Inhibition of Signal Transducer and Activator of Transcription 3 (STAT3) reduces neonatal hypoxic-ischaemic brain damage

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Abstract

Hypoxic-ischaemic encephalopathy is a leading cause of child death, with high mortality and morbidity, including cerebral palsy, epilepsy and cognitive disabilities. Hypoxia-ischaemia (HI) strongly up-regulates Signal Transducer and Activator of Transcription 3 (STAT3) in the immature brain. Our aim was to establish whether STAT3 up-regulation is associated with neonatal HI-brain damage and evaluate the phosphorylated STAT3-contribution from different cell types in eliciting damage. We subjected postnatal day seven mice to unilateral carotid artery ligation followed by 60 min hypoxia. Neuronal STAT3-deletion reduced cell death, tissue loss, microglial and astroglial activation in all brain regions. Astroglia-specific STAT3-deletion also reduced cell death, tissue loss and microglial activation, although not as strongly as the deletion in neurons. Systemic pre-insult STAT3-blockade at tyrosine 705 (Y705) with JAK2-inhibitor WP1066 reduced microglial and astroglial activation to a more moderate degree, but in a pattern similar to the one produced by the cell-specific deletions. Our results suggest that STAT3 is a crucial factor in neonatal HI-brain damage and its removal in neurons or astrocytes, and, to some extent, inhibition of its phosphorylation via JAK2-blockade reduces inflammation and tissue loss. Overall, the protective effects of STAT3 inactivation make it a possible target for a therapeutic strategy in neonatal HI.

Keywords: astroglia, microglia, neonatal hypoxia-ischaemia, neuroprotection, STAT3.

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middle cerebral artery occlusion (Lei et al. 2011). The neuroprotective effects of estradiol (Dziennis et al. 2007) and IL6 (Jung et al. 2011) depend on inhibition of pSTAT3. Therapeutic hypothermia also reduces pSTAT3 levels (Choi et al. 2011).

In addition to neurons, in adult animal models of ischaemia, activated STAT3 is also present in blood vessel endothelia, astrocytes and microglia (Planas et al. 1996; Justicia et al. 2000; Choi et al. 2011), potentially associating it with neuroinflammation and the associated tissue repair (Yi et al. 2007). Although some studies have looked into the activation and up-regulation of STAT3 following neonatal HI relating it to the balance between pro- and anti-inflammatory cytokines (Shrivastava et al. 2013), the overall function and precise role of this transcription factor in different cell types of the neonatal injured brain remains unclear. Using global inhibition of STAT3 phosphorylation and brain cell type-specific deletion of the STAT3 gene, the study reveals a critical role of neuronal and astroglial STAT3 in promoting neuronal damage and glial activation in response to neonatal HI-insult.

Materials and methods

Animal strains and breeding strategy

The animals used in the WP1066 inhibitor experiments were from the C57/B16 strain obtained from Charles River (Kent, UK). Mice carrying floxed STAT3 allele (STAT3<sup>F/F</sup>) on SVJ129 background (Takeda et al. 1998) were kindly provided by Prof. Shizuo Akira from the Institute for Molecular and Cellular Biology, Osaka University, and were initially maintained as heterozygotes (STAT3<sup>F/wt</sup>) by crossing them with C57/B16 obtained from Charles River (Kent, UK). Animals expressing cre recombinase driven by synapsin promoter (syn::Cre, C57/B16 strain) were provided by Dr Axel Behrens from the Mammalian Genetics Laboratory, Cancer Research UK, and animals expressing cre recombinase under the control of glial fibrillary acidic protein promoter (GFAP::Cre, FVB strain) were from Jackson Labs (Kent, UK). Animals expressing cre recombinase under the control of glial fibrillary acidic protein promoter (GFAP::Cre, FVB strain) were provided by Dr Axel Behrens from the Mammalian Genetics Laboratory, Cancer Research UK, and animals expressing cre recombinase under the control of glial fibrillary acidic protein promoter (GFAP::Cre, FVB strain) were from Jackson Labs (Kent, UK).

For western blot analysis, the animals were sacrificed at 1 h following HI. Western blot analysis was performed as previously described (Nateri et al. 2005). For immunoblotting, we used antibody against pSTAT3 (Y705) (New England Biolabs, Hitchin, UK).

DNA isolation and genotyping

DNA extraction was performed with the ‘Wizard’ Genomic DNA purification system according to manufacturer’s instructions (Promega, Southampton, UK), using tail tips taken before the perfusion. Specific oligonucleotide primers (Invitrogen, Paisley, UK) were used for genotyping.

STAT3 <sup>flox</sup> forward primer: 5’-CCTGAAGACCAAGTTCATCCTCTGAG-3’

STAT3 <sup>flox</sup> reverse primer: 5’-CACACAAAGCCCATCAAACCTGCTGGTCTCC-3’

GFAP-cre forward primer: 5’-ACTCTCTTCTAAAAGCCCT-3’

GFAP-cre reverse primer: 5’-ATACCTCTGTGTGATCGACCGG-3’

Synapsin-cre forward: 5’-AGCTTCAGCCACGGAGCATG-3’

Synapsin-cre reverse: 5’-TCGGTGCATCGACGGTAATG-3’

HI Insult

All animal experiments and care protocols were carried out according to the UK Animals (Scientific Procedures) Act 1986 and approved by the Home Office. The ARRIVE guidelines were followed. All experiments involved postnatal day 7 mice (P7) bred in house.

The surgical procedures were performed as previously described (Kendall et al. 2006). Briefly, male and female postnatal day 7 (P7) mice were anaesthetised with isoflurane (5% induction, 1.5% maintenance). The left common carotid artery was permanently occluded with 8/0 polypropylene suture and the wound closed with tissue glue. The mice were left to recover at 36°C and returned to the dam for 2 h. The pups were then placed in a hypoxia chamber and exposed to humidified 8% oxygen/92% nitrogen (3 L/min) at 36°C for 60 min.

In the rodent brain at P7, some cell types are at a developmental stage similar to a mid-third-trimester human fetus or new-born infant, with complete cortical neuronal layering, inviolated germinal matrix, and slightly myelinated white matter (Vannucci and Vannucci 1997). The rodent HI model at P7, though slightly preterm, presents phenotypical similarities to the grey and white matter injury observed in humans, i.e. tissue loss, cell-death-mediated apoptosis, microglia-mediated immune response and astroglisis as well as alteration in neurobehavioural performance (Vannucci and Vannucci 1997).

Pharmacological treatment

The JAK2 inhibitor WP1066 (Calbiochem, Watford, UK) was dissolved in 100% dimethyl sulfoxide (DMSO) and administered intraperitoneally at two doses of 40 mg/kg body weight (BW) (Iwamaru et al. 2007; Horiguchi et al. 2010) 20 min prior and then again directly after HI, as the mean half-life of WP1066 is 4.5 h (Madden et al. 2006). The WP1066-treated animals received two injections of 0.25 μL/g BW. The control groups received a corresponding amount of DMSO. The animals were left to survive for 48 h and the brains were collected for histological analysis.

Western blot analysis

For western blot analysis, the animals were sacrificed at 1 h following HI. Western blot analysis was performed as previously described (Nateri et al. 2005). For immunoblotting, we used antibody against pSTAT3 (Y705) (New England Biolabs, Hitchin, UK).
Tissue sample preparation
For histological assessment, the animals were sacrificed at 48 h post-HI by intraperitoneal injection of pentobarbital and perfused with 30 mL 4% paraformaldehyde in phosphate buffered saline (PBS). The brains were then removed, post-fixed in 4% paraformaldehyde in PBS for 1 h at 4°C, and cryoprotected for 24 h in phosphate-buffered 30% sucrose solution as described before (Möller et al. 1996). The brains were then frozen on dry ice, cut on a cryostat into sequential 40-μm sections and stored at −80°C until required.

Immunohistochemistry and histological analysis
Five cryosections from each brain (400 μm apart), obtained at 48 h post-HI, were rehydrated in distilled water and stained using immunohistochemistry as previously described (Möller et al. 1996). Briefly, the sections were incubated overnight with primary antibodies summarized in Table 1, for 2 h with biotinylated goat anti-rabbit, -rat or -hamster (1 : 100, Vector, Peterborough, UK) secondary antibody, followed by incubation with Avidin-Biotinylated horseradish peroxidase Complex (Vector) and visualization with diaminobenzidine/H2O2 (Fisher Scientific, Loughborough, UK). αX integrin immunoreactivity was enhanced with Co/Ni. Overall, GFAP immunoreactivity was detected with the rabbit polyclonal anti-GFAP antibody. However, for the double immunofluorescence with pSTAT3 in Fig. 1j and n we used rat monoclonal anti-GFAP antibody. However, for the double immunofluorescence with pSTAT3 in Fig. 1j and n we used rat monoclonal anti-GFAP antibody, to avoid cross-staining with the rabbit polyclonal antibody against pSTAT3 (see Table 1).

Five further cryosections from each brain with the same spacing were stained using Terminal transferase mediated d-UTP nick end labelling (TUNEL) (Roche, Burgess Hill, UK). The staining procedure followed the manufacturer protocol with Co/Ni enhancement. Overall, GFAP immunoreactivity was detected with the rabbit polyclonal anti-GFAP antibody. However, for the double immunofluorescence with pSTAT3 in Fig. 1j and n we used rat monoclonal anti-GFAP antibody, to avoid cross-staining with the rabbit polyclonal antibody against pSTAT3 (see Table 1).

Five more sections per brain with the same spacing were stained with cresyl violet (Nissl).

For the double immunofluorescence with pSTAT3 in Fig. 1h, i, l and m, we used Neurofilament-H (h, i, l and m) as a major component of the neuronal cytoskeleton and thus a neuronal marker, GFAP (j and n) and αM (k and o), and followed the above-described protocol replacing the biotinylated secondary antibodies with fluorescently labelled ones.

Infarct volume measurement
The cresyl violet-stained sections were scanned and imported into Optimas 6.5 image analysis software (Bothell, WA, USA). The areas of intact staining in cortex, pyriform cortex, hippocampus, striatum, thalamus and external capsule were outlined and measured bilaterally. The percentage tissue loss was then calculated by converting the measured injured and uninjured areas into square millimetres and then converting the volume by multiplying by 400 μm. The sum of these volumes was then used to calculate the percentage of surviving brain tissue as injured/uninjured volume × 100 (Kendall et al. 2006).

pSTAT3 immunoreactivity
To analyse the pSTAT3 immunoreactivity following HI, we counted the number of positive cells bilaterally in three different optical fields at ×40 magnification in cortex and hippocampus at 0, 15 min, 1, 2, 4, 8, 16, 24, 48, 72, and 96 h following HI (n = 4 animals per group). The counts were then averaged per animal and per group.

AlphaM score
Immunohistochemistry for αM integrin as a marker of early microglial activation (Kloss et al. 1999; Hristova et al. 2010; Kendall et al. 2011a; Lange et al. 2014), was performed as previously described (Ohno et al. 1995; Kendall et al. 2006). Semi-quantitative scores were allocated to each brain region (cortex, pyriform cortex, hippocampus, striatum, thalamus and external capsule) by an observer blinded to the treatment of the groups (Table 2).

AlphaX and TUNEL
Late microglial activation and phagocytosis was assessed through immunohistochemistry for αX integrin (Kloss et al. 1999; Hristova et al. 2010). To analyse the αX integrin and TUNEL immunoreactivity at 48 h following HI, the number of positive cells for each of the stains was counted bilaterally in three different optical fields at ×20 magnification in cortex, pyriform cortex, hippocampus, striatum, thalamus and external capsule. The counts were then averaged per animal and per group.

Table 1: List of antibodies used for immunohistochemistry

| Antibody against  | Concentration | Host species     | Secondary antibody     | Obtained from                        |
|-------------------|---------------|------------------|------------------------|--------------------------------------|
| αM integrin subunit | 1 : 5000      | Rat              | Goat anti-rat          | Serotec, Kidlington, UK              |
|                   |               |                  |                        | Cat Nr MCA711                         |
| αX integrin subunit | 1 : 400       | Hamster          | Goat anti-hamster      | Pierce, Paisley, UK                  |
|                   |               |                  |                        | Cat Nr MA111C5                        |
| GFAP              | 1 : 6000      | Rabbit polyclonal| Goat anti-rabbit       | Dako, Ely, UK                        |
|                   |               |                  |                        | Cat Nr 20334                          |
| GFAP              | 1 : 6000      | Rat monoclonal   | Goat anti-rabbit       | Zymed, Loughborough, UK              |
|                   |               |                  |                        | Cat Nr 13-0300                        |
| pSTAT3 (Y705)     | 1 : 400       | Rabbit           | Goat anti-rabbit       | New England Biolabs, Hitchin          |
|                   |               |                  |                        | Cat Nr 9131                          |
| Neurofilament-H   | 1 : 10000     | Chicken          | Goat anti-chicken      | Abcam, Cambridge, UK                 |
|                   |               |                  |                        | Cat Nr ab4680                         |
Optical luminosity

GFAP is expressed not just by the bodies, but is also registered in the processes of astroglial cells. In order to quantify the total intensity of the GFAP staining in the tissue, we used optical luminosity values as a well-established technique (Möller et al. 1996). Images for both ipsilateral and contralateral sides were captured with a Sony AVT-Horn 3CCD colour video camera (24bit RGB, 760 x 570 pixel resolution) in three different optical fields in cortex, pyriform cortex, hippocampus, striatum, thalamus and external capsule, as well as the surrounding glass at ×20 magnification. We used Optimas 6.5
Fig. 1 pSTAT3 increase in cerebral cortex and hippocampus following HI-insult in P7 mice. (a and b) Western Blots for pSTAT3 (Y705) in cerebral cortex (a) and hippocampus (b) from naïve C57Bl/6 animals, animals with unilateral carotid occlusion/ischemia (Occ) or hypoxia (Hyp) – 60 min exposure to 8% oxygen and 1 h recovery; and animals with unilateral occlusion followed by 60 min hypoxia (Hi) and 1 h recovery. Tubulin protein levels served as controls. L and R – left and right hemispheres in naïve and hypoxic animals, (c) and (i) – contralateral and ipsilateral in animals with unilateral occlusion only, and in those with Hi-insult. The strong and bilateral pSTAT3 increase in cortex and hippocampus is only present following HI-insult (a and b). No STAT3 activation is observed in cortex or hippocampus of naïve or animals subjected to occlusion (Occ) or hypoxia alone (Hyp). (c, d and f) HI-insult strongly increased ipsilateral (c) and contralateral (d) pSTAT3 immunoreactivity in cortex and hippocampus (rostral part of parietal isocortex) at 1 h post-HI. Occlusion alone (Occ) produces no change in pSTAT3 immunoreactivity (f). (c.1 and d.1) Magnified images of the dotted boxes in cerebral cortex (c) and the CA3 region of hippocampus (d), respectively. (e and g) Number of pSTAT3 (Y705)+ cells in ipsilateral and contralateral cerebral cortex (e) and hippocampus (g) 0–96 h post-HI (Mean ± SEM, n = 4 animals per group). Note the bilateral peak at 1 and 4 h in cortex (e) and at 1–2 h in hippocampus (g) in the animals with unilateral occlusion followed by 60 min HI compared to unilateral occlusion alone (Occ) or naïve animals (t-test, ipsilateral cortex: p = 0.0063 for 1 h, p = 0.046 for 4 h; contralateral cortex: p = 0.0015 for 1 h). (h–o) Immunofluorescence for chick polyclonal anti-neurofilament-H (NFH, h, i, l, m), rat monoclonal anti-γ-3 fibrillary acidic protein (GFAP, j and n) and rat monoclonal anti-αM integrin subunit (k and o) in cerebral cortex at 1 h following neonatal HI, in red, superimposed on the rabbit polyclonal anti-pSTAT3 (Y705) fluorescence in green and nuclear DAPI fluorescence in blue. Note the co-localization of pSTAT3 and NFH (i and m, full arrows) and the lack of such co-localization with GFAP+ astroglia (n, empty arrows) and αM+ microglial cells (o, empty arrows), suggesting neuronal expression of pSTAT3 at this time point post-HI. Scale bars: c, d, f = 400 μm; c.1, d.1, h–o = 50 μm.

Table 2 Semi-quantitative scores for αM integrin

| Score | Microglial appearance |
|-------|----------------------|
| 0     | No activation        |
| 1     | Focal microglial activation |
| 2     | Mild phagocytic activation affecting < 50% of the region |
| 3     | Phagocytic activation affecting > 50% of the region |
| 4     | Total phagocytic activation |

software to obtain the mean and standard deviation (SD) for optical luminosity values (OLV). SD was subtracted from the mean for each image and the resulting value was subtracted from the values obtained for the surrounding glass. (Möller et al. 1996).

Statistics
Statistical significance was assessed through repeated testing using Mixed Linear Model with SPSS 13.0 software (IBM Corporation, Armonk, New York, USA), treating region as the repeated measure. For each outcome, several regions of the brain were examined. With repeated measures such as these, it is likely that the observations from a single subject will be correlated, therefore the first stage of the analysis included the observations from all the regions tested in a single mixed model with a random subject effect, to produce an estimate of the treatment effect and associated inference that accounts for the correlations in the data arising from the repeated measures. Further post hoc Student t-tests were carried out to assess evidence for subregional differences, p < 0.05. For each outcome, the overall effect from the linear mixed model is reported, followed by the results from the individual regional t-tests. All data are presented as Mean ± SEM.

Results
STAT3 phosphorylation following HI–insult in the neonatal brain
The protein levels of pSTAT3 (Y705) were assessed through western blot analysis. Compared to littermate controls (naïve, after unilateral carotid occlusion or after hypoxia alone), postnatal day 7 (P7) mouse pups (C57/Bl6) demonstrated bilateral pSTAT3 (Y705) up-regulation in cortex and hippocampus, at 1 h following a 60 min HI-insult (Fig. 1a and b). In both regions and on both sides, the total protein STAT3 levels in animals exposed to combined HI-injury remained unchanged when compared to naïve littermate controls (data not shown).

Similar HI-mediated pSTAT3 (Y705) increase was also observed through immunohistochemistry. Figures 1c and d reveal a bilateral increase in pSTAT3 (Y705) immunoreactivity observed on the occluded (Fig. 1c and c.1) and non-occluded (Fig. 1d and d.1) side, in the cortex (Figs 1c and c.1) and hippocampus (Figs 1c,d and d.1) at 1 h following 60 min HI-insult. Carotid artery ligation alone did not result in detectable pSTAT3 immunoreactivity (Fig. 1f).

In terms of pSTAT3 + cell density, this bilateral up-regulation was detected in both cortex and hippocampus as early as 1 h (Fig. 1e and g) and was still detectable at lower levels up to 24 h following HI-insult (n = 4 animals per time point). Cortical pSTAT3 + counts (Fig. 1e) revealed a biphasic, bilateral increase with peaks at 1 and 4 h, and almost no pSTAT3 + cells at 2 h post-HI. The hippocampus (Fig. 1g) demonstrated a single-bilateral peak at 1 h with lower variable levels in both hemispheres at later time points. To determine the identity of the pSTAT3 + cells at 1 h following neonatal HI insult, pSTAT3 immunoreactivity was combined with fluorescent immunostaining for neurofilament-H (NFH), GFAP or αM integrin subunit, and counter-stained for nuclear 4’,6-diamidino-2-phenylindole (DAPI) fluorescence in dorsoparietal cortex. As shown in Figures 1h and i, and l and m there was clear co-localization with neuronal NFH (red), but not with astroglial GFAP (Fig. 1j and n) or with microglial αM (Fig. 1k and o). We did not observe microglial and astroglial co-localization at 1h post-HI, but such co-localization has been demonstrated at later
time-points, i.e., 3–72 h, by other groups (Shrivastava et al. 2013; D’Angelo et al. 2015).

Neuronal deletion of pSTAT3 reduces brain damage following HI-insult in the neonate

To explore the functional role of HI-induced STAT3 activation, animals expressing one gene copy of cre recombinase under the control of the synapsin promoter were crossed twice with mice carrying 2 copies of floxed STAT3 gene (STAT3FP/FP), to obtain animals with homozygous CNS neuron-specific deletion of STAT3 (STAT3ΔS) and STAT3FP/FP littermate controls, on a mixed C57/Bi6xFVJ129 background.

As shown in Fig. 2a and b neuronal deletion of both copies of the floxed STAT3 gene (STAT3ΔS) strongly reduced western blot levels of pSTAT3 (Y705) in hippocampus and cortex compared to STAT3FP/FP littermate controls at 1 h following a 60 min HI-insult (Fig. 2a and b). However, unlike the C57/Bi6 wild-type mice (Fig. 1a and b), the relative pSTAT3 levels on the non-occluded, contralateral side of the forebrain were lower, both in STAT3ΔS and in STAT3FP/FP littermates.

To determine the biological impact of neuron-specific STAT3 deletion, we next examined the effects on TUNEL+ cell death and brain tissue volume, on reactive astrogliosis and on different markers or microglial activation at 48 h following a 60 min HI-insult. Figures 2e, g and h show that Syn::cre-mediated deletion of STAT3 (STAT3ΔS) markedly reduced the number of TUNEL+ cells compared to STAT3FP/FP littermate controls (Fig. 2e, g and h). The TUNEL+ cells displayed the typical pyknotic nuclear morphology (Fig. 2g-l) in optical sections (OLV, Mean ± SEM) on the ipsilateral (i) and contralateral (c) side of the forebrain were lower, both in STAT3ΔS and in STAT3FP/FP littermates.

The inserts in (k) and (l) show higher magnifications of the dotted regions in rostro-parietal isocortex. Note the intense staining and the round, phagocytic microglia activation in the STAT3FP/FP (o-insert, hippocampus), compared to the STAT3ΔS brains exhibiting a ramified phenotype (s-insert). Neuron-specific deletion of STAT3 reduced mTDL microglial activation across all 6 examined forebrain regions, with significant, individual decrease (t-test) in isocortex (p = 0.002) and thalamus (p = 0.001) in (i), and in contralateral hippocampus (p = 0.018) in (j). Mixed Linear Model, treating region as a repeated measure revealed p = 0.001 for the ipsilateral, and p = 0.049 for the contralateral side. Numbers of mTDL cells per 20× eye-field (Mean ± SEM) on the ipsilateral (m) and contralateral, non-occluded side (n), and their ipsilateral distribution in STAT3FP/FP (o) and STAT3ΔS (p) animals. Note the intense staining and the round, phagocytic morphology of the mTDL cells in the STAT3ΔS animals (o-insert, hippocampus). Neuron-specific STAT3 deletion significantly reduced the number of mTDL cells with individual decrease (t-test) in isocortex (p = 0.006), pyriform cortex (p = 0.002), hippocampus (p = 0.010), striatum (p = 5 × 10⁻⁶), and thalamus (p = 0.0009) in (m); and in contralateral pyriform cortex (p = 0.010) in (n). Mixed Linear Model, treating region as a repeated measure revealed p = 0.0001 for the ipsilateral and p = 0.013 for the contralateral side. (q-s) Activation of mTDL microglia – ipsilateral mTDL microglial activation score (q, Mean ± SEM) and low magnification ipsilateral overview in STAT3ΔS (r) and STAT3FP/FP (s) animals. Note the strong microglial activation in STAT3ΔS animals with mTDL cells showing phagocytic morphology at high magnification (r-insert, hippocampus), compared to the STAT3ΔS brains exhibiting a ramified phenotype (s-insert).
animals compared to their STAT3F/F littermates (p < 0.05 in t-test).

A similar effect of the Syn::cre-mediated deletion of STAT3 was also observed for forebrain tissue loss, i.e. the reduction of the volume on the ipsilateral versus the non-occluded contralateral side. Figure 2c shows large infarct in mid-lateral isocortex (09:00-11:00 segment) and hippocampus of the STAT3F/F animal (Fig. 2c) and its sparing in the
The effect on TUNEL+ cells is shown in Fig. 3a (Mixed Linear Model, treating region as a repeated measure, \( p = 0.02 \)), with individual significant decrease of 30–87% in hippocampus, thalamus and external capsule (\( p < 0.05 \) in \( t \)-test). However, despite the apparently consistent lower average tissue loss values across the examined brain regions (Fig. 3a, b and c) in \( STAT3^{AG} \) animals, astroglial deletion of \( STAT3 \) did not have a significant effect on ipsilateral brain tissue loss (Mixed Linear Model, treating region as a repeated measure, \( p = 0.217 \)). Likewise, microglial activation based on the \( \alpha \)M integrin immunoreactivity (Fig. 3k) had a trend towards reduced levels in all the examined regions but overall was not significantly affected (\( p = 0.229 \)).

In contrast, astroglial pSTAT3 deletion strongly reduced HI-induced up-regulation of GFAP-immunoreactivity in both ipsi- and contralateral brain regions (Fig. 3g,h, and i and Fig. 3j, respectively). The extent of this reduction was beyond that observed with \( STAT3^{AS} \) (Fig. 2k and l). Analysis with Mixed Linear Model, treating region as a repeated measure revealed \( p = 0.0001 \) on the ipsilateral and 0.0042 on the contralateral side, with individual significant decrease of 50–70% in all of the six regions on the occluded and 16–65% in four of the regions on the contralateral, non-occluded side (\( p < 0.05 \) in \( t \)-test).

**Inhibition of \( STAT3 \) Y705-phosphorylation reduces HI-mediated glial activation**

Due to the early up-regulation of pSTAT3 post-HI (Fig. 1), the JAK2 inhibitor WP1066 was injected intraperitoneally just before and immediately after 60 min hypoxia to prevent \( STAT3 \) activation during the latent period. Compared to vehicle (DMSO)-treated control animals, intraperitoneal application of WP1066 (80 \( \mu \)g/g BW) significantly reduced the protein levels of pSTAT3 (Y705) at 1 h post-HI in both cortex (Fig. 4a and c) and hippocampus (Fig. 4b and c) on the ipsilateral side.

Pre- and post-insult treatment with 80 \( \mu \)g/g WP1066 did not have significant effect on ipsilateral brain tissue loss at 48 h post-HI in Mixed Linear Model, treating region as a repeated measure (\( p = 0.257 \)) (Fig. 4d–f). There was also no significant effect of WP1066-treatment on the number of TUNEL+ cells (data not shown). However, reactive astrogliosis and microglial activation on the occluded side were affected. In the case of astroglial GFAP immunoreactivity (Fig. 4g–i), analysis with Mixed Linear Model, treating region as a repeated measure revealed \( p = 0.047 \), with individually significant decrease of 28% in external capsule (\( p < 0.05 \) in \( t \)-test). Microglial activation (Fig. 4j–l) based on \( \alpha \)M integrin immunoreactivity was significantly reduced in animals treated with WP1066 (Mixed Linear Model, treating region as a repeated measure, \( p = 0.041 \)), with individually significant decrease of 51% in ipsilateral hippocampus (\( p < 0.05 \) in \( t \)-test). The insert in Fig. 4d shows the typical...
Fig. 3 Astroglial deletion of STAT3 with Cre recombinase under the control of GFAP promoter (STAT3$^{ΔG}$) reduces cell death and reactive astrogliosis. All graph data are shown as Mean ± SEM.

(a) Ipsilateral brain tissue volume loss was not significantly affected despite the consistent lower average tissue loss values across the examined brain regions in the STAT3$^{ΔG}$ animals. (b and c) Ipsilateral forebrain Nissl staining (Cresyl-Violet, at rostral parietal level) of STAT3F/F (b) and STAT3$^{ΔG}$ (c). (d) Reduction in the number of TUNEL$^+$ dying cells (per 20$^x$ eye-field) at 48 h following HI, with significant, individual decrease (t-test) across all 6 ipsilateral forebrain regions – isocortex ($p = 0.0006$), pyriform cortex ($p = 0.003$), hippocampus ($p = 0.0002$), striatum ($p = 0.003$), thalamus ($p = 0.0003$) and external capsule ($p = 1 \times 10^{-5}$). Mixed Linear Model, treating region as a repeated measure revealed $p = 0.001$. (e and f) Low magnification ipsilateral overview in STAT3$^{ΔG}$ (h) and STAT3$^{ΔG}$ (i) animals. The insert in H shows higher magnification of the dotted region in rostro-parietal isocortex. Note the reduced levels of GFAP immunoreactivity in the STAT3$^{ΔG}$ animals. (j) Strong reduction in contralateral reactive astrogliosis with significant, individual decrease (t-test) in contralateral isocortex ($p = 0.010$), hippocampus ($p = 0.003$), thalamus ($p = 0.040$) and external capsule ($p = 6 \times 10^{-5}$). Mixed Linear Model, treating region as a repeated measure revealed $p = 0.004$. (k) Ipsilateral microglial activation score based on the αM integrin immunoreactivity was decreased in all examined regions but overall was not significantly affected. STAT3$^{ΔG}$ ($n = 15$) and STAT3$^{ΔG}$ ($n = 12$) in all assessments. $^*p<0.05$. Scale bars: e, f, h and i = 600 μm; inserts=30 μm; b and c = 1200 μm.

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phenotype of strongly αM immunoreactive, phagocytic microglia in the vehicle (DMSO) control group, compared to ramified microglia with reduced αM immunoreactivity in the WP1066-treated animal (Fig. 4e-insert).

Discussion

As shown in the current study, in a Rice-Vannucci model of severe HI insult in postnatal (P7) mice, neuronal deletion of...
STAT3 inhibition in neonatal hypoxia ischaemia

Fig. 4 Jak2-inhibition with WP1066 reduces STAT3-phosphorylation (Y705), reactive astrogliosis and microglial activation following neonatal HI. (a–c) Western Blots (a and b) of pSTAT3 (Y705) immunoreactivity in cerebral cortex (a) and hippocampus (b) and their densitometric quantification (c) in arbitrary units (AU) against a tubulin control (Mean ± SEM) at 1 h after 60 min HI-insult. Intraperitoneal WP1066 injection at a combined dose of 80 μg/kg BW (20 min prior and directly after 60 min HI-insult), reduces pSTAT3 (Y705) levels in both cortex and hippocampus in the ipsi- (i) and contralateral (c) hemisphere (n = 6), when compared to the vehicle (DMSO) injected littermate controls (n = 5). *Significant effect for WP1066 versus DMSO (unpaired t-test) for ipsilateral cortex (p < 0.005) and ipsilateral hippocampus (p < 0.005). (d, e, g, h, j and k) Nissl staining of whole forebrain (d and e) and ipsilateral immunoreactivity for astrogl GFAP (g and h) and αM+ microglia at rostro-parietal level (j and k) 48 h after HI-insult in animals injected with vehicle (DMSO) (d, g, j) or WP1066 (e,h and k). The inserts in (g and h) show WP1066-induced reduction in GFAP immunoreactivity in external capsule and in (j and k) the disappearance of αM+ microglial phagocytes in the hippocampal CA3 subregion. (f, l, and k) Quantitative assessment of WP1066 effect vs DMSO (n = 9 per group) on ipsilateral brain tissue volume loss (f), GFAP immunoreactivity in OLV (i) and αM microglial activation score (l). All graph data are shown as Mean ± SEM. In the Mixed Linear Model, treating region as a repeated measure did not reveal significant effect for volume loss (f), but demonstrated reduced astrogliosis (i, p < 0.019) and microglial activation score (l, p < 0.049) with significant, individual decrease (t-test) in external capsule (GFAP-immunoreactivity, i, p = 0.040) and in hippocampus (microglia, l, p = 0.048). Scale bars: d and e = 1200 μm, g, h, j and k = 300 μm; inserts = 30 μm.

STAT3 (STAT345) clearly reduced forebrain cell death and tissue loss, as well as microglial and astrogliosis activation. Astrogliosis-specific STAT3 deletion (STAT34G) blocked reactive astrogliosis and attenuated cell death, but had a more moderate effect on tissue loss and active microgliosis. A combined pre-insult and immediate post-insult blockade of JAK2 with systemically applied WP1066 inhibited the HI-induced forebrain STAT3(705)-phosphorylation and reduced HI-brain damage based on some evidence for astrogial and microglial post-HI response. However, this effect was weak and did notlead to a statistically significant reduction in brain tissue loss. Overall, our data suggest a critical role for STAT3, and possibly also a contribution in neonatal HI-brain damage via Tyr705 phosphorylation.

There are significant differences in the response of different mouse strains to the Rice-Vannucci model of neonatal HI (Sheldon et al. 1998; Rocha-Ferreira et al. 2015), with C57Bl6 demonstrating an increasing degree of injury with increasing duration of hypoxia, and SVJ129 demonstrating relative resistance to injury. As both the cell-specific deletion strains used in our experiments were on mixed C57Bl6 background with either FVB (astroglial) or SVJ129 (neuronal), and because of the reported susceptibility of the C57Bl6 strain to the neonatal HI model, we chose to use the latter for the STAT3 inhibitor experiments.

The increase of pSTAT3 (Y705) in the current study, was specific for HI, peaking at 1–4 h following insult (Fig. 1e and g). We did not observe STAT3 activation in the brains of naive, animals subjected to hypoxia alone or of those with unilateral carotid occlusion alone (Fig. 1a and b), suggesting a HI-dependent pattern of STAT3 activation (Shrivastava et al. 2013). Interestingly, the early pSTAT3 presence at 1 h following HI was specific for neurons but not for the GFAP+ astrocytes (Fig. 1j and n) or the αM+ microglia (Fig. 1k and o), preceding the previously observed up-regulation from 3 to 72 h post-HI in micro- and astroglial populations (Shrivastava et al. 2013), and suggesting an additional, time-dependent and cell-specific pattern of STAT3 activation. The pSTAT3-localization around neuronal cell body-near processes (Fig. 1h and i and l and m) could be explained with a previously described role for this retrogradely transported axonal molecule in injury-induced axonogenesis of hippocampal neurons (Ohara et al. 2011).

Total STAT3 protein levels remained unaffected and similar to the expression observed in naive animals (data not shown), implying that the functional effect of STAT3 is probably elicited by some post-translational modification and/or translocation, cytoplasmic translation and nuclear transcription (Nicolas et al. 2012; Haghikia et al. 2014). Most of the immediate/early HI damage in the Rice-Vannucci model occurs on the occluded side (obvious from Nissl histology in Fig. 2c and d or phagocytic, αX+ microglia in Fig. 2m–p). However, pSTAT3-activation is bilateral (Figs 1a and b and 2a and b) with contralateral intensity possibly depending on the strain background. This bilateral activation suggests that STAT3 is not a directly pathogenic effector molecule but rather exerts a permissive, but nevertheless substantial, predisposing role in mediating HI-injury. Although the contralateral side in the Rice-Vannucci model is often used as intra-animal control reference for ipsilateral damage (Vannucci et al. 1988; Towfighi et al. 1994; Skoff et al. 2007; Shrivastava et al. 2012), some studies report bilateral change in the expression of some cytokines, for example Hypoxia Inducing Factor alpha and P-Akt (Shrivastava et al. 2012), suggesting that hypoxia can regulate some mediators contributing, but not sufficient to cause long-term damage. The same study reports some degree of delayed contralateral atrophy in hippocampus and corpus callosum (Shrivastava et al. 2012). Severe HI-insult in the Rice-Vannucci model following unilateral carotid occlusion and prolonged (60 + min) hypoxia is associated not only with substantial acidosis in the territory of the ipsilateral medial cerebral artery but also with very pronounced contralateral pH drop (Kendall et al. 2011b), which could contribute to the rapid STAT3 activation on the
non-occluded side observed in the current study. Moreover, the elevated, contralateral levels of pSTAT3, and the fact that neuronal as well as astrogial STAT3 deletion inhibit contralateral astrogliosis (Figs 2j and 3d) and microgliosis (Fig. 2n) also points to a more long-term involvement in pathways underlying delayed contralateral atrophy (Shrivastava et al. 2012).

We used 3 strategies to interfere with brain STAT3 function, i.e. removal of STAT3 gene expression in neurons (STAT3fl), in GFAP+ astroglia (STAT3fl) and blocking JAK2-mediated Y705-phosphorylation with WP1066. The pharmacological inhibition produced a relatively moderate effect. Compared to vehicle (DMSO)-treated controls, the combined pre-HI and immediate post-HI intraperitoneal injection of WP1066 significantly reduced the levels of pSTAT3 in cortex and hippocampus (Fig. 4a–c), demonstrating that WP1066 does penetrate the blood brain barrier at a physiologically active dose (Hussain et al. 2007). It is possible that this reduction is due to an incomplete, approximately 70% decrease in Y705-phosphorylation thus permitting some residual activity, compared to complete removal in neurons or astrocytes expressing cre recombinase. Moreover, there are several different ways involved in STAT3 activation – phosphorylation at Y705 and S727, dimerization, nuclear translocation, etc. (Haghikia et al. 2014) – and interference with just one could produce a moderate result. Interestingly, cell-specific removal of the floxed STAT3 gene, as well as pharmacological inhibition result in partial overlap of the histopathology phenotypes, but there are pronounced differences in the magnitude of their impact suggesting either direct or secondary effects.

Removal of STAT3 in astrocytes resulted in a consistent, general decrease in reactive astrogliosis across different regions of the ipsilateral and contralateral hemisphere, underscoring the essential role of this transcription factor in astroglial activation and induction of GFAP (Fukuda et al. 2007; Herrmann et al. 2008; Hong and Song 2014). Previous studies have suggested that reactive astrogliosis is neuroprotective and enhances repair. Astrogial STAT3 deletion interferes with synaptic plasticity on regenerating facial motor neurons via astrocyte TSP1 (Tyzack et al. 2014), delays oligodendrogial maturation in endotoxin-induced neonatal white matter damage (Nobuta et al. 2012), and increases inflammation and lesion volume, with attenuated motor recovery in spinal cord injury (Herrmann et al. 2008). However, in the current model, deletion of astroglial STAT3 resulting in suppression of astrogliosis was associated with clearly reduced TUNEL+ cell death in the ipsilateral hippocampus, thalamus and external capsule (Fig. 3d,e and f). Although the effects were less prominent than with neuron-specific deletion (Fig. 2e, g and h), and the decrease in tissue loss and microglial activation did not reach the statistical significance needed for repeated measures, in our study reduced astrogliosis in HI was associated with decrease in cell death and did not increase inflammation or tissue loss, thus contradicting the effects observed in other experimental brain pathologies (Herrmann et al. 2008; Nobuta et al. 2012).

In contrast to GFAP promoter-driven deletion, pSTAT3-deletion via the neuronal synapsin promoter produced a general decrease of cell death across all examined regions of the ipsilateral hemisphere, and significantly reduced tissue loss in cerebral isocortex and striatum, and overall ipsilateral forebrain volume loss. It also resulted in a significant reduction in microglial αM activation score and the number of αX+ phagocytes. Microglia do not express synapsin during embryonic or post-natal development (Kügler et al. 2001). The neuronal effects on reactive astrogliosis are fairly moderate (Fig. 2i and j), and the decrease in microgliosis in STAT3fl animals did not reach statistical significance (Fig. 3k). Therefore, the observed reduction in microglial activation in the STAT3fl animals is probably a consequence of reduced neuronal signalling and/or the strongly decreased cell death and tissue loss. Following neonatal HI-injury activated microglia, similarly to other pathologies, are a major marker of damage (Ohno et al. 1995) and source of inflammatory cytokines contributing to cell death, tissue loss and astrogial activation, as well as an important contributor to endogenous defence mechanisms through effective elimination of apoptotic neurons (Faustino et al. 2011). Activated microglial cells produce interleukin-6 (IL6) (Lambertsen et al. 2012), a strong astrogial activator in vitro (Benveniste et al. 1995) and in vivo (Campbell et al. 1993; Galiano et al. 2001). Moreover, elevated levels of microglial IL6 following neonatal HI-injury correlate with increase in pSTAT3+ astrocytes (Shrivastava et al. 2013). Interestingly, WP1066 strongly inhibits microglial IL6 production (Lambertsen et al. 2012), which in the current study could contribute to the WP1066-dependent reduction in reactive astrogliosis.

Overall, neuronal and astroglial STAT3 are clearly involved in the pathways underlying cell death, tissue loss and gliosis following neonatal HI but they do differ with respect to the target of their effect. Y705-phosphorylation does contribute to HI histopathology; however a better understanding of this and the other pathway(s) through which STAT3 activation is involved in HI-brain damage would considerably improve the therapeutic potential of interfering with STAT3 in a clinical setting.

Acknowledgments and conflict of interest disclosure

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All experiments were conducted in compliance with the ARRIVE guidelines.

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