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nNOS inhibition during profound asphyxia reduces seizure burden and improves survival of striatal phenotypic neurons in preterm fetal sheep

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Abstract

Basal ganglia injury after hypoxia-ischemia remains common in preterm infants, and is closely associated with later cerebral palsy. In the present study we tested the hypothesis that a highly selective neuronal nitric oxide synthase (nNOS) inhibitor, JI-10, would improve survival of striatal phenotypic neurons after profound asphyxia, and that the subsequent seizure burden and recovery of EEG are associated with neural outcome. 24 chronically instrumented preterm fetal sheep were randomized to either JI-10 (3 ml of 0.022 mg/ml, n=8) or saline (n=8) infusion 15 min before 25 min complete umbilical cord occlusion, or saline plus sham-occlusion (n=8). Umbilical cord occlusion was associated with reduced numbers of calbindin-28k-, GAD-, NPY-, PV-, Calretinin- and nNOS-positive striatal neurons ($p < 0.05$ *vs*. sham occlusion) but not ChAT-positive neurons. JI-10 was associated with increased numbers of calbindin-28k-, GAD-, nNOS-, NPY-, PV-, Calretinin- and ChAT-positive striatal neurons ($p < 0.05$ *vs*. saline+occlusion). Seizure burden was strongly associated with loss of calbindin-positive cells ($p < 0.05$), greater seizure amplitude was associated with loss of GAD-positive cells ($p < 0.05$), and with more activated microglia in the white matter tracts ($p < 0.05$). There was no relationship between EEG power after 7 days recovery and total striatal cell loss, but better survival of NPY-positive neurons was associated with lower EEG power. In summary, these findings suggest that selective nNOS inhibition during

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asphyxia is associated with protection of phenotypic striatal projection neurons and has potential to help reduce basal ganglia injury in some premature babies.

Keywords

Asphyxia; basal ganglia; nNOS; neuroprotection; preterm fetus; seizures

1. Introduction

Cerebral palsy (CP) of perinatal origin remains a major problem (Committee on Understanding Premature Birth and Assuring Healthy Outcomes, 2007). There is increasing evidence that it results from antenatal hypoxia-ischemia (HI) in about 70–80 % of cases (Graham et al., 2008), particularly in association with premature birth (Robertson et al., 2007). Although white matter injury (WMI) remains the predominant pattern of brain injury after HI in preterm infants (Bax et al., 2006), injury to the basal ganglia is very common and is the second most frequently injured region (Barkovich and Sargent, 1995; Bax et al., 2006; de Vries et al., 1998; Gilles et al., 1998; Leijser et al., 2004; Logitharajah et al., 2009; Paneth et al., 1990). The presence of basal ganglia injury is much more predictive of CP and other motor problems, as well as subsequent epilepsy and learning difficulties problems than injury in other regions such as a ‘watershed’ distribution (Harteman et al., 2013; Martinez-Biarge et al., 2011), and WMI (Himmelmann and Uvebrant, 2011). Thus, it is important to find new therapies that specifically protect the basal ganglia from HI injury.

Consistent with the clinical patterns, asphyxia induced by complete umbilical cord occlusion in preterm fetal sheep is associated with significant basal ganglia and white-matter injury, but not cortical injury after 3 and 7 days recovery (Bennet et al., 2007; Drury et al., 2014). We have recently shown that prophylactic therapy with a novel and potent inhibitor of neuronal nitric oxide synthase (nNOS) was associated with protection of neurons in the caudate nucleus (Drury et al., 2013). This supports previous findings in rabbit kits that selective nNOS inhibition during profound prenatal asphyxia was associated with reduced CP (Ji et al., 2009; Yu et al., 2011). There is some evidence that the basal ganglia is highly active during seizures (Clozel et al., 1985) and that seizures are associated with striatal injury at term (Brambrink et al., 1999). It is unclear whether basal ganglia damage is associated with seizures following hypoxia-ischemia in the very immature brain.

In the present study, we tested the hypothesis that prophylactic selective inhibition of nNOS with a highly specific agent (JI-10) (Drury et al., 2013) would protect phenotypic striatal neurons following profound asphyxia in 0.7 gestation preterm fetal sheep, when brain development is broadly consistent with 28 to 32 weeks in humans, before the development of cortical myelination (McIntosh et al., 1979). We examined the secondary hypothesis that reduced seizure activity would be associated with improved neural outcome.

2. Methods

2.1 Instrumentation

All procedures were approved by the Animal Ethics Committee of The University of Auckland following the New Zealand Animal Welfare Act, and the Code of Ethical Conduct for animals in research established by the Ministry of Primary Industries, Government of New Zealand. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Twenty-four Romney/Suffolk fetal sheep were operated on at 98–99 days of gestation (term = 147 days). Food, but not water was withdrawn 18 h before surgery. Ewes were given 5 ml of Streptocin (procaine penicillin (250,000 IU) and dihydrostreptomycin (250 mg/ml); Stockguard Labs Ltd, Hamilton, N.Z.) intramuscularly 30 min prior to the start of surgery. Maternal weight was recorded. Anesthesia was induced by i.v. injection of propofol (5 mg/kg; AstraZeneca Limited, Auckland, New Zealand), and general anesthesia maintained using 2–3% isoflurane (Medsource, Ashburton, N.Z.) in O_2. Under anesthesia, a 20 g i.v. catheter was placed in a maternal front leg vein and the ewes were placed on a constant infusion saline drip to maintain maternal fluid balance. Ewes were ventilated, and the depth of anesthesia, maternal heart rate, SpO_2, end-tidal isoflurane and CO_2, and respiration were constantly monitored by trained anesthetic staff.

As previously described (Drury et al., 2013), catheters were placed in the left fetal femoral artery and vein, right brachial artery and vein, and the amniotic sac. A 3S ultrasonic blood flow probe (Transonic Systems Inc., Ithaca, NY, USA) was placed around the left carotid artery to measure carotid blood flow (CaBF), as an index of global cerebral blood flow (Bennet et al., 1999; Gonzalez et al., 2005; van Bel et al., 1994). EEG electrodes (AS633-5SSF, Cooner Wire Co., Chatsworth, CA, USA) were placed on the dura over the parasagittal parietal cortex (5 mm and 10 mm anterior to bregma and 5 mm lateral). Electrodes were placed to measure the fetal electrocardiogram (ECG). An inflatable silicone occluder was placed around the umbilical cord of all fetuses (In Vivo Metric, Healdsburg, CA, USA). All fetal leads were exteriorized through the maternal flank and a maternal long saphenous vein was catheterized to provide access for post-operative care and euthanasia. 80 mg gentamicin (Rousell, Auckland, New Zealand) was administered into the amniotic sac as the uterus was closed.

Post-operatively all sheep were housed in separate metabolic cages with access to water and food *ad libitum*, together in a temperature-controlled room (16 ± 1 °C, humidity 50 ± 10 %) with a 12 h light/dark cycle. A period of at least 4 days post-operative recovery was allowed before experiments commenced, during which time antibiotics were administered i.v. to the ewe daily for four days (600 mg benzylpenicillin sodium; Novartis Ltd, Auckland, New Zealand, and 80 mg gentamicin sulphate; Pfizer Pty Ltd, Perth, Australia). Fetal catheters were maintained patent by continuous infusion of heparinized saline (20 U/ml at 0.2 ml/h) and the maternal catheter maintained by daily flushing. JI-10 (Ji et al., 2010) was dissolved with sterile saline (0.022 mg/ml) and a total of 0.066 mg was given, to achieve 100x the Ki of 0.0077 μmol/l determined in previous experiments, with estimated fetal weight 1.5 kg, and a circulating fetal blood volume of 120 ml/kg (Brace, 1983). All containers and syringes

for JI-10 were first rinsed with 0.1 % bovine serum albumin in sterile saline, after filtering through a bacterial filter.

2.2 Recordings

Fetal mean arterial blood pressure (Novatrans II, MX860; Medex Inc., Hilliard, OH, USA), fetal heart rate derived from the ECG (Drury et al., 2013), CaBF, and EEG were recorded continuously from −24 h to 168 h after umbilical cord occlusion. The analogue fetal EEG signal was low pass filtered with the cut-off frequency set with the −3 dB point at 30 Hz, and digitized at 256 Hz (using analogue to digital cards, National Instruments Corp., Austin, TX, USA). The intensity and frequency were derived from the intensity spectrum signal between 0.5 and 20 Hz. Data from left and right EEG electrodes were averaged. Power in the Delta (0–3.9 Hz), Theta (4–7.9 Hz), Alpha (8–12.9 Hz), and Beta (13–22 Hz) spectral bands was calculated as previously described (Keogh et al., 2012a). The continuous EEG recording was assessed for continuity by quantifying the percentage of time per minute EEG amplitude was >25 μV.

2.3 Experimental protocol

Experiments were conducted at 103–104 days gestation. Fetuses were randomly assigned to JI-10+occlusion (n = 8) or saline+occlusion (n = 8). Sham occlusion fetuses received saline infusions and no occlusion (n = 8). JI-10 was administered to the fetal brachial vein 15 min prior to umbilical cord occlusion as 0.022 mg/ml in 3 ml, over 10 min. Saline+occlusion fetuses received the same volume of saline.

Fetal asphyxia was induced by rapid inflation of the umbilical cord occluder for 25 min with sterile saline of a defined volume known to completely inflate the occluder as determined in pilot experiments with a flow probe placed around the umbilical cord (Bennet et al., 1999). Successful occlusion was confirmed by observation of a rapid onset of bradycardia, and by pH and blood gas measurements. The duration of occlusion was chosen as one that we have previously reported to represent an acute, severe, near-terminal insult (Bennet et al., 2007). All occlusions or sham occlusions were undertaken between 0900 and 1000 h.

Fetal arterial blood was taken at 30 min prior to occlusion, 5 and 17 min during asphyxia, and then 10 min, 1, 2, 4, 6, 24, 48, 72, 96, 120, 144, and 168 h post-asphyxia for pH and blood gas determination (Ciba-Corning Diagnostics 845 blood gas analyzer and co-oximeter, MA., USA) and for glucose and lactate measurements (YSI model 2300, Yellow Springs, Ohio, USA). After the last blood sample, ewes and fetuses were killed by an i.v. overdose of pentobarbitone sodium (9 g) to the ewe (Pentobarb 300; Chemstock International, Christchurch, New Zealand). Fetal brains were perfusion-fixed in situ with 0.9% saline solution followed by 10% phosphate-buffered formalin (500 ml, from 2 m height), and then removed and fixed for a further three days before processing and paraffin embedding (Bennet et al., 2007).

2.4 Histopathology

Slices (10 μm thick) were cut using a microtome (Leica Jung RM2035). Slides were dewaxed in xylene and rehydrated in decreasing concentrations of ethanol. Slides were

washed in 0.1 mol/L phosphate buffered saline (PBS). Antigen retrieval was performed in citrate buffer (with the exception of EDTA for GAD) using the pressure cooker technique in an antigen-retrieval system (EMS Antigen 200 Retriever, Emgrid, Australia). Endogenous peroxidase quenching was performed by incubation in 1 % H_2O_2 in methanol for anti-neuronal nuclei monoclonal antibody (NeuN), Calbindin-28k, nNOS, GAD, ChAT, Parvalbumin (PV), Neuropeptide Y (NPY), Calretinin and ionized calcium binding adaptor molecule-1 (Iba-1). Blocking was performed in 3 % normal horse serum (NHS) for NeuN and Iba-1 and normal goat serum (NGS) for Calbindin-28k, nNOS, ChAT, GAD, PV, NPY, and Calretinin for 1 h at room temperature. Sections were labeled with 1:400 mouse anti-NeuN (Chemicon International, Temecula, CA, USA), 1:200 goat anti-Iba-1 (Abcam, via Sapphire Bioscience, Hamilton, New Zealand), 1:200 rabbit anti-Calbindin-28k (Swant® Ltd, Rte de l'ancienne Papeterie, Marly, Switzerland), 1:500 rabbit anti-nNOS (Abcam), 1:200 mouse anti-ChAT (Abacus ALS, Auckland, New Zealand), 1:200 rabbit anti-GAD (Abcam), 1:200 rabbit anti-PV(Swant®), 1:500 rabbit anti-NPY(Abcam), and 1:1000 rabbit anti-Calretinin(Swant®) overnight (2 nights for GAD) at 4 °C. Sections were incubated in biotin-conjugated secondary 1:200 horse anti-mouse in 3% NHS (NeuN and ChAT) or 1:200 goat anti-rabbit IgG (Vector Laboratories, Burlingame, USA) in 3.5% NGS for 2 h at room temperature. Slides were then incubated in 1:200 ExtrAvidin® (Sigma-Aldrich Pty. Ltd.) in PBS for 2 h at room temperature and then allowed to react in diaminobenzidine tetrachloride (DAB) (Sigma-Aldrich Pty. Ltd.). The reaction was stopped by washing in dH_2O, the sections were then dehydrated and mounted.

Brain regions of the forebrain used for analysis included the mid-striatum (comprising the caudate nucleus and putamen) and the frontal subcortical white matter (comprising the intragyral, IGWM, and periventricular, PVWM, regions) on sections taken 23 mm anterior to stereotaxic zero (Gluckman and Parsons, 1983). Neuronal (NeuN) and microglial (Iba-1) changes were quantified by light microscopy at 40 x magnification (Nikon eclipse 80i, Scitech ltd, Preston, Victoria, Australia) and NIS Elements Br 4.0 software (Nikon Instruments Inc., Melville, N.Y., U.S.A.) using seven fields in the striatum (four in caudate nucleus, three in putamen), and two fields in the white matter (one intragyral, one periventricular) (Dean et al., 2006). Total microglia were counted as all cells with Iba-1 immunostaining. For assessment of activated microglia Iba-1 cells showing an amoeboid morphology with no cell processes were counted.

Estimates of total striatal phenotypic neuron density were quantified by light microscopy using Stereoinvestigator software (Microbrightfield Bioscience (MBF), Williston, VT, USA). The caudate nucleus and putamen were outlined at 2x magnification and neurons were counted at 10x magnification. A grid of 1200 × 1200 μm was placed at a random rotation on the outlined area. Cells touching the bottom and right hand boundaries were counted whilst those touching the top and left were excluded using a fractionator probe. Neurons were assessed according to morphological characteristics and were excluded if cells showed a condensed nucleus or fragmented appearance (Pozo Devoto et al., 2006). For each animal, average scores across both hemispheres from two sections were calculated for each region. Counts were made by an assessor blinded to the treatment groups.

2.5 Data analysis

Off-line analysis of the physiological data was performed using customized Labview programmes. The raw EEG was assessed for epileptiform activity. Seizures were identified visually and defined as the concurrent appearance of sudden, repetitive, evolving stereotyped waveforms in the EEG signal lasting more than 10 sec and of an amplitude greater than 20 μV (Scher et al., 1993b). Seizures were analyzed to determine total number, mean amplitude, onset, and burden, defined as the total time spent having seizures.

Data were analyzed using SPSS 15.0 for Windows (SPSS, Chicago, Il, USA). For between group comparisons two-way analysis of variance (ANOVA) for repeated measures was performed. Multiple regions within one structure (i.e. caudate nucleus and putamen in the striatum) were tested as a repeated measure. Where significance was found with ANOVA for group or region*group, Tukey's pairwise comparisons were used to compare selected groups. Non-parametric Mann-Whitney U-tests were used where appropriate. Linear or non-linear regression was used as appropriate to compare total EEG power, alpha power, CaBF over the 6^{th} hour and the final day of recovery, and seizure activity with histological outcome as appropriate. Statistical significance was accepted as $p < 0.05$. Data are presented as mean ± SEM unless otherwise stated.

3. Results

3.1 Baseline and umbilical cord occlusion

Baseline blood gases, acid-base status and glucose-lactate values were not different between groups. Umbilical cord occlusion was associated with marked fetal hypoxia, hypercarbia, and acidosis, bradycardia, hypotension, peripheral vasoconstriction, cerebral hypoperfusion, EEG suppression, and increased cortical impedance, similarly to our previous report (Drury et al., 2013). There were no differences during occlusion between the saline and JI-10 groups.

3.2 Recovery

EEG power was suppressed for more than 36 h in both groups, with similar loss of high frequency activity in both groups, before progressively recovering to baseline values by approximately 96 h. Electrographic seizures developed 354 (329–403) min (median (interquartile range)) after saline+occlusion, with a marked further delay after JI-10+occlusion (588 (453–895) min, $p < 0.05$). There was no significant difference in the total number of seizures during the recovery period (saline+occlusion: 58 (48–80) vs. JI-10+occlusion: 69 (14–81)) or the duration (67 (57–86) vs. 65 (47–81) sec) or peak amplitude of individual seizures (170 (162–211) vs. 155 (128–188) μV). CaBF was significantly suppressed below sham occlusion values (31.0 ± 4.9 ml/min) in both occlusion groups (6 hours after asphyxia, saline+occlusion: 18.3 ± 1.5 vs. JI-10+occlusion: 21.1 ± 3.5 ml/min, N.S.), as previously described in this model (Bennet et al., 2006; Drury et al., 2013). CaBF was not different to saline control values from day 3 to day 7, with no difference between occlusion groups (day 7, saline+occlusion: 30.5 ± 2.0 vs. JI-10+occlusion: 36.7 ± 6.9 ml/min, N.S.).

3.3 Histopathology

Occlusion was associated with marked loss of NeuN-positive neurons in the caudate nucleus (sham: 111 ± 2 vs. saline+occlusion: 86 ± 5 cells per high power field, $p < 0.05$) and putamen (95 ± 2 vs. 76 ± 6, $p < 0.05$) compared to sham occlusion. JI-10+occlusion was associated with significantly greater overall neuronal survival in the caudate nucleus (105 ± 4, $p < 0.05$), but no significant effect in the putamen (71 ± 7, N.S.).

Iba-1 positive microglia were markedly increased after occlusion in both the IGWM (saline +occlusion: 84.0 ± 7.0 vs. sham occlusion: 43.1 ± 5.3 cells per high power field, $p < 0.05$) and the PVWM (89.4 ± 7.5 vs. 44.3 ± 7.2, $p < 0.05$). JI-10+occlusion did not affect microglial induction compared to saline+occlusion in either the IGWM (87.5 ± 19.3 cells per high power field, N.S.) or PVWM (98.6 ± 20.4, N.S.). Similarly, numbers of Iba-1 positive microglia showing an amoeboid morphology were increased after occlusion in both the IGWM (saline+occlusion: 13.2 ± 3.2 vs. sham occlusion: 8.1 ± 1.7 cells per high power field, $p < 0.05$) and the PVWM (17.0 ± 3.4 vs. 9.3 ± 2.3, $p < 0.05$). There was no significant change in amoeboid microglia after JI-10+occlusion compared to saline+occlusion in either the IGWM (22.5 ± 11.5 cells per high power field, N.S.) or PVWM (25.2 ± 11.3, N.S.).

Occlusion was associated with a significant loss of neurons immunopositive for calbindin, calretinin, NPY, parvalbumin, GAD and nNOS in both the caudate nucleus and putamen (Figure 1, $p < 0.05$ saline+occlusion vs. sham occlusion), but not for ChAT (N.S.). JI-10 was associated with significant protection in both regions for calbindin, calretinin, NPY, parvalbumin, GAD and nNOS positive neurons ($p < 0.05$ compared to saline+occlusion), and there were significantly more ChAT immunopositive neurons in the caudate nucleus in the JI-10 group compared to the saline group ($p < 0.05$). There were no differences between the sham occlusion and JI-10+occlusion groups for any immunostaining (N.S.).

3.4 Correlations

CaBF at 6 h was positively associated with the number of NeuN-positive neurons in the caudate nucleus (linear, $R^2 = 0.33$, $p < 0.05$), but no other region or immunostaining (N.S. for all). There was a significant relationship between CaBF at 7 d and total and amoeboid Iba-1 immunostaining ($R^2 = 0.34$ for both total IGWM and PVWM ($p < 0.05$) but no other histological measure (N.S.).

Total numbers of seizures was associated with greater total neuronal loss in the putamen (linear, $R^2 = 0.37$, $p < 0.05$), and with greater loss of calbindin-positive neurons in the caudate nucleus (non-linear, $R^2 = 0.33$, $p < 0.05$), and putamen (non-linear, $R^2 = 0.43$, $p < 0.05$), and with greater loss of NPY in the putamen (non-linear, $R^2 = 0.34$, $p < 0.05$) but no other region or immunostaining (N.S.). Seizure burden was inversely associated with total number of surviving neurons in the caudate nucleus (NeuN positive, linear, $R^2 = 0.29$, $p < 0.05$), and with calbindin in the caudate nucleus (non-linear, $R^2 = 0.33$, $p < 0.05$) and putamen (non-linear, $R^2 = 0.39$, $p < 0.05$), and with NPY in the putamen (non-linear, $R^2 = 0.35$, $p < 0.05$) but not with any other region or immunostaining (N.S. for all). Seizure amplitude was positively associated with the number of Iba-1 positive cells with amoeboid morphology in the IGWM (non-linear, $R^2 = 0.40$, $p < 0.01$) and PVWM (non-linear, $R^2 =$

0.61, $p < 0.001$), negatively with GAD positive cells in the putamen (non-linear, $R^2 = 0.31$, $p < 0.05$) and PV positive cells in the caudate (non-linear, $R^2 = 0.27$, $p < 0.05$) but not with total Iba-1 or any other immunostaining. There was no relationship between seizure onset and any histological outcome.

Total EEG power and alpha EEG power at day 7 were negatively associated with NPY in the caudate (non-linear, $R^2 = 0.67$ and 0.6 respectively, $p < 0.005$), and total EEG power with NPY in the putamen (non-linear, $R^2 = 0.23$, $p < 0.05$). EEG continuity >25 μV at 7 d recovery was negatively associated with ChAT in the caudate nucleus (non-linear, $R^2 = 0.36$, $p < 0.05$), and with NPY in the caudate and putamen (non-linear, $R^2 = 0.71$ and 0.33 respectively, $p < 0.05$) There was no other relationship between EEG continuity and histological outcome (N.S. for all). Consistent with increased seizures, total EEG power at 6 h was negatively associated with calbindin and NPY in the caudate nucleus (non-linear, $R^2 = 0.59$ and 0.22 respectively, $p < 0.05$) and putamen (non-linear, $R^2 = 0.45$ and 0.27 respectively, $p < 0.05$), with GAD, nNOS, and calretinin in the putamen (0.38, 0.23, 0.27 and 0.29 respectively, $p < 0.05$). Similarly, alpha power at 6 h was negatively associated with calbindin in the caudate nucleus (non-linear, $R^2 = 0.42$, $p < 0.05$), with NeuN in the caudate nucleus (non-linear $R^2 = 0.44$, $p < 0.01$), and with NPY in the putamen (non-linear, $R^2 = 0.31$, $p < 0.05$).

4. Discussion

The present study demonstrates for the first time that potent and selective inhibition of nNOS during and early after profound asphyxia significantly reduced loss of striatal phenotypic neurons in the preterm fetus. Greater EEG power at 6 h, reflecting greater electrographic seizure activity, was associated with worse neural outcome and greater loss of phenotypic striatal neurons. Interestingly, although there was no relationship between recovery of EEG power after 7 days recovery and total striatal cell survival, better survival of NPY-positive neurons was paradoxically associated with lower EEG power, consistent with a significant role for NPY-expressing interneurons in inhibiting cortical EEG activity.

Basal ganglia injury is highly implicated in the pathogenesis of athetoid CP (Barkovich and Sargent, 1995; Bax et al., 2006; Bell et al., 2005; de Vries et al., 1998; Gilles et al., 1998; Graham et al., 2008; Leijser et al., 2004; Logitharajah et al., 2009; Paneth et al., 1990; Robertson et al., 2007). To our knowledge this is the first study to report the effects of profound umbilical cord occlusion on phenotypic striatal neurons at 7 d recovery in the preterm fetus. In previous studies in preterm rabbit kits nNOS inhibition was associated with a significant reduction in cerebral palsy (Yu et al., 2011). Similar to our previous study at 3 d recovery after asphyxia there was a significant reduction in calbindin-28 k, GAD, and nNOS immunopositive cells, but no significant effect on ChAT cholinergic interneurons (George et al., 2007). JI-10 treatment was associated with greater ChAT immunostaining in the caudate compared to the saline treated fetuses after occlusion.

Previously, we showed that JI-10 was associated with neuroprotection in the caudate nucleus but not in the putamen (Drury et al., 2013), whereas in the present study we found significant overall improvement in number of surviving phenotypic neurons in both the

caudate and the putamen. The difference between the studies may reflect minor differences in insult or drug treatment as we previously demonstrated a similar trend to the present study. In contrast with the improvement in neuronal survival, nNOS inhibition did not modulate overall microglial induction after asphyxia or numbers of amoeboid microglia, suggesting that nNOS inhibition does not affect the inflammatory reaction to asphyxial brain injury. Microglia are highly reactive. Similarly to the present study, microglia have been repeatedly reported to become less ramified after injury, with rounded “amoeboid” cell bodies (Walker et al., 2014). Although microglial activation is often thought to be damaging, there is increasing evidence that in the early stages of injury microglia mediate cytotoxic responses, with release of pro-inflammatory cytokines, but that this is followed by a phenotypic shift toward immune regulation, and injury resolution (Mallard et al., 2014; Mosser and Edwards, 2008). In the present study greater total and amoeboid microglial induction at day 7 was associated with reduced carotid blood flow, consistent with a phenotypic shift away from production of cytotoxic factors (Mosser and Edwards, 2008). Strikingly, depletion of microglia before neonatal focal stroke increased injury, suggesting that microglial activation is an important contributor to the endogenous brain defenses of the developing brain (Faustino et al., 2011). Thus, the present findings suggest that neuroprotection can be achieved independent from the microglial reaction.

In terms of neuroprotection, the present study demonstrates a similar effect to post-asphyxial hypothermia (George et al., 2007), but with additional protection of GAD positive cells with JI-10. Hypothermia can suppress nitric oxide free radicals (Thoresen et al., 1997), has been shown to reduce nNOS activity (Karabiyikoglu et al., 2003), and protect calbindin-28k and nNOS positive striatal neurons (George et al., 2007). The vulnerability of the calbindin-28k and GAD populations may relate to their susceptibility to pathological calcium exposure (Freund et al., 1990; George et al., 2007), while the vulnerability of the nNOS population may relate to excitotoxin vulnerability (Figueredo-Cardenas et al., 1997). There is some evidence that the ChAT cholinergic interneuron population is relatively resistant to exogenous glutamate-agonist mediated depolarization (Calabresi et al., 1990). Further, ChAT-positive interneurons have been shown to have greater expression of superoxide dismutase (Inagaki et al., 1991; Medina et al., 1996), consistent with no significant reduction compared to the sham occlusion group. However, there was significantly more ChAT immunostaining in the JI-10 treated group compared to the saline treated group, suggesting that the effect of JI-10 may extend beyond prevention of free radical damage. Consistent with this there was a significant improvement in the GAD population that was not protected by hypothermia. Hypothermia can suppress excitotoxicity and free radical damage (Drury et al., 2010), suggesting that either this is required earlier (i.e. before hypothermia is generally started), or is an additional protective mechanism of JI-10.

Seizures occur in 58–132 per 1000 live births in preterm infants (Lanska et al., 1995; Scher et al., 1993a). It is unclear whether seizures *per se* cause new brain injury, or are a manifestation of evolving injury (Cole et al., 2002; Ferriero, 2004). In preterm neonates with hypoxic-ischemic encephalopathy 65% of infants with seizures had severe outcome or died, whereas absence of seizures was associated with good outcome (Logitharajah et al., 2009; Shah et al., 2010). There is a strong but non-linear relationship between seizures after

ischemic injury and neural survival at term equivalent (Williams et al., 1992). The relationship between seizure burden and outcome in preterm infants is unclear, although seizures are independently associated with an increased risk of CP in <32 week preterm infants (Murphy et al., 1997).

During seizures, blood flow to the basal ganglia is increased more than to the cortex, cerebellum, and brainstem, suggesting a high metabolic demand and thus electrical activity (Clozel et al., 1985). Miller and colleagues showed that in term infants seizures were associated with increased lactate/choline in the basal ganglia, after adjusting for the severity of injury, suggesting that seizures *per se* were associated with metabolic stress (Miller et al., 2002). In the model used in the present study, electrographic seizures are closely associated with the onset of secondary mitochondrial deterioration (Bennet et al., 2006), and we showed in a previous study that JI-10 was associated with a delay in the onset of seizures and mitochondrial deterioration. Potentially, loss of GABAergic interneurons in the basal ganglia could mediate disinhibition, facilitating seizures. Consistent with this, there was a strong negative correlation between seizure amplitude and GAD immunostaining in the putamen.

In near-term fetal sheep there is a non-linear positive correlation between EEG recovery at 7 d post-insult and cortical neuronal survival (Davidson et al., 2012). Reduced high frequency EEG activity is associated with reduced neurodevelopment in preterm infants (Scher et al., 1996), and increased low frequency activity is associated with injury in chorioamnionitis (Gavilanes et al., 2010), and with increased microglial activation, but no WMI in preterm fetal sheep (Keogh et al., 2012a). A shift to lower frequency activity but not total power was associated with WMI in preterm infants (Inder et al., 2003). There was a negative association between total power and alpha power at +6 h and neural outcome. Presumably this was related to seizure activity at this time (Dean et al., 2006; Keogh et al., 2012b; Yawno et al., 2007). Consistent with this, excessive alpha rhythmicity was associated with seizures and sharp waves in term infants (Hrachovy and O'Donnell, 1999). We have previously shown a relationship between EEG recovery at 7 d and neural outcome in term fetal sheep (Davidson et al., 2012), we show in the present study that there was no relationship between total, low, or high frequency EEG power at 7 d recovery with neural outcome. This is consistent with limited cortical injury in this paradigm (Drury et al., 2014; Drury et al., 2013), and that EEG power recovered to baseline by 7 d.

Intriguingly, in contrast, greater loss of NPY-positive interneurons was strongly associated with lower total and alpha EEG power. This effect appears to be specific since there was no relationship between EEG power and overall neuronal survival or numbers of calbindin-positive neurons. NPY inhibits glutamate release and seizure activity in rodents through the Y2 receptors (El Bahh et al., 2005) and, similarly, inhibited excitatory responses in slices from epileptic human dentate gyrii (Patrylo et al., 1999). Further, in adult rats, local NPY injection in the basal forebrain is associated with suppression of higher frequency EEG activity (Toth et al., 2005). Thus, these findings strongly suggest that loss of NPY-expressing interneurons in the present study was associated with loss of normal inhibition of cortical EEG activity.

In summary, selective and potent nNOS inhibition during asphyxia and the latent phase was associated with significant protection of striatal phenotypic neurons. There was a significant association between seizure activity and greater loss of neurons, and also between greater EEG power and lower neuronal counts, consistent with seizures at this time. In summary, these findings suggest that selective nNOS inhibition during asphyxia is associated with protection of phenotypic striatal projection neurons and has potential to help reduce basal ganglia injury in some premature babies.

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Glossary

CaBF	carotid arterial blood flow
ChAT	choline acetyl transferase
CP	cerebral palsy
EEG	electroencephalography
GAD	glutamic acid decarboxylase
HI	hypoxia-ischemia
nNOS	neuronal nitric oxide synthase
NPY	neuropeptide Y
PV	parvalbumin WMI, white matter injury.

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- nNOS inhibition significantly improved survival of striatal GABAergic interneurons
- Neuroprotection after nNOS inhibition was independent from microglial induction
- nNOS inhibition delayed onset of post-asphyxial seizures
- Seizure amplitude and numbers of GAD-positive neurons were inversely related
- Improved survival of NPY-positive neurons was associated with reduced EEG power

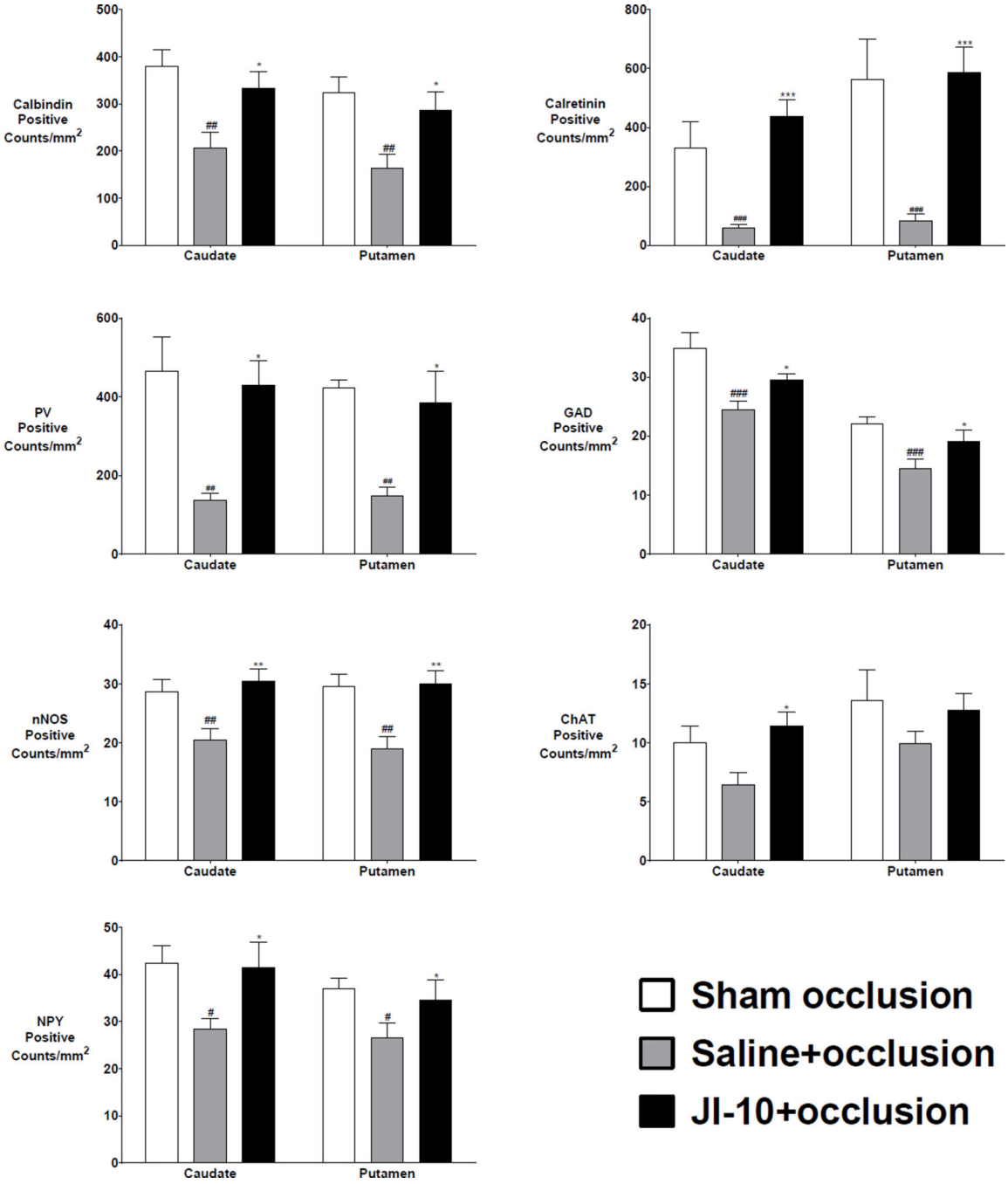

Figure 1.

Phenotypic striatal neuron density in the caudate nucleus and putamen. Data are mean ± SEM. Compared with repeated measures ANOVA and Tukey's pairwise comparisons where significance was found between groups. ChAT showed an interaction between region and group and thus regions were analyzed separately. # $p < 0.05$ for sham occlusion vs. saline +occlusion, ## $p < 0.005$ for sham occlusion vs. saline+occlusion, ### $p < 0.001$ for sham occlusion vs. saline+occlusion, * $p < 0.05$ for saline+occlusion vs. JI-10 +occlusion, ** $p <$

0.005 for saline+occlusion vs. JI-10 +occlusion, *** $p < 0.001$ for saline+occlusion vs. JI-10 +occlusion.

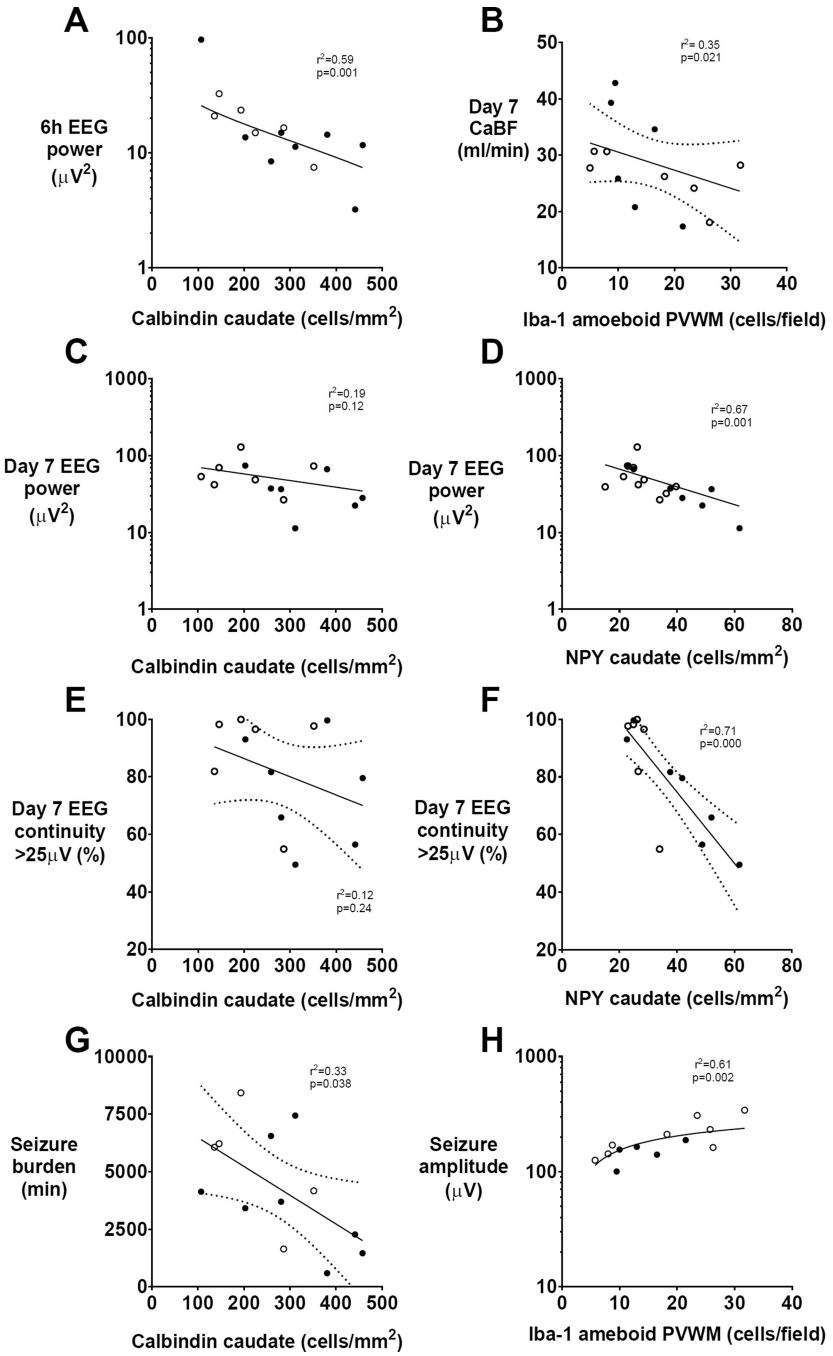

Figure 2.

Representative photomicrographs of phenotypic striatal neurons in the caudate nucleus from sham occlusion, saline+occlusion and JI-10+occlusion groups at 40x magnification. Arrows with tail show examples of cells counted and arrowheads show examples that were not counted. In addition to overall cell loss, occlusion was associated with regression of spinous processes, which appeared to be restored by JI-10 treatment. Scale bar is 100 μm.

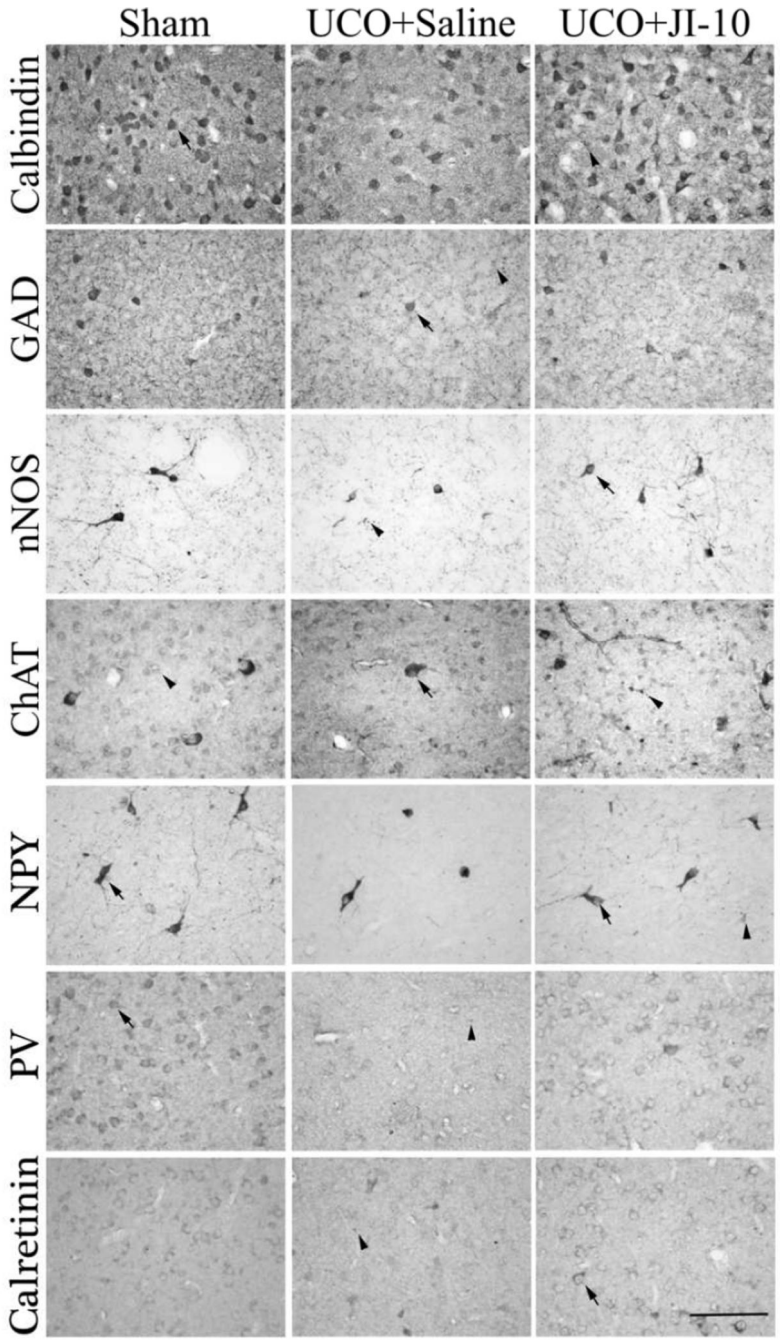

Figure 3.

Representative photomicrographs of phenotypic striatal neurons in the putamen from sham occlusion, saline+occlusion and JI-10+occlusion groups at 40x magnification. Arrows with tail show examples of cells counted and arrowheads show examples that were not counted. Morphologically, in addition to overall cell loss, occlusion was associated with regression of spinous processes, which appeared to be restored by JI-10 treatment. Scale bar is 100 μm.

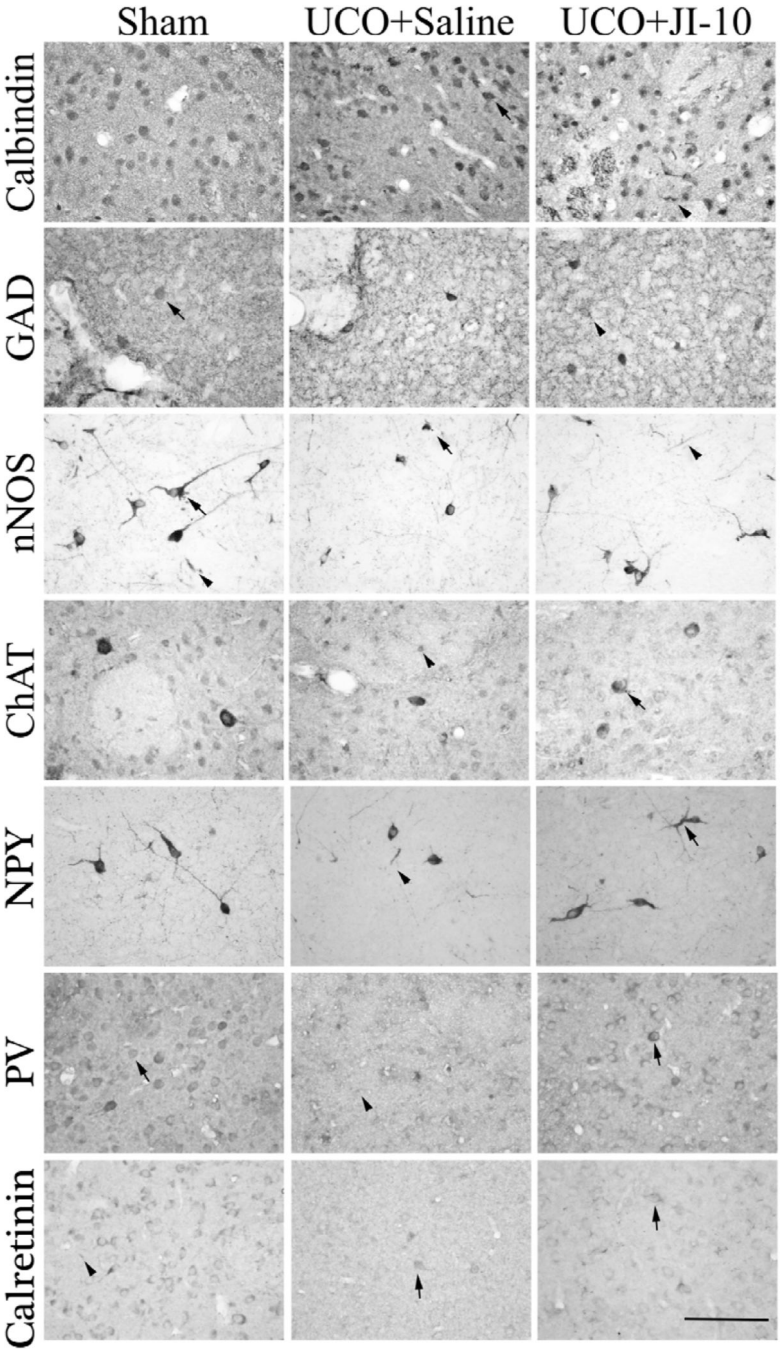

Figure 4.

Representative examples of neurophysiological correlations with histopathology. Panel A: 6 h total EEG power with calbindin in the caudate nucleus; Panel B: CaBF over day 7 of recovery with amoeboid Iba-1 immunostaining in the periventricular white-matter (PVWM); Panel C: total EEG power on day 7 of recovery with calbindin in the caudate nucleus; Panel D: total EEG power on day 7 of recovery with NPY in the caudate nucleus; Panel E: EEG continuity on day 7 of recovery with calbindin in the caudate nucleus; Panel F: EEG continuity on day 7 of recovery with NPY in the caudate nucleus; Panel G: seizure burden,

i.e. total duration of seizures with calbindin in the caudate nucleus; Panel H: seizure amplitude with amoeboid (activated) microglia in the PVWM. Data were analyzed with linear (Panels E, F and G) or non-linear regression (Panels A–D, and H) as appropriate and both occlusion groups were combined in the model. Linear regressions lines are shown with their respective 95% confidence interval. Where significance was found it is shown with the R^2 value on the graph. Open circles: saline+occlusion; closed circles: JI-10 plus occlusion.