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Sustained effects of neonatal systemic lipopolysaccharide on IL-1 β and Nrf2 in adult rat substantia nigra are partly normalized by a *Spirulina* enriched diet

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Abstract

Background/Aim—Neonatal infection can sensitize the adult substantia nigra (SN) to secondary insults, causing a decrease in antioxidant capacity which may lead to Parkinson's disease in adults. We studied, prolonged effect of systemic infection by (i.p.) administration of lipopolysaccharide (LPS) on interleukin 1 beta (IL-1 β), the antioxidant regulators nuclear factor-erythroid 2-related factor 2 (Nrf2) and peroxisome proliferator-activated receptor gamma coactivator-1 alpha (PGC-1 α) in rat SN.

Method and Results—Five-day old rat pups were treated with LPS (i.p. 2 mg/kg). After 65 days, the mRNA level of IL-1 β was significantly increased in parallel with decreases in mRNAs of the rate limiting catalytic subunit of glutathione synthesis, γ glutamylcysteine ligase (γ GCLC), Nrf2 and brain-derived neurotrophic factor (BDNF). Protein levels of γ GCLC and Nrf2 were decreased while IL-1 β protein was significantly increased. These LPS-induced long term changes were correlated with a decrease in phosphorylated AKT (pAKT) and phosphorylated-inactive GSK3 β (pGSK3 β). In another set of experiment, a *Spirulina* (0.1%) containing diet was given to lactating mothers 24 h before LPS treatment of the pups. The *Spirulina* supplemented diet decreased IL-1 β protein expression in SN and elevated the mRNA level of the rate limiting catalytic subunit of glutathione synthesis, γ glutamylcysteine ligase (γ GCLC), Nrf2 protein, PGC-1 α protein and pAKT.

Conclusion—Early life infection can negatively affect Nrf2, pAKT and pGSK3 β for a long time in SN. A diet enriched in antioxidant and anti-inflammatory phytochemicals can partly restore some but not all the effects on the antioxidant defense, possibly via normalizing effects on pAKT.

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Disclosure Statement

The authors declare that they have no conflicts of interest.

Keywords

neuroinflammation; Nrf2; antioxidant system; Spirulina

Introduction

The initiating factors behind sporadic Parkinson's disease (PD) are not clear but may relate to dysfunctional redox homeostasis. This idea is based partly on the finding that the tissue concentration of glutathione, the main water-soluble antioxidant in brain, is dramatically decreased in the substantia nigra from PD patients. Interestingly such decreases are also demonstrated in tissue from patients suffering from incidental Lewy body disease which is considered to represent presymptomatic PD [1–3]. Ageing is the largest risk factor in sporadic PD. Likely reasons are decreased levels of glutathione and increased levels of proinflammatory cytokines in the ageing brain [4,5].

One relatively new idea is that pre- and perinatal infection can predispose for development of sporadic PD in adulthood. Infection in utero may lead to loss of DA-neurons in the adult substantia nigra whereas perinatal infection appear to make the adult DA-cells in the substantia nigra vulnerable to normally sub-lethal insults and levels of toxins. [6–8]. It appears that the early increase in IL-1 β is important as neonatal administration of IL-1 receptor antagonist, which dramatically reduced LPS-mediated motor behavioral deficits, also increased the number of tyrosine hydroxylase (TH) immunoreactive neurons in the substantia nigra of postnatal day (PND) 70 rats [8]. The authors suggested that IL-1 β may play a pivotal role in mediating a chronic neuroinflammation status by a single LPS exposure in early postnatal life [8]. However, the mediator between inflammation and increased vulnerability has not been settled.

One inducible regulator of enzymes involved in redox-homeostasis is nuclear factor-erythroid 2-related factor 2 (Nrf2). Activated Nrf2 is translocated to the nucleus and can initiate transcription of hundreds of protective genes, including the subunits for rate limiting enzymes in glutathione synthesis γ -glutamylcysteine ligase. Nrf2 activation by various agents have been shown to protect against experimental PD and genetic deletion of Nrf2 make animals more vulnerable to PD inducing agents [9,10].

Another factor that has been suggested to be important in PD is the transcriptional cofactor, peroxisome proliferator-activated receptor γ coactivator-1 α (PGC-1 α). Activation of PGC-1 α causes upregulation of mitochondrial proteins and anti-oxidant systems localized to mitochondria [11]. In a recent genome-wide meta-analysis of gene sets (groups of genes that encode the same biological pathway or process) it was shown that patients with symptomatic Parkinson's have low levels of PGC-1 α and genes regulated by PGC-1 α [12]. It was demonstrated that upregulation of PGC-1 α could counteract pathology in cellular models of PD.

We have shown earlier in *in vitro* and *in vivo* studies that inflammation by LPS can cause a down-regulation in the Nrf2-system up to 3 days after treatment [13–15]. Here we used a model where LPS is injected i.p. in PND5 rat pups, a treatment which by unknown

mechanisms makes the DA-neurons of substantia nigra vulnerable to a normally sub-toxic dose of rotenone [6] 65 days later. The main hypothesis of our study was that this elevated vulnerability could be due to decreased Nrf2/PGC-1 α systems even 65 days following the LPS injection. As a possible intervention strategy we evaluated the effects of a diet enriched in Spirulina shown earlier to have antioxidant activity both *in vivo* and *in vitro* [16]. Spirulina contains antioxidant and anti-inflammatory phytochemicals; for example beta-carotene [17], phycocyanin and phycocyanobilin [18]. The effects of a Spirulina enriched diet on the Nrf2/PGC-1 α systems in substantia nigra pars compacta *in vivo* have to our knowledge not been evaluated earlier.

Material and Methods

Animal Treatment and Tissue Preparation

Sprague-Dawley rats from our in-house colony were housed in a 12-hour light-dark cycle and bred at Experimental Biomedicine (University of Gothenburg, Gothenburg, Sweden) with free access to food and drinking water. Animal experiments were approved by the Ethical Committee of Gothenburg (No. 264–2009 and 205–2012) and followed the guidelines for the care and use of experimental animals and the European Communities Council Directive of 24 November 1986 (86/609/EEC). At PND5, rat pups were injected intraperitoneally (i.p.) with saline or LPS (LPS isolated from *E. coli* 055:B5, List Biological Laboratories, INC, Campbell, CA, USA) 2 mg/kg dissolved in saline. Brain tissue was collected 65 days following injection. In one set of experiments rat dams were fed normal diet or diet containing 0.1 % Spirulina extract (Earthrise Nutrionals, Irvine, CA, USA) beginning 24 h prior to the LPS injection of the pups and continued until PND 70 after the rats were separated from their mothers. At PND 70 rats were anesthetized by isoflurane (Baxter) and sacrificed by decapitation. Substantia nigra was dissected out of rat brain were dissected free and immediately frozen in liquid nitrogen, and stored at –80 °C.

Quantitative real time PCR

Substantia nigra which was dissected 65 days following saline or LPS injection were used for total RNA extraction by using RNeasy Lipid Tissue Mini Kit (Qiagen, Solna, Sweden). Total RNA was measured in a spectrophotometer at 260 nm absorbance (NanoDrop 2000/2000c (Thermo Scientific)). Total RNA (500ng) from each sample was used for first strand cDNA synthesis according to the manufacturer's instructions (QuantiTect Rev. Transcription Kit (Qiagen, Solna, Sweden)). In order to determine mRNA expression the cDNA samples were further processed by quantitative real time PCR (qRT-PCR). Each PCR reaction (20 μ l) contained 10 ng of cDNA in 5 μ l volume, 10 μ l Quanti Fast SYBR Green PCR Master Mix (Qiagen, Solna, Sweden), 2 μ l PCR primers and 3 μ l of H₂O to make final reaction volume of 20 μ l. A list of the PCR primers (Qiagen, Solna, Sweden) used are listed in in Table 1.

The amplification protocol comprised an initial 5 min denaturation at 95 °C, followed by 40 cycles of denaturation for 10 s at 95 °C and annealing/extension for 30 s at 60 °C on a LightCycler 480 (Roche, Stockholm, Sweden). Melting curve analysis was performed to ensure that only one PCR product was obtained. For quantification and estimation of

amplification efficiency, a standard curve was created using increasing concentrations of cDNA. The amplification transcripts were quantified with the relative standard curve and normalized against the geomean of reference genes Beta-glucuronidase (GUSB), Hypoxanthine-guanine phosphoribosyltransferase (HPRT1) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Protein sample preparation

The protein containing fraction from the silica column (first flow through) in the RNA extraction steps was used for acetone precipitation of proteins as described by the manufacturer. Briefly, acetone precipitation of proteins was completed as follows: four times the sample volume of ice cold acetone was added to each sample placed in a compatible tube. Tubes were vortexed and placed for at least 30 min in -20°C , the tubes were centrifuged for 10 minutes at 13,000–15,000 rpm. The acetone fraction was removed and ice-cold buffer (50 mM Tris, pH 7.3; 2 mM EDTA, 1% protease inhibitor cocktail) was added to each sample and homogenization was performed briefly by sonication (Branson Sonifier 250).

Western Blot Analysis

Protein homogenate was mixed with 1: 3 (v/v) Laemmli sample buffer (Bio-Rad, Hercules, Calif., USA) plus 5% (w/v) β -mercaptoethanol and boiled for 5 min. An equal amount of protein (20 μg) was resolved on 8–16% Criterion™ TGX Stain-Free™ Gel (Bio-Rad, Hercules, Calif., USA) and electrophoresis was done by using 10 $\times$ Tris/Glycine/SDS premixed electrophoresis buffer (Bio-Rad, Hercules, Calif., USA) following dilution to 1 $\times$ with at 300 V for 21 min. For blotting gels further processing was performed by using Trans-Blot® Turbo™ Midi Nitrocellulose Transfer Packs (Bio-Rad, Hercules, Calif., USA) and electroblotting at 25 V for 7 min. The membranes were blocked for 1 h at room temperature in 5 % (w/v) dry skimmed milk (Semper Mjöl, Sundyberg, Sweden) in TBS with 0.1 % Tween 20 (TBST). The membranes were then incubated overnight at 4°C with the corresponding primary antibodies in 5 % bovine serum albumin-TBST, thoroughly washed with TBST solution and incubated with the correspondent secondary antibodies for 1 hr at room temperature. Lastly, the blots were rinsed with TBST and the peroxidase reaction was developed by enhanced chemiluminescence SuperSignal® West Dura Extended Duration Substrate (Thermo Scientific, Rockford, Ill., USA). Blots were stripped in stripping Buffer (Tris base 62.7 mM, SDS 69.5 mM, β -mercaptoethanol 111.6 mM, pH 6.7) and were reused sequentially. Images were captured with a Fujifilm Image Reader LAS-1000 Pro version 2.6 (Fujifilm, Stockholm, Sweden), and band intensities (density arbitrary units) corresponding to immunoblot detection of protein samples were quantified using the Fujifilm Multi Gauge version 3.0 software (Fujifilm, Stockholm, Sweden).

Antibodies

Anti-Nrf2 antibody (1:500) was from R&D Diagnostics (Minneapolis, Minn., USA). Anti-AKT and anti-phosphorylated AKT antibody (1:1000), Anti-GSK3 β (1:500), anti-pGSK3 β (1:500) was from Cell Signaling Technology (Massachusetts, USA). Anti-PGC-1 α (1:500), anti- α -tubulin (1:2000), anti- γ -glutamylcysteine ligase catalytic subunit (γ GCLC) (1:500) antibodies were from Santa Cruz Biotechnology (Heidelberg, Germany). Anti-IL-1 β

antibody (1:500) was purchased from Bio-Rad AbD Serotec, (Dusseldorf, Germany). Peroxidase-conjugated anti-rabbit (1:5000) and anti-mouse (1:5000) secondary antibodies were from Vector Laboratories (Burlingame, Calif., USA).

Statistical Analysis

Results are presented as means $\pm$ standard error of the mean (SEM). For analysis of the interaction of diet on LPS treatment, two-way ANOVA analysis was performed by the SAS PROC MIXED using the SAS v.9.3 (SAS Inc., Cary, N.C., USA). This was followed by Bonferroni post-hoc test. Significance levels were at *p 0.05, **p 0.01, ***p 0.001. The Bonferroni post hoc test was performed by using GraphPad software.

Results

A Spirulina enriched diet normalized elevated IL-1 β protein in substantia nigra

We first analysed IL-1 β mRNA as well as IL-1 β protein expression which both were increased 65 days after LPS injection in PND 5 rats (Fig. 1A, B, C). Feeding the dams 24 h before the pups were injected with LPS with a diet enriched with 0.1% Spirulina had no effects on the levels of IL-1 β mRNA but restored the levels of IL-1 β protein (Fig. 1B). No effects were observed on mRNA expression for TNF α (data not shown).

The NRF2-system in substantia nigra of 70 day old rats was decreased by neonatal LPS, effects that were restored by a Spirulina enriched diet

The Nrf2 mRNA expression was decreased 65 days after injection of LPS (Fig. 2A). The mRNA of the Nrf2 down-stream gene γ GCLC was also decreased, whereas the reduction of γ GCLM mRNA expression did not reach significance (Fig. 2 B,C). The Spirulina enriched diet elevated mRNA of γ GCLM but left other gene targets of Nrf2 unchanged (Fig. 2A–D). The protein levels of Nrf2 and γ GCLC in substantia nigra of LPS injected animals were reduced compared to saline injected animals (Fig. 3 A,B). The Spirulina diet normalized the effects of LPS on Nrf2 (Fig. 3A). There was a trend for PGC-1 α protein levels to be reduced by LPS and following maternal administration of the Spirulina diet, the levels were normalized (Fig. 3C).

GSK-3 β and AKT are putative mediators of decreased Nrf2 in substantia nigra after LPS

LPS treatment in PND 5 pups led to decreased levels of the active phosphorylated form of AKT (pAKT) and of the inactive form of GSK-3 β (phosphorylated, pGSK-3 β) in PND 70 rats (Fig. 4A–D). The decreased level of pAKT by LPS treatment was restored by the Spirulina enriched diet while the effect on GSK-3 β was less clear. In the original report on this model [6], it was shown that the level of tyrosine hydroxylase (TH) was decreased in substantia nigra 65 days following the injection of LPS in 5 day old rats [6]. In our study an apparent decrease in TH did not reach significance (Fig. 4E, F).

Discussion

Increase in IL-1 β and decrease in Nrf2 in the adult substantia nigra after neonatal systemic LPS

Here we show that systemic LPS in PND 5 rats caused an increase of IL-1 β and decrease in Nrf2 in substantia nigra that is sustained after 65 days. The effect on IL-1 β is in agreement with earlier studies using this model [6] and appears to be region specific as the effect of LPS was normalized in cortex after 65 days [19]. The decrease in Nrf2 has to our knowledge not been demonstrated earlier and could be an important mechanism behind the elevated vulnerability in the substantia nigra after neonatal infection [6].

We found down-regulation of both mRNA and protein of Nrf2 in substantia nigra 65 days following peripheral LPS. A dysfunctional Nrf2 system was further indicated by the decrease in the mRNA and protein of one of the subunits of the rate limiting enzyme in glutathione synthesis, γ GCLC, whereas the apparent decrease in γ GCLM mRNA did not reach significance. Likewise, others have shown that mRNA of BDNF, which can be regulated by Nrf2, was decreased after neonatal LPS exposure [20,21]. From these results we argue that one mechanism by which increased neuroinflammation can mediate increased vulnerability is via a decreased Nrf2 function. This prolonged effect on Nrf2 is evident in the substantia nigra but not in cortex [19], which, at least partly, could explain the increased vulnerability to toxins of substantia nigra in animals subjected to neonatal inflammation [9] and could potentially be an important determinant behind sporadic Parkinson's disease (PD).

Earlier studies have indirectly suggested a prolonged effect on the Nrf2 system of *prenatal* inflammation where LPS given to gravid rats leads to changes in the homeostasis of glutathione in the offspring 4 and 17 months later [22]. Down-regulation of the Nrf2-system by genetic deletion makes animals more sensitive to drugs and other manipulations that induce Parkinson-like behavior in animals [23]. Interestingly, treadmill exercise reversed the MPP⁺ induced down-regulation of the Nrf2-system in rat nigrostriatal dopaminergic neurons further indicating that the Nrf2 system is critical for protection of DA-neurons in substantia nigra [24]. In human postmortem material of Parkinson's patients, Nrf2 showed strong expression in the nuclei of surviving neurons [25]. The authors suggested that this indicates that Nrf2 was induced in surviving neurons, but that this was not effective enough to protect the already dead neurons from dying and/or that in the already dead neurons the Nrf2 system had been dysfunctional [25]. An association has been found between a haplotype in the Nrf2-gene and risk to develop PD [26–28]. This indicates that variations in the NFE2L2 gene could contribute to the pathology of Parkinson's disease [29]. In addition, Nrf2 levels decrease with aging, which is the main risk factor for PD and several other neurodegenerative diseases [30]. These studies together suggest that a decreased Nrf2 function following inflammation may be one factor mediating sporadic PD.

A diet enriched in Spirulina partly restores the negative effects of systemic neonatal LPS

Previous studies using diets enriched with Spirulina showed enhanced recovery of tyrosine hydroxylase positive neurons in the substantia nigra following an intrastriatal 6-OHDA lesion model of PD [31] and in an α -synuclein model of PD [32]. In these studies rats were

fed a Spirulina enriched diet for 30 days before the onset of the Parkinson inducing protocol. In our study the pregnant mother were fed the Spirulina diet one day before treatment of the pups with LPS and the rats were continued to be fed with Spirulina diet after the separation from the mother until PND70. Spirulina treatment of rats undergoing MCAO showed that oral Spirulina protects *in vivo* against the oxygen-deprivation induced pathology [33]. The mechanism behind the positive effects of Spirulina enriched diet was suggested to be related to elevated levels of anti-oxidant enzymes and the reduced form of glutathione. In another study using a glioma culture it was demonstrated that Spirulina elevate the level of mRNA of BDNF likely via HO-1 [34] which indicates that Nrf2 could have been activated. In our study we found that the Spirulina enriched diet normalized or partly normalized the levels of Nrf2 protein and elevated the level of PGC-1 α protein. The LPS-induced enhanced protein level of IL-1 β but not the mRNA level was decreased by the Spirulina enriched diet, which points towards post-translational modifications and/or changed proteolysis induced by the diet. Whether these putative modifications are linked to the changes in the function of the inflammasome, proteolysis or to miRNA that may reduce IL-1 β protein needs further studies [35]. The persistent upregulation of IL-1 β mRNA in 70 day old rats after a single dose of LPS in 5 day old rats indicate epigenetic mechanisms as the cause for the upregulation. One possible mechanism is that the inflammation cause hypomethylation of the IL-1 β promoter as recently described in old and SIRT1 deficient animals [36].

Decreased active AKT and increased active GSK-3 β are possible mediators of the elevated Nrf2/ PGC-1 α in adult substantia nigra after neonatal LPS

It has earlier been shown that neonatal infection can cause decreased BDNF and increased IL-1 β mRNA induction in specific regions of hippocampus following fear conditioning in the adult animal [37]. The basal levels were not effected, only the induced level. In our study the basal level of mRNA for BDNF was reduced as has been found earlier in substantia nigra of PD patients [38,39]. One of the down-stream effects of BDNF is activation of AKT. Here we found a decrease in the level of phosphorylated AKT after LPS which in turn can be the mechanism behind the decrease in phosphorylated GSK-3 β as GSK-3 β is a substrate of AKT. A decrease in pGSK-3 β is equivalent to an increased activity [15]. In our earlier studies we have shown that active GSK-3 β and inactivation of AKT decrease the Nrf2-system in astroglia after treatment with medium from LPS activated microglia [15]. Active GSK-3 β has earlier been suggested as an important factor in development of PD via phosphorylation of various targets including α -synuclein [40]. In addition to down-regulation of Nrf2 by GSK-3 β this enzyme could also decrease PGC-1 α which will lead to a decrease in the number of mitochondria and reduced anti-oxidant systems in mitochondria [41]. The Spirulina enforced diet normalized the LPS induced decrease in pAKT whereas the effects on pGSK-3 β was less clear. A possible improvement of the intervention could thus be to combine an enriched diet with a specific inhibitor of GSK-3 β .

In conclusion, neonatal LPS caused a prolonged decrease in Nrf2 and increase in IL-1 β in substantia nigra that was partly restored to normal levels by a diet enriched in Spirulina. The diet also increased the protein level of the anti-oxidant transcriptional cofactor PGC-1 α . The mechanisms behind the negative effects of LPS on Nrf2 and the partly restorative effects of

the diet on Nrf2 and PGC-1 α may relate to the observed changes in the AKT/GSK-3 β signaling system (Fig. 5).

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Abbreviations

BCA	Bicinchoninic acid assay
BDNF	Brain-derived neurotrophic factor
DA	dopamine
γGCLC	gamma-glutamylcysteine ligase catalytic subunit
γGCLM	gamma-glutamylcysteine ligase modulatory subunit
γGCS	glutamylcysteine synthetase
Gapdh	glyceraldehyde 3-phosphate dehydrogenase
GSK3β	glycogen synthase kinase-3 beta
Gusb	betaglucuronidase
GSH	glutathione
GPx	glutathione peroxidase
GR	glutathione reductase
IL-1β	Interleukin 1 beta
Hprt1	hypoxanthine-guanine phosphoribosyltransferase
Keap1	Kelch-like ECH-associated protein 1
LPS	lipopolysaccharide
Nrf2	nuclear factor-erythroid 2-related factor 2
PD	Parkinson's disease
PGC-1α	peroxisome proliferator-activated receptor gamma coactivator-1 alpha
qRT-PCR	quantitative real time PCR
SIRT1	Sirtuin1
SP0.1%	Spirulina 0.1%

TNF α tumor necrosis factor- α

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Figure 1.

Systemic LPS (PND5) induced changes in IL-1 β mRNA and protein in substantia nigra (PND 70) and the effects of a Spirulina enriched diet (see Materials and Methods for details). Quantitative real time RT-PCR expression data is expressed as relative expression of IL-1 β mRNA normalized to geometric mean of GAPDH, GUSB and HPRT1 mRNA. For IL-1 β mRNA Fig.1A, two-way ANOVA did not show a significant interaction between diet and treatment, but did reveal main effects of diet and LPS treatment. Bonferroni post-hoc analysis comparing Saline (n = 19) vs LPS (n = 18) and LPS vs LPS+SP0.1% (n = 12) groups are shown. Densitometric analysis of IL-1 β , protein expression is shown in Fig.1B. Data are plotted as ratio of the protein of interest /tubulin. Representative blots are shown in Fig. 1C. Two-way ANOVA showed a statistically significant interaction between diet and LPS treatment for IL-1 β protein, $p = 0.0181$, Bonferroni post-hoc analysis comparing Saline (n = 10) vs LPS (n = 9) and LPS vs LPS+SP0.1% (n = 12) groups are shown. *denotes statistical significance (** $p < 0.01$). Values are expressed as means $\pm$ SEM.

Figure 2.

The effects of neonatal LPS on Nrf2 and Nrf2-regulated mRNA expression in substantia nigra in PND70 rats and the effects of a Spirulina enriched diet (for details see Materials and Methods). Quantitative real time RT-PCR expression data is expressed as relative expression of target genes normalized to geometric mean of GAPDH, GUSB and HPRT1 mRNA. Two-way ANOVA showed a statistically significant interaction between diet and LPS treatment for: Nrf2 in Fig. 2A, $p = 0.0306$ and BDNF in Fig. 2D, $p = 0.0034$, two-way ANOVA did not show a significant interaction between diet and treatment, but did reveal main effects of diet and LPS treatment for GCLC mRNA, Fig. 2B and GCLM mRNA Fig. 2C. Bonferroni post-hoc analysis comparing Saline ($n = 19$) vs LPS ($n = 18$) and LPS vs LPS+SP0.1% ($n = 12$) groups are shown, *denotes statistical significance (ns-not significant). Values are expressed as means $\pm$ SEM.

Figure 3.

The effect of neonatal LPS on Nrf2 protein, Nrf2-related protein expression in substantia nigra of PND 70 rats and the effects of a Spirulina enriched diet (for details see Material and Methods). Densitometric analyses of Nrf2, γGCLC and PGC-1α protein expression are shown in Fig.3 A, B, C, respectively. Data are plotted as ratio of the protein of interest/ tubulin. Representative blots are shown in Fig. 3D. Two-way ANOVA showed a statistically significant interaction between diet and LPS treatment for: Nrf2 in Fig. 3A, $p = 0.0003$. The two-way ANOVA did not show statistically significant interaction between diet and treatment but did reveal main effects of diet and LPS treatment for: γGCLC and PGC-1α. Bonferroni post-hoc analysis comparing Saline ($n = 10$) vs LPS ($n = 9$) and LPS vs LPS +SP0.1% ($n = 12$) groups are shown, *denotes statistical significance (* $p < 0.05$; ** $p < 0.01$; ns-not significant). Values are expressed as means $\pm$ SEM.

Figure 4.

The effects of neonatal LPS on Nrf2 regulating kinases AKT and GSK-3 β and tyrosine hydroxylase (TH) and in substantia nigra of PND70 rats and the effects of a Spirulina enriched diet. Densitometric analyses of phosphoAKT (pAKT), phospho-GSK3 β (pGSK3 β) and TH protein expression are shown in Fig.4 A, C, E. Data are plotted as ratio of the pAKT/total AKT (Fig. 4A), pGSK3 β /total GSK3 β (Fig. 4C) and TH/tubulin (Fig. 4E). Representative blots are shown in Fig. 4 B, D, F; respectively. Two-way ANOVA showed a statistically significant interaction between diet and LPS treatment for: phospho-Akt in Fig.

4A, $p = 0.004$ and for phospho-GSK3 β in Fig. 4C, $p = 0.0320$; however the two-way ANOVA did not show statistically significant interaction between diet and treatment for TH. Bonferroni post-hoc analysis comparing Saline ($n = 10$) vs LPS ($n = 9$) and LPS vs LPS + SP0.1% ($n = 12$) groups are shown, *denotes statistical significance (* $p < 0.05$; ** $p < 0.01$; ns-not significant). Values are expressed as means $\pm$ SEM.

Figure 5.

Putative relationship between IL-1β and Nrf2. Systemic LPS cause prolonged IL-1β upregulation in Substantia Nigra pars compacta (SNigra PC) and decreased protein and mRNA of Nrf2 and its down-stream gene products γGCLC and BDNF. The mechanistics indicated have not been proven but may involve: decreased pAKT and increased active GSK-3β. The action of Spirulina is likely multifactorial including decreased neuroinflammation, activation of AKT and possibly direct activation of Nrf2.

Table 1

List of primers used for qRT-PCR

Primer name	Catalog number
Rn_IL-1 β _1_SG QuantiTect Primer Assay	QT00181657
(Nrf2) Rn_RGD:620360_1_SG QuantiTect Primer Assay	QT00183617
Rn_GCLC_1_SG QuantiTect Primer Assay	QT00178878
Rn_GCLM_1_SG QuantiTect Primer Assay	QT00180103
Rn_BDNF_1_SG_1_SG QuantiTect Primer Assay	QT00375998
Rn_GUSB_1_SG QuantiTect Primer Assay	QT01082053
Rn_GAPDH_1_SG QuantiTect Primer Assay	QT00199633
Rn_HPRT1_2_SG QuantiTect Primer Assay	QT00365722