Published in final edited form as:
Neurosci Lett. 2008 February 27; 432(3): 243–247.

Brain prostaglandin formation is increased by α-synuclein gene-ablation during global ischemia

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Abstract

We have previously demonstrated that α-synuclein (Snca) gene ablation reduces brain arachidonic acid (20:4n-6) turnover rate in phospholipids through modulation of endoplasmic reticulum-localized acyl-CoA synthetase activity. Although 20:4n-6 is a precursor for prostaglandin (PG), Snca effect on PG levels is unknown. In the present study, we examined the effect of *Snca* ablation on brain PG level at basal conditions and following 30 sec of global ischemia. Brain PG were extracted with methanol, purified on C_{18} cartridges, and analyzed by LC-MS/MS. We demonstrate, for the first time, that *Snca* gene ablation did not affect brain PG mass under normal physiological conditions. However, total PG mass and masses of individual PG were elevated ~2-fold upon global ischemia in the absence of Snca. These data are consistent with our previously observed reduction in 20:4n-6 recycling through endoplasmic reticulum-localized acyl-CoA synthetase in the absence of Snca, which may result in the increased 20:4n-6 availability for PG production in the absence of Snca during global ischemia and suggest a role for Snca in brain inflammatory response.

Keywords

alpha-synuclein; prostaglandin; brain fatty acids; arachidonic acid; acyl-CoA synthetase; neuroinflammation

INTRODUCTION

α-Synuclein (Snca) is widely distributed in neurons [22;24;28;31], astrocytes [9;33], oligodendroglia [33;43], and microglia [3;36] and accounts for 0.1 - 1% of neuronal cytosolic protein in nervous system [21;45]. Snca overexpression and mutations are associated with familial Parkinson disease [25;38;46;54], although aggregates containing Snca are hallmark of a number of neurodegenerative disorders [19;27;47;48;50]. Despite the close association with neurodegenerative diseases, the physiological function of Snca is poorly defined.

While Snca may have a number of diverse roles in the nervous system, a number of studies suggests its role in brain fatty acid metabolism. Snca facilitates palmitic acid and arachidonic acid (20:4n-6) uptake in astrocytes [9] and in brain [14;15], although it has no affect on

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docosahexaenoic acid (22:6n-3) uptake both in astrocytes and brain [9;16], indicating a fatty acid selective affect. More importantly, we have recently demonstrated a functional interaction of Snca with microsomal acyl-CoA synthetases in brain, accounting for the profound reduction in 20:4n-6 incorporation and turnover in phospholipids in *Snca*$^{-/-}$ mice [15]. Conversely, 22:6n-3 incorporation and turnover is increased in these mice as a result of metabolic compensation for the decrease in 20:4n-6 brain metabolism [16]. Because 20:4n-6 is a precursor for prostaglandins (PG), we hypothesize that reduced recycling of 20:4n-6 back into phospholipid pool may result in the increased availability of 20:4n-6 for PG formation upon 20:4n-6 release during ischemia, thus increasing brain PG mass upon stimulation.

To address the potential role for Snca in brain prostaglandin formation, we measured brain prostaglandin levels following global ischemia in *Snca*$^{-/-}$ and *Snca*$^{+/+}$mice. *Snca* gene deletion did not alter basal brain PG levels, however all measured PG masses were increased ~2-fold upon global ischemia as compared to wild-type animals. These data are consistent with our proposed hypothesis and demonstrate that Snca has a key role in modulating PG formation, suggesting a role in brain inflammatory response.

This study was conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals (NIH publication 80-23) and under an animal protocol approved by the IACUC at the University of North Dakota (Protocol #407-9). α-Synuclein gene-ablated mice (*Snca*$^{-/-}$) were generated from 129/SvEv strain by gene targeted deletion [8]. Male mice (25-30 g) were maintained on standard laboratory chow diet and water *ad libitum*. In both groups, the ages of the mice were between 9-11 months.

Fasted, male mice were anesthetized with halothane (1-3%) and killed either by decapitation or by head-focused microwave irradiation (2.8 kW, 1.35 s; Cober Electronics, Inc, Norwalk, CT) to heat denature enzymes *in situ*. The whole brain was removed, frozen in liquid nitrogen, and pulverized under liquid nitrogen temperatures to a fine, homogeneous powder. The total time of global brain ischemia was 30 s in non - microwaved brains, while brain basal levels were assessed in mice immediately killed using head-focused microwave irradiation.

Brain PG were extracted with methanol and purified on a C_{18} column as described previously [29;39]. Briefly, 20 mg of non-microwaved or 100 mg of microwaved brain tissue powder was homogenized in 3 ml of 15% methanol at pH=3 containing 0.005% of butylated hydroxytoluene (BHT), and PGE_2d_4 and 6-keto-$PGF_{1\alpha}d_4$ as internal standards. The tissue debris were removed by centrifugation and supernatant was loaded onto C_{18} Sep-Pak classic cartridges (Waters, Corporation, Milford, MA) that were prewashed with methanol and water. The cartridges were then washed with 20 mL of 15% methanol following with 20 mL of water, and then the PG were eluted with 10 mL of methyl formate (spectral grade, Acros Organics, Pittsburg, PA). The methyl formate was removed under a stream of nitrogen and PG were then dissolved in acetonetrile for analysis.

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 -4250 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. PGE_2, PGD_2, $PGF_{2\alpha}$, and TXB_2 were quantified using PGE_2d_4 as the internal standard and 6-keto-$PGF_{1\alpha}$ was quantified using 6-keto-$PGF_{1\alpha}d_4$ as the internal standard.

All statistical comparisons were calculated using a one-way ANOVA followed by a Tukey-Kramer post-hoc test using Instat II (Graphpad, San Diego, CA). Statistical significance was defined as <0.05. All values are expressed as mean ± SD.

Snca gene ablation did not affect basal levels of total (Figure 1) and individual PG (Figure 2). However, the total and individual PG mass in *Snca*$^{-/-}$ brains was elevated ~2-fold as compared to wild-type brains upon stimulation with 30 s of global ischemia (Figure 1 and 2).

The observed 4-to 20- fold elevation of brain PG levels upon a global ischemia modeled by decapitation is consistent with previously reported values [2;7]. Increased PG formation is the result of dramatic 20:4n-6 release from phospholipids following cerebral ischemia [2;5-7;10; 13] through activation of phospholipases and diacylglycerol lipases [18;26;34;53]. This released 20:4n-6 is used by COX1 and COX2 for PG formation, thereby acting as a proinflammatory mediator. As a possible protective mechanism against neuroinflammation following ischemia, 20:4n-6 is recycled back into brain phospholipid pool via its initial conversion to acyl-CoA by acyl-CoA synthetases [41;42]. As the result of acceleration of 20:4n-6 recycling following ischemia [41], brain 20:4n-6-CoA mass is increased, while 22:6n-3-CoA mass is decreased after decapitation [12;42], indicating fatty acid selectivity of the recycling mechanism following brain ischemia. Because Snca specifically stimulates 20:4n-6 recycling by activation 20:4n-6-CoA formation through acyl-CoA synthetases mechanism [15;16], the recycling of a released 20:4n-6 following ischemia would be depressed in the *Snca*$^{-/-}$ brains. The reduced recycling of 20:4n-6 in *Snca*$^{-/-}$ brains would lead to increased substrate availability for PG formation, thereby leading to the observed increase in the PG levels in *Snca*$^{-/-}$ brains (Figure 1 and 2). Importantly, all of the PG analyzed were increased to the same extent in *Snca*$^{-/-}$ brains, further supporting our assumption that the increased PG levels in *Snca*$^{-/-}$ brains was the result of increased substrate availability for COX rather than modulation of specific PG-synthetases by Snca.

The effect of Snca on PG formation following ischemia suggests that Snca has a role in the brain physiological response to injury and downstream processes such as neuroinflammation. The proposed role for Snca in suppressing neuroinflammatory response is not without evidence. First, *Snca*$^{-/-}$ microglia have an activated phenotype that secretes elevated levels of proinflammatory cytokines upon stimulation with proinflammatory stimuli [3]. This phenotype may be the result of elevated phospholipase D (PLD) activity because PLD is involved in promoting a reactive state in microglia [4;11;32;40;44] and because Snca tonically inhibits PLD activity *in vitro* [1;23;37]. We have demonstrated an increase in palmitic acid (16:0) turnover in brain phosphatidylcholine pools in *Snca*$^{-/-}$ mice [14], consistent with an increase in turnover due to the absence of PLD inhibition by Snca. The increase in TNFα secretion in cultured Snca$^{-/-}$ microglia [3] may result in increased astrocyte PG formation because TNFα increases astrocyte 20:4n-6 release and downstream PG formation [51]. In addition, Parkinsonism is associated with a maintained presence of reactive microglia [30;35;49]. Importantly, mutant forms of Snca, that are associated with familial forms of Parkinsonism

[25;38;54], do not restore 20:4n-6-CoA synthetase activity in *Snca* ablation which we show is critical for 20:4n-6 recycling [15]. Collectively, this suggests a link between the functions of Snca and neuroinflammation associated with Parkinsonism. Second, *Snca* expression is significantly increased during cerebral ischemia and hypoxia [17;20;52]. Although this is not direct evidence, our proposed role for Snca in regulating brain 20:4n-6 metabolism and downstream of PG is consistent with this observation. Ischemic/hypoxic conditions are characterized by increased PG formation; hence ischemia-induced increases in Snca levels may serve as a protective mechanism to down regulate brain PG levels. Third, the level of Snca is upregulated in neurons, astrocytes and oligodendrocytes via induced transcription of mRNA in a model of multiple sclerosis [36]. This is important because inflammation is increased during this disease process, once again indicating a link between Snca and neuroinflammatory response.

Taken together, our results indicate that *Snca* gene deletion increases brain PG formation following 30 s of global ischemia. This is consistent with our previously observed reduction in 20:4n-6 recycling through endoplasmic reticulum-localized acyl-CoA synthetase in the absence of Snca, which would result in the increased 20:4n-6 availability for PG production in the absence of Snca. During pathological events such as ischemia where 20:4n-6 level is increased, the absence of Snca would provide more substrate for downstream PG formation as observed herein, suggesting Snca is an important regulator of brain PG formation during such events. This impact of Snca may be exacerbated in its absence via a dysregulation of PLD-mediated signaling in microglia, leading to increased cytokine release [3], resulting in a downstream elevation in PG formation in astrocytes [51]. More than likely, in the absence of Snca, multiple lipid-mediated signaling cascades in different cell populations in the brain are altered, resulting in our observed increase in PG formation during ischemia.

Acknowledgments

We thank Dr Carole Haselton for her excellent technical assistance. We thank Cindy Murphy for the typed preparation of the manuscript. This work was supported by a project (EJM) on a COBRE grant from NIH, P20 RR17699.

Abbreviations

Snca, alpha-synuclein; 20:4n-6 arachidonic acid; 22:6n-3 docosahexaenoic acid; PG, prostaglandins; CoA, coenzyme A; BHT, butylated hydroxytoluene; COX, cyclooxygenase.

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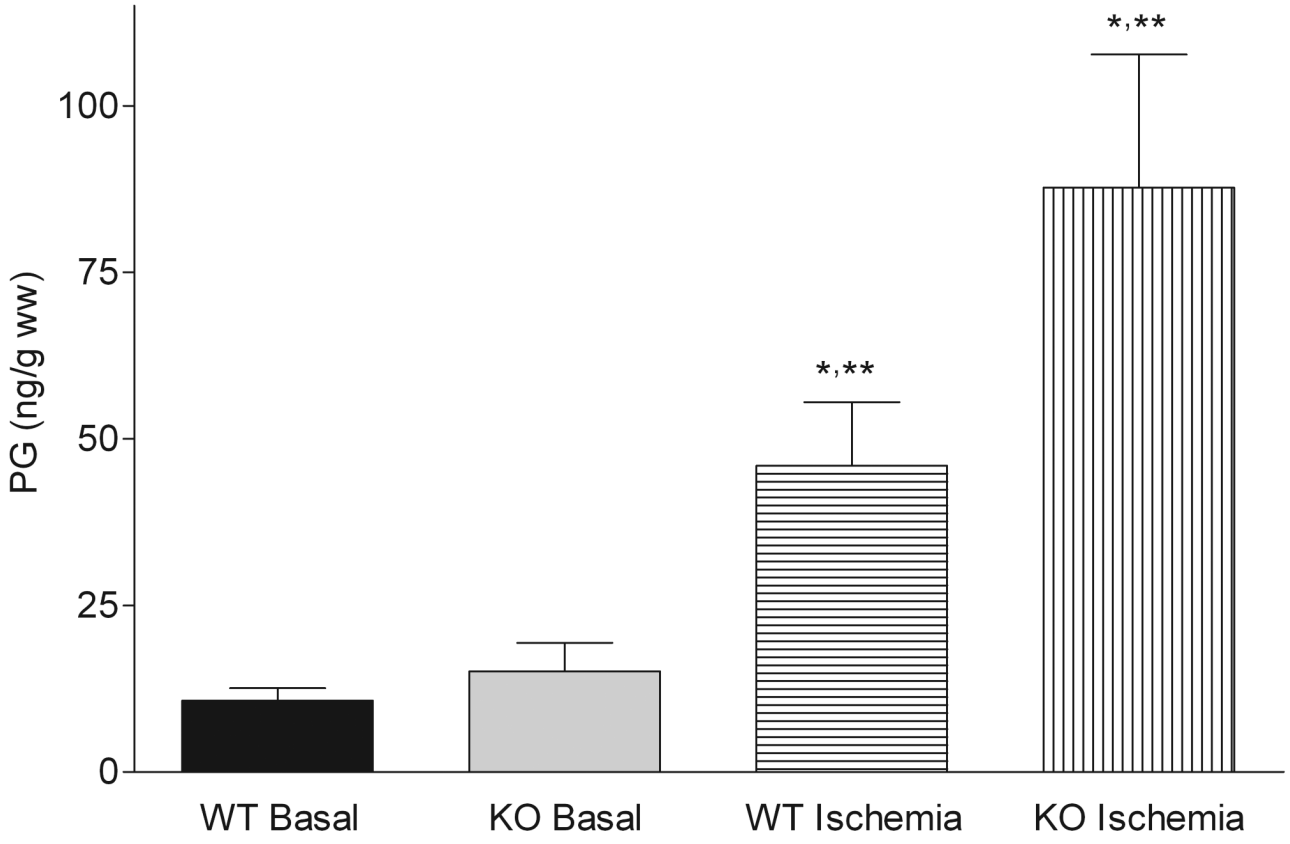

Figure 1. α-Synuclein gene ablation increases total prostaglandin mass following 30 sec of global ischemia

Wild type and α-synuclein gene ablated mice were subjected to either 30 seconds of global ischemia or the brains were fixed *in situ* using head-focused microwave irradiation (basal PG levels). Brain PG were extracted with methanol, purified on C_{18} cartridges, and analyzed by LC-MS/MS. Values are means ± SD. PG-prostaglandins; WT-wild type mice; KO-α-synuclein gene ablated mice; * - significantly different from WT, $p< 0.05$; ** - significantly different from WT and KO basal levels, $p< 0.05$.

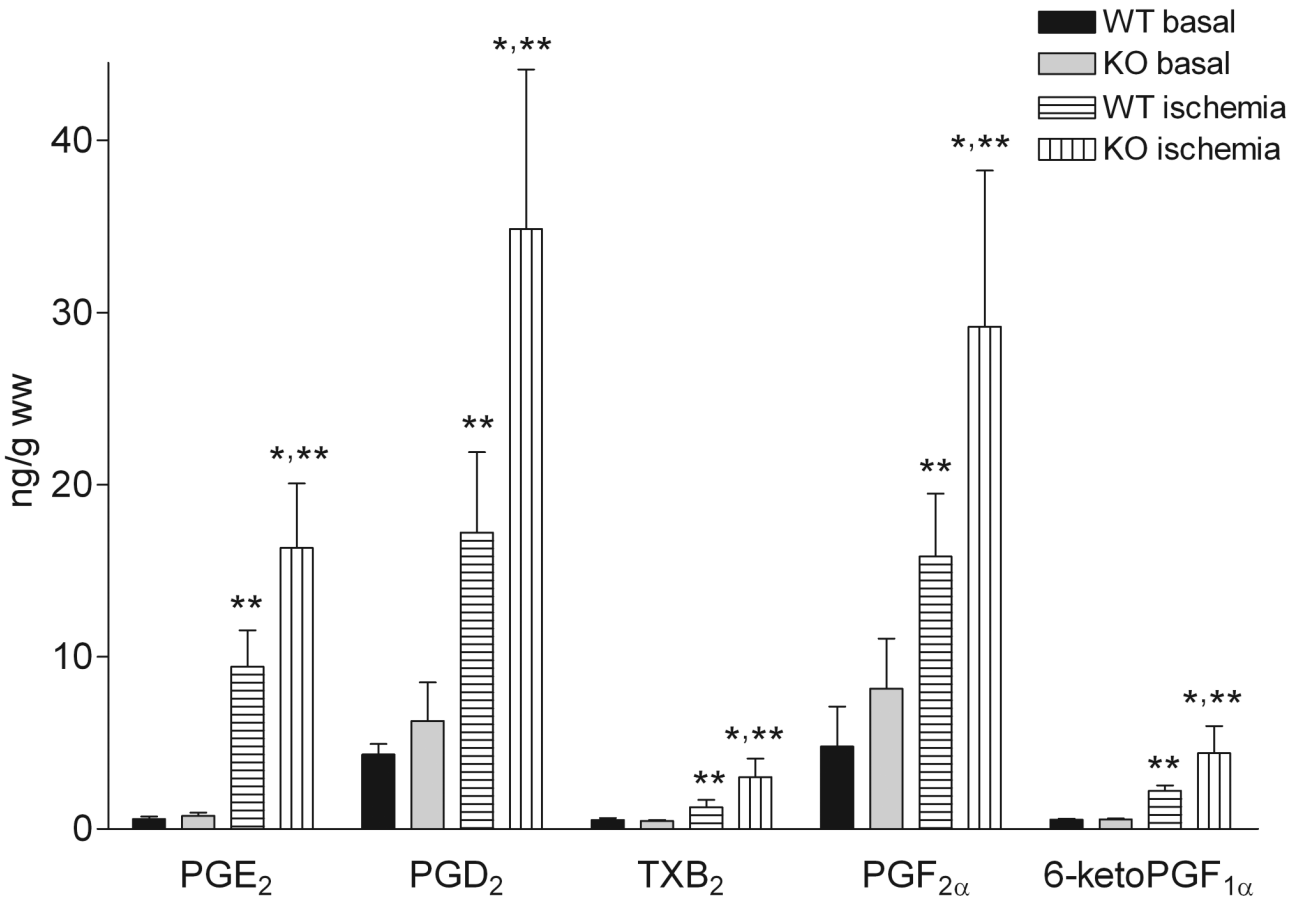

Figure 2. α-Synuclein gene ablation increases prostaglandin mass following 30 sec of global ischemia

Wild type and α-synuclein gene ablated mice were subjected to either 30 seconds of global ischemia or the brains were fixed *in situ* using head-focused microwave irradiation (basal PG levels). Brain PG were extracted with methanol, purified on C_{18} cartridges, and analyzed by LC-MS/MS. Values are means ± SD. PG-prostaglandins; WT-wild type mice; KO-α-synuclein gene ablated mice; * - significantly different from WT, $p< 0.05$; ** - significantly different from WT and KO basal levels, $p< 0.05$.