analyte. The quadrupole mass spectrometer was operated at unit resolution.

Statistical Analysis

For comparison of prostaglandins or protein levels in untreated *Snca*$^{-/-}$ and wild type cells (Fig. 1) mean values (±SD) were determined and a two-sided, unpaired Student's t-test was used to assess statistical significance ($P < 0.05$). For all other data, mean values (±SD) for each experiment were determined and values statistically different from controls were calculated using either one-way ANOVA with Tukey–Kramer post-hoc comparison or two-way ANOVA with Holm-Sidak all pairwise multiple comparison procedure (when both genotype and drug conditions were being compared). Sigmastat software version 3.5 was used for analysis.

Animal Care

Animal care and use was approved by the Institutional Animal Care and Use Committee of the University of North Dakota.

Results

Snca$^{-/-}$ Microglia Displayed Increased Protein Levels of Select Lipid-Associated Enzymes

Prior reports have documented α-synuclein-lipid interaction and effects of α-synuclein on lipid metabolism [10–12,

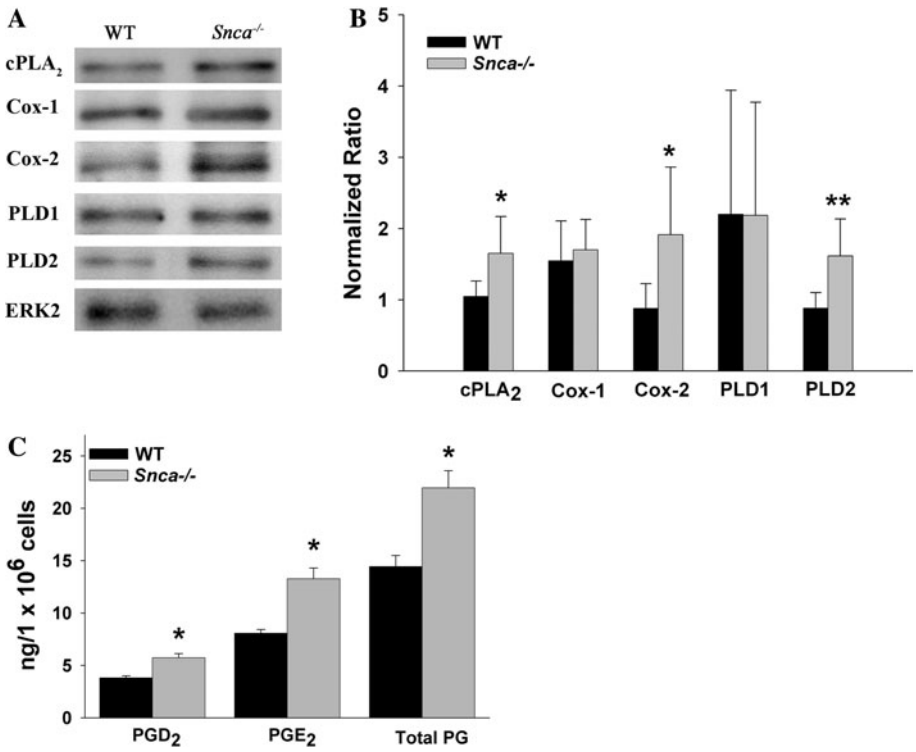

Fig. 1 Microglia from *Snca*$^{-/-}$ mice displayed statistically higher prostaglandin and protein levels of select enzymes involved in lipid-mediated activation. Microglia were isolated from 14 days in vitro mixed glia cultures and plated onto tissue culture plastic. **a** Cells were lysed and proteins resolved by 10% SDS–PAGE. Lysates were Western blotted using anti-cPLA$_2$, Cox-1, Cox-2, PLD1, PLD2, or ERK2 (loading control) antibodies. **b** Optical density of cPLA$_2$, Cox-1, Cox-2, PLD1 and PLD2 from five independent culture preps derived from different littermates were normalized against respective ERK2 levels and averaged (±SD). *$P < 0.05$, **$P < 0.01$ from respective wild type (WT). **c** Prostaglandins (PG) were extracted from the medium of the wild type (four independent cell cultures) and *Snca*$^{-/-}$ microglia (three independent cell cultures) and analyzed as described in the methods. Total PG is the sum of prostaglandin E$_2$(PGE$_2$) prostaglandin D$_2$ (PGD$_2$), prostaglandin F$_{2\alpha}$, thromboxane B$_2$, and stable prostacyclin metabolite 6-keto-prostaglandin F$_{1\alpha}$. Prostaglandin mass was normalized by cell numbers and expressed as mean ± SD. *$P < 0.05$ from respective wild type. Data were analyzed via two-sided, unpaired Student's *t*-test

32–37]. Indeed, α-synuclein overexpression [24] and interaction with PLD [5, 23–25] results in inhibition of PLD activity. These robust α-synuclein-dependent changes suggest that microglia from *Snca*$^{-/-}$ mice would have altered levels or activity of enzymes involved in lipid-mediated signaling. Interestingly, Western blot analyses demonstrated increased protein levels of several enzymes, cPLA$_2$, Cox-2 and PLD2, but not PLD1 or Cox-1 from *Snca*$^{-/-}$ versus wild type cultures (Fig. 1).

In order to asses whether the increased protein levels of PLD2, cPLA$_2$, and Cox-2 correlated with increased enzyme activity, we chose to assess prostaglandin levels in *Snca*$^{-/-}$ microglia compared to wild type cells. Consistent with the increased protein levels, under basal conditions there was a 1.6-fold increase in PGE$_2$ mass and a 1.5-fold increase in PGD$_2$ mass in microglia from *Snca*$^{-/-}$ mice compared to wild type mice (Fig. 1). The total PG mass was also elevated in microglia from *Snca*$^{-/-}$ mice 1.5-fold, with the majority of the total PG mass accounted for by PGE$_2$ and PGD$_2$. These data demonstrated that α-synuclein regulates the levels and activity of a distinct pool of enzymes associated with lipid-mediated signal transduction in microglia.

Butanol Attenuated Increased Protein Levels of cPLA$_2$ and Cox-2 But Not PLD2 in Snca$^{-/-}$ Microglia

Because cPLA2 and subsequent arachidonic acid metabolism by enzymes like Cox-2 have been demonstrated to be downstream of PLD activity in immune cells [38, 39] it was possible that changes in PLD activity might be auto-regulating PLD2 expression and feed-forward regulating the increases in cPLA$_2$ or Cox-2 protein levels. To assess whether the changes in protein levels were due to PLD activity, PLD-mediated generation of phosphatidic acid was attenuated by treating cells with increasing concentrations of butanol [40, 41]. A 1.0% butanol treatment significantly reduced both cPLA$_2$ and Cox-2 protein levels in the *Snca*$^{-/-}$ microglia compared to untreated *Snca*$^{-/-}$ cells (Fig. 2). In fact, cPLA$_2$ protein levels in 1.0% butanol

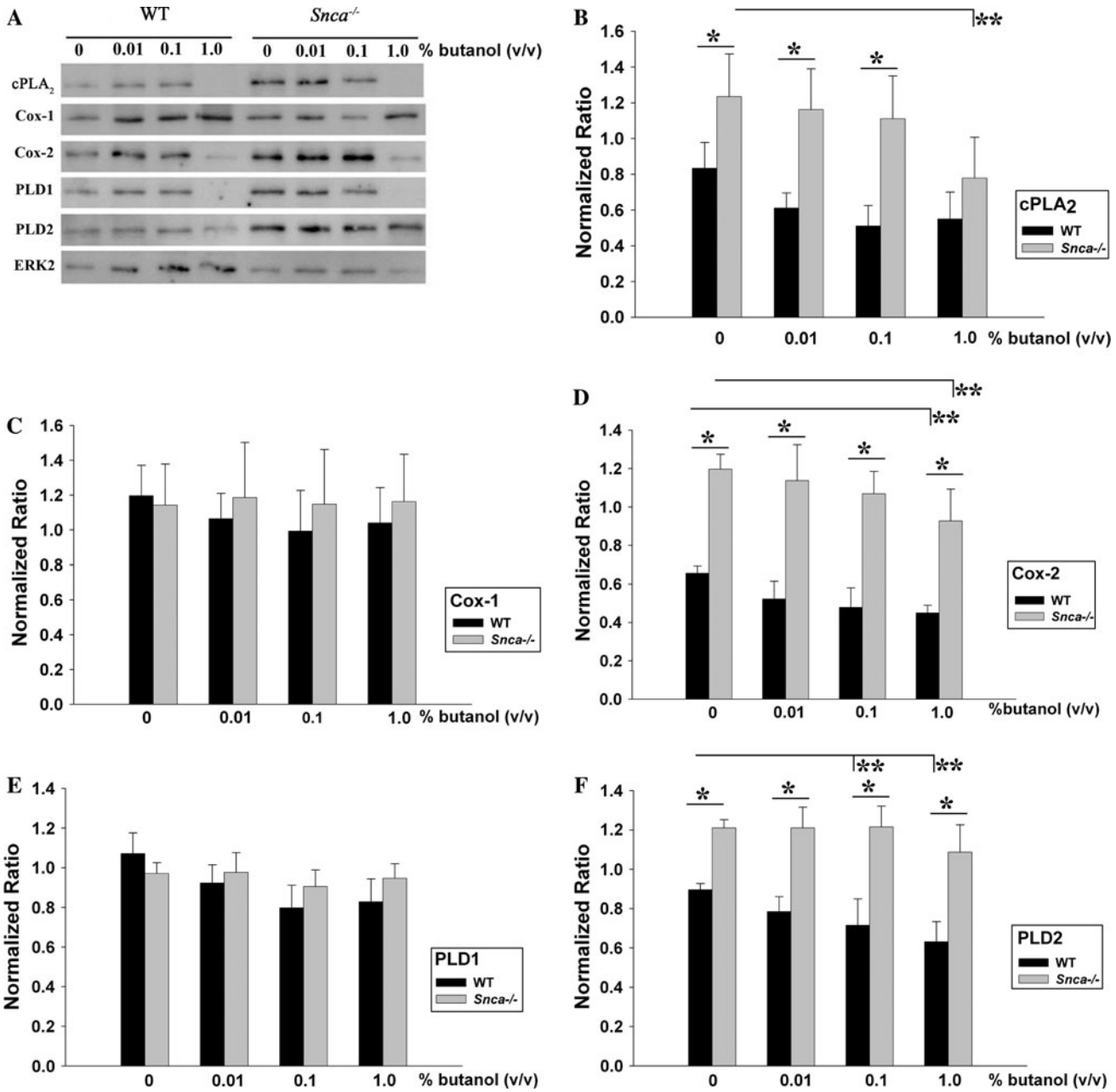

Fig. 2 Butanol treatment reduced protein levels of lipid-mediated activation enzymes in *Snca*$^{-/-}$ microglia. Microglia were isolated from 14 days in vitro mixed glia cultures and plated onto tissue culture plastic and treated with or without 0.01, 0.1 or 1.0% butanol (v/v) for 24 h. **a** Cells were lysed and proteins resolved by 10% SDS–PAGE. Lysates were Western blotted using anti-cPLA$_2$, Cox-1, Cox-2, PLD1, PLD2, or ERK2 (loading control) antibodies. Optical density of **b** cPLA$_2$, **c** Cox-1, **d** Cox-2, **e** PLD1 and **f** PLD2 from five independent culture preps derived from different littermates were normalized against respective ERK2 levels and averaged (±SD). Data were analyzed by a two-way Analysis of Variance with Holm-Sidak all pairwise multiple comparison procedure. *$P < 0.05$, **$P < 0.01$

treated *Snca*$^{-/-}$ microglia were reduced to wild type cell levels, while levels in wild-type microglia were unaffected (Fig. 2). However, Cox-2 protein levels in *Snca*$^{-/-}$ microglia did not reach those found in wild type cells. Butanol treatment did not alter PLD2 protein levels in *Snca*$^{-/-}$ microglia, although PLD2 protein levels were attenuated by butanol treatment of wild type cells (Fig. 2). These data suggested that increased PLD2 expression in *Snca*$^{-/-}$ microglia is a result of loss of α-synuclein expression and the observed increase in cPLA$_2$ and partially Cox-2 protein levels was, in part, due to elevated PLD activity.

Snca$^{-/-}$ Microglia Secreted Increased Levels of the Proinflammatory Cytokine, TNF-α, in a PLD-Dependent Manner

Based upon the observation that elevated cPLA2 and Cox-2 protein levels were partially associated with PLD-mediated signaling, we next determined whether another component of a reactive phenotype, cytokine secretion, was also dependent upon PLD activity. Secretion of the pro-inflammatory cytokine, TNF-α, was quantified from microglial medium from *Snca*$^{-/-}$ and wild type cultures with and without butanol pretreatment. Basal levels of secreted TNF-α were significantly higher in medium from unstimulated *Snca*$^{-/-}$ microglia compared to wild type cells (Fig. 3). The highest concentration of butanol, 1.0% v/v, attenuated TNF-α secretion levels from both wild type and *Snca*$^{-/-}$ microglia, although concentrations from *Snca*$^{-/-}$ cells remained significantly higher than their treated wild type counterparts (Fig. 3). Importantly, butanol exhibited no toxic effect on either cell type (Fig. 3). These data demonstrated that another parameter of increased microglial activation in the *Snca*$^{-/-}$ cells, cytokine secretory phenotype, was also only partially mediated via PLD activity.

Snca$^{-/-}$ Microglia Demonstrated a Decrease in Phagocytic Ability

Based upon the evidence of some PLD-mediated changes in *Snca*$^{-/-}$ microglia it was hypothesized that another PLD activity associated behavior, phagocytosis, might be altered in α-synuclein deficient microglia. In order to quantify changes in microglial phagocytic ability, uptake of FITC-labeled *E. coli* bioparticles was assessed from wild type and *Snca*$^{-/-}$ microglia. Consistent with our prior report [17], *Snca*$^{-/-}$ cells had diminished ability to take up the bioparticle compared to wild type microglia (Fig. 4). A butanol concentration of 0.01% (v/v) was sufficient to decrease uptake in wild type cells to the level of *Snca*$^{-/-}$ microglia without having any effect on *Snca*$^{-/-}$ microglia uptake (Fig. 4). Higher concentrations of butanol, 1.0% (v/v), were required to exert any effect on *Snca*$^{-/-}$ microglia which normalized their uptake to that of wild type treated cells and the no treatment controls (Fig. 4). These data demonstrated that even though *Snca*$^{-/-}$ microglia displayed components of a PLD-mediated reactive phenotype, this did not include a PLD-associated increase in phagocytic ability. Therefore, α-synuclein expression likely regulates microglial phenotype through a larger mechanism than simply increased PLD-mediated signaling events.

Snca$^{-/-}$ Microglia Demonstrated PLD-Independent Neurotoxicity in Co-Culture

Based upon the increased cytokine secretory phenotype of *Snca*$^{-/-}$ microglia, we hypothesized that these cells may be also secreting neurotoxic factors. In particular, since we have already demonstrated that microglial secreted TNFα contributes to neurotoxicity in culture [42] we expected the *Snca*$^{-/-}$ microglia to be neurotoxic. To test this hypothesis, *Snca*$^{-/-}$ microglia were co-cultured with cortical neurons in the absence or presence of increasing concentrations of butanol. As expected, untreated *Snca*$^{-/-}$ microglia were

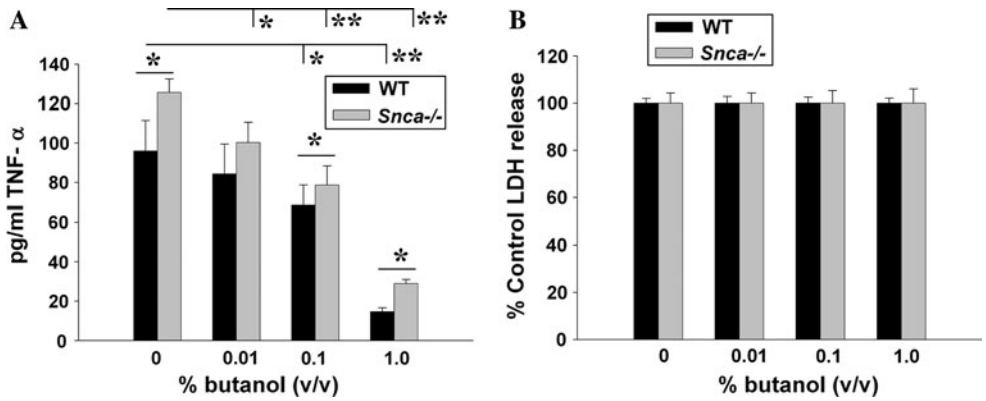

Fig. 3 Microglia from *Snca*$^{-/-}$ mice secreted increased amounts of the proinflammatory cytokine, TNF-α, that was attenuated by butanol treatment. Microglia were isolated from wild type and *Snca*$^{-/-}$ mixed glia cultures and plated 24 h in the absence or presence of 0.001, 0.01, or 1.0% butanol (v/v). **a** Conditioned medium from the cells was collected and secreted TNF-α concentrations were determined using a commercial ELISA. **b** Viability of the treated cells was determined by quantitating lactate dehydrogenase release (LDH) into the medium. Secreted values were normalized to respective secretion levels from unstimulated control wild type or *Snca*$^{-/-}$ microglia and graphed as percent control release. Graphs are representative of four independent experiments. Each experiment was performed with eight replicates per condition and averaged (±SD). Data were analyzed by a two-way Analysis of Variance with Holm-Sidak all pairwise multiple comparison procedure. *$P < 0.05$, **$P < 0.01$

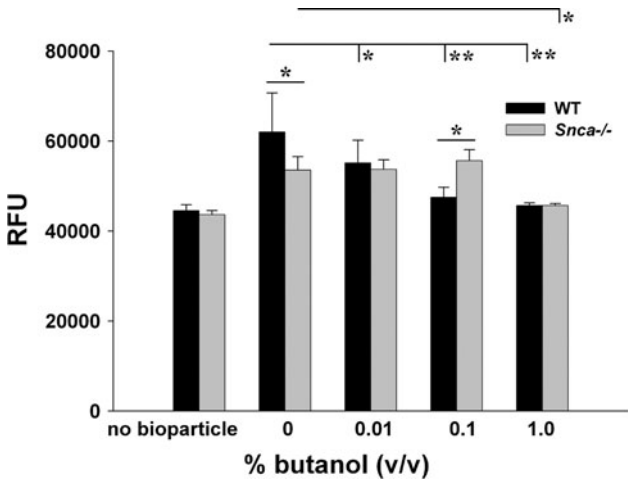

Fig. 4 *Snca*$^{-/-}$ microglia displayed decreased phagocytic ability. Microglia from *Snca*$^{-/-}$ and wild type mice were cultured as mixed glia for 14 days in vitro. Microglia were isolated at 14 days and plated with or without 0.01, 0.1, or 1.0% butanol (v/v) overnight. Microglia were then incubated with or without FITC-labeled *E. coli* bioparticles (0.25 mg/ml) for 3 h. After the incubation, the media was removed and the signal from unphagocytosed or extracellular membrane associated FITC-labeled bioparticles was quenched by rinsing with 0.25 mg/ml trypan blue solution. Fluorescence intensity of internalized, phagocytosed bioparticles was measured via a fluorescent plate reader (480 nm excitation and 520 nm emission) and averaged (±SD). Each condition was performed with eight replicates and graph is representative of three independent experiments. Data were analyzed by a two-way Analysis of Variance with Holm-Sidak all pairwise multiple comparison procedure. *$P < 0.05$, **$P < 0.01$

potently neurotoxic in neuron-microglia co-cultures compared to untreated wild type microglia (Fig. 5). However, butanol pretreatment did not reduce the neurotoxic capacity of *Snca*$^{-/-}$ microglia (Fig. 5). Therefore, although *Snca*$^{-/-}$ microglia had elevated neurotoxic secretion compared to wild type cells, attenuating PLD-dependent generation of phosphatidic acid with butanol did not impact this difference in spite of being able to decrease TNF-α secretion (Fig. 3). These data suggest that *Snca*$^{-/-}$ microglia were neurotoxic through a mechanism that does not involve elevated PLD-dependent activity. This again demonstrated that the reactive *Snca*$^{-/-}$ phenotype was only partially mediated via PLD activity.

Discussion

Numerous reports have demonstrated the ability of microglia and macrophage to become activated via stimulation by both monomeric and fibrillar aggregate α-synuclein [43–49]. The consequence of these stimulations invariably demonstrate changes in microglial phenotype favoring a reactive state that includes a range of changes such as increased secretion of cytokines [46], increased proinflammatory protein levels [46, 50], increased adhesion ability [18], increased transmigration [18], and increased secretion of neurotoxins [51]. In addition, over-expression of α-synuclein can drive microglia to acquire a reactive, migratory phenotype [18]. These findings have supported a hypothesis that extracellular α-synuclein promotes microglial-dependent proinflammatory changes that contribute to the degeneration of neurons in Parkinson's disease (PD). Indeed, reactive microglia are a histological characteristic of PD brains, correlating with levels of deposited α-synuclein [52].

It is important to point out that our efforts have focused on identifying changes in microglial phenotype that result from the absence of α-synuclein expression. This approach offers insight into the normal role of this protein in regulating microglial behavior rather than further elucidating the role that extracellular α-synuclein may play in activating microglia during Parkinson's disease. However, it is still possible that endogenous expression of α-synuclein leads to microglial secretion of the protein allowing it to act in an autocrine fashion to regulate microglial behavior. In this way, a portion of the phenotype changes we have observed in *Snca*$^{-/-}$ microglia may be due to loss of autocrine stimulation with α-synuclein. Indeed, recent data demonstrating that α-synuclein can be secreted further supports the possibility that this protein may serve as an activating extracellular ligand for microglia [53, 54]. However, it is likely that the protein has some role in modulating the behavior of these cells independent of any extracellular ligand-type stimulation. Microglial activation both in vitro [18, 49] and in vivo [55] correlates with increased α-synuclein levels suggesting that the protein has a role in phenotype changes. Based upon our prior work demonstrating that lack of α-synuclein expression altered microglial behavior towards a type of proinflammatory state [17], the current study has further defined the mechanistic role of α-synuclein in regulating this microglial phenotype.

We report for the first time that decreased α-synuclein expression promotes microglial activation characterized by increased protein levels of three distinct enzymes involved in lipid-mediated signaling, $cPLA_2$, Cox-2, and PLD2 with no increase observed in PLD1 and Cox-1 levels. Moreover, increased levels of $cPLA_2$, partially Cox-2, but not PLD2 were attenuated by inhibition of PLD-dependent signaling. This suggests that PLD activity partially regulates $cPLA_2$ and Cox-2 expression in *Snca*$^{-/-}$ microglia, while the increase in PLD2 protein levels was apparently a consequence of loss of α-synuclein function. To the best of our knowledge, prior work has not demonstrated that α-synuclein expression regulates expression of any of these three enzymes, in spite of the fact that α-synuclein has the ability to negatively regulate PLD2 activity [5, 23–25]. It is

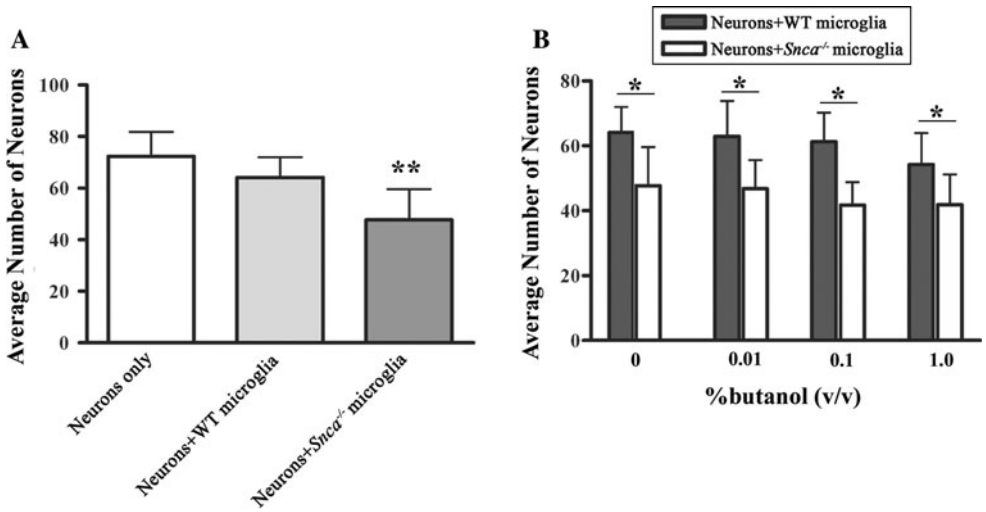

Fig. 5 Butanol treatment did not attenuate the neurotoxic action of *Snca*$^{-/-}$ microglia. Microglia were isolated from 14 days in vitro mixed glia cultures plated onto cell culture inserts and co-cultured with 14 days in vitro cortical neurons, with or without 0.01, 0.1, or 1.0% butanol (v/v) for 72 h. After the 72 h incubation, neurons were fixed in 4% paraformaldehyde and immunostained with antibodies recognizing the neuronal marker protein, MAP2. Viable neurons were counted and defined as MAP2 positive cells with visible nuclei and immunostained processes that were at least two times the length of the cell body. Average numbers of MAP2 positive neurons (±SD) were graphed **a** with our without co-cultured microglia from wild type and *Snca*$^{-/-}$ microglia alone or **b** with added concentrations of butanol. Experiments were performed with eight replicates per condition, repeated two-five times. Data in panel **a** were analyzed by analyzed by one-way Analysis of Variance with Tukey–Kramer post-hoc comparison. $^{**}P < 0.01$ from respective neuron + wild type (WT) microglia. Data in panel **b** were analyzed by two-way Analysis of Variance with Holm-Sidak all pairwise multiple comparison procedure. $^{*}P < 0.05$

important to point out that our findings demonstrated that α-synuclein can dually regulate PLD2 via expression as well as the prior reported binding-dependent mechanism of regulating activity. It is also worth noting that all of these enzymes are active participants in lipid-mediated signaling consistent with a plethora of studies from our group as well as others demonstrating that α-synuclein not only associates with lipid membranes, but that its expression alters lipid function and metabolism [10–12, 32–37, 56]. For example, our own work demonstrates that α-synuclein is critical in maintaining arachidonic acid metabolism in the brain and that its absence results in reduced brain recycling of arachidonic acid and increased PG formation in *Snca*$^{-/-}$ brains relative to wild type brains following ischemic insult [12]. This reduction in recycling is entirely consistent with the observed increase in basally secreted levels of PG observed from *Snca*$^{-/-}$ microglia compared to wild type cells. These data not only offer insight into an expanded physiology of this protein but also provide a caution against strategies that might seek to limit α-synuclein expression during Parkinson's disease as this may inadvertently drive a microglial-mediated proinflammatory, neurodegenerative environment in the brain.

Similarly, attenuated α-synuclein expression led to increased TNFα secretion from the *Snca*$^{-/-}$ microglia compared to wild type cells. The highest concentration of butanol treatment (1.0%) was sufficient to attenuate TNFα secretion from wild type and *Snca*$^{-/-}$ cells versus their respective untreated controls. However, it was not sufficient to lower TNFα secretion from *Snca*$^{-/-}$ cells to levels released from similarly treated wild type cells suggesting that additional changes beyond PLD activity were involved in the elevated cytokine secretion. Nevertheless, this demonstration of a partial PLD-dependent increase in TNF-α secretion from *Snca*$^{-/-}$ cells was consistent with our observation of increased PLD2 expression. The fact that α-synuclein tonicly inhibits PLD2 activity [23, 25], and the well characterized role of PLD activity in regulating cytokine secretion from phagocytes [20, 57], enhances the importance of our findings. It is important to point out that the concentrations of butanol employed were not toxic to the microglia (Fig. 3) and have been demonstrated in several reports as concentrations appropriate for attenuating PLD-dependent generation of active lipid signaling molecules such as phosphatidic acid [40, 41, 58].

It was curious that *Snca*$^{-/-}$ microglia displayed diminished phagocytic ability for the bioparticles compared to wild type cells in spite of the fact that PLD-dependent changes in cytokine secretory phenotype and cPLA$_2$ and Cox-2 expression were occurring in these cells. PLD activity has a confirmed role in regulating phagocytosis with both PLD1 and PLD2 serving important and distinct roles [22, 59, 60]. Therefore, our expectation was that an increased PLD-dependent phagocytic ability would also be

a defining characteristic of the *Snca*$^{-/-}$ phenotype. The data of diminished phagocytic uptake indicates that *Snca*$^{-/-}$ cells have some additional problem with the phagocytic machinery that is fundamentally substantial enough to be unaffected by the increased PLD2 expression. There are a multitude of proteins involved in the phagocytic uptake process [61–63] and any number of which could be altered in expression or function. In addition, it is possible that the increase in PGE_2 levels in *Snca*$^{-/-}$ microglia competed with any PLD-mediated effects on phagocytosis such that the net effect was attenuated uptake. Prior work has shown not only that PGE_2 stimulates increased microglial Cox-2 and not Cox-1 levels [64] but it also limits certain types of phagocytosis [65].

An earlier study has provided findings complementary to ours demonstrating that α-synuclein expression positively regulates receptor-mediated, clathrin dependent endocytic uptake and vesicular recycling in neuronal cell lines [66]. Although α-synuclein is likely to have an important role in neuronal synaptic function, the current data set indicates that additional membrane uptake mechanisms, such as that employed by professional phagocytes like microglia, are also intimately regulated by α-synuclein expression or function. Therefore, a role for α-synuclein in regulating endocytosis or exocytosis appears broadly important across cell types and particular vesicle forms thus making this protein important for cell types throughout the body and brain.

It is interesting to note that we [27] as well as others [67–69] have previously reported that microglial secreted TNF-α can exert toxic effects on cultured neurons suggesting that the elevated levels of this cytokine in the medium should contribute to enhanced toxicity of *Snca*$^{-/-}$ microglia. However, despite a profound reduction in TNF-α secretion by addition of n-butanol, the neurotoxic capacity of the *Snca*$^{-/-}$ microglial secretions was not attenuated. These data suggest that *Snca*$^{-/-}$ microglia secrete additional factors in a PLD-independent manner that were responsible for neuron death. Again, our results indicate that therapeutic attenuation of α-synuclein expression in PD may inadvertently initiate an unwanted neurotoxic, proinflammatory change in microglial phenotype.

Although this study has not focused on the biology of Parkinson's disease, per se, our findings are of relevance to disease. Increased numbers of reactive microglia are an important component of the histological findings from PD brains [70, 71]. Furthermore, the number of reactive microglia reportedly increase with disease duration suggesting active participation in disease progression [72]. Our findings indicate that absence of α-synuclein expression leads to a reactive microglial phenotype although the direct implications to disease mechanism must be defined. For example, it will be important to determine the effects of mutant α-synuclein expression on the reactive state of microglia as mutations in synuclein have been shown to cause familial forms of PD [73–75]. One possibility is that mutant forms of synuclein will lead to a loss of function similar to the phenotype we have observed in *Snca*$^{-/-}$ microglia. This is not without precedence as wild type but not mutant α-synuclein rescues brain long-chain acyl-CoA synthetase activity in microsomal preparations from *Snca*$^{-/-}$ brains demonstrating a pivotal role for α-synuclein in brain arachidonic acid metabolism [12, 34]. However, it is also possible during disease that overexpression of or mutations in α-synuclein will each lead to a unique microglial phenotype [18]. Further understanding of the role α-synuclein plays in regulating microglial phenotype, as well as the effect of mutant α-synuclein expression, may provide therapeutic targets to attenuate the neuroinflammatory processes seen in the PD brain.

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