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Different apoptotic mechanisms are activated in male and female brains after neonatal hypoxia–ischaemia

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Abstract

Sex-related brain injury was evaluated after unilateral hypoxia–ischaemia (HI) in C57/BL6 mice on postnatal day (P) 5, 9, 21 or 60, corresponding developmentally to premature, term, juvenile and adult human brains. There was no sex difference in brain injury when the insult was severe, as evaluated by pathological scoring or tissue loss, but when the insult was moderate, adult (P60) females displayed less injury. In the immature (P9) male brains, neurones displayed a more pronounced translocation of apoptosis-inducing factor (AIF) (loss of AIF from the mitochondrial fraction and increase in nuclear AIF) after HI, whereas the female brain neurones displayed a stronger activation of caspase 3 (more pronounced loss of pro-caspase 3, increase in cleaved caspase 3 and increase in caspase 3 enzymatic activity). Two other mechanisms of injury, peroxynitrite-induced formation of nitrotyrosine and autophagy, were no different between males and females at P9. These data show that the CNS is more resistant to HI in adult females compared with males, whereas no sex differences were found in the extent of injury in neonatal mice. However, critical sex-dependent differences were demonstrated *in vivo* with regard to cellular, apoptosis-related mechanisms.

Keywords: apoptosis-inducing factor, brain development, caspase, cell death, hypoxia–ischaemia, sex.
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Sex-related differences in brain injury and outcome following cerebral stroke or trauma have been considered attributable to the differences in brain structure and function induced by oestrogen (MacLusky and Naftolin 1981). In adult rodents, females sustain less injury than males after experimental ischaemia (Hurn and Macrae 2000). This resistance is acquired after puberty (Payan and Conrad 1977) and is lost after menopause, in accordance with the putative protective effect of sex steroids, especially oestrogen (Hurn and Macrae 2000). It has been proposed that oestrogen has tissue antioxidant properties (Hall *et al*. 1991) and increases cerebral perfusion (Hurn *et al*. 1995). Oestradiol has also been demonstrated to offer protective effects against kainic acid-induced neuronal damage in immature females (Hilton *et al*. 2003).

Sex differences have also been reported in neonatal models of hypoxic–ischaemic brain injury in rats (Bona *et al*. 1998) and mice (Hagberg *et al*. 2004). The differences between males and females in the neonatal model are unlikely to involve exposure to hormones, but sex-related differentiation of the brain occurs in critical phases during embryonic and postnatal life in ways that might affect

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Abbreviations used: AIF, apoptosis-inducing factor; Ac-DEVD-AMC, N-Acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin; C/Cont, control; CL, contralateral hemisphere; Cyt *c*, cytochrome *c*; DEVD, Asp-Glu-Val-Asp; HI, hypoxia–ischaemia; IL, ipsilateral hemisphere; iNOS, inducible nitric oxide synthase; LC3, light chain 3; MAP, microtubule-associated protein; nNOS, neuronal nitric oxide synthase; P, postnatal day; PAR, poly(ADP-ribose); PARP, PAR polymerase; PBS, phosphate-buffered saline.

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vulnerability to injury (Becu-Villalobos *et al*. 1997). There is also evidence of innate sex differences at the cellular level (Zhang *et al*. 2002; Du *et al*. 2004).

It remains unknown, however, at what time point during postnatal development the sex dependency of vulnerability is established. Furthermore, even though the extent of hypoxic–ischaemic damage does not differ between males and females during the neonatal period, we hypothesized that the *in vivo* mechanisms related to the apoptotic cascade are sex dependent. Poly(ADP-ribose) polymerase (PARP) was recently found to be critical for neonatal hypoxic–ischaemic brain injury in males but not in females (Hagberg *et al*. 2004), and the effects of PARP may be mediated by apoptosis-inducing factor (AIF) (Yu *et al*. 2002). The aims of this study were to compare brain injury between males and females at different postnatal ages, with emphasis on the term neonatal brain, and to evaluate to what extent caspase-dependent and -independent mechanisms are related to sex in neonatal mice.

Materials and methods

Induction of hypoxia–ischaemia (HI)

Unilateral HI was induced in C57/BL6 mice on postnatal day (P)5, P9, P21 and P60 essentially according to the Rice–Vannucci model (Rice *et al*. 1981; Hagberg *et al*. 2004). Mice were anaesthetized with halothane (3.0% for induction and 1.0–1.5% for maintenance) in a mixture of nitrous oxide and oxygen (1 : 1), and the duration of anaesthesia was < 5 min. The left common carotid artery was cut between double ligatures of polypropylene sutures (6/0). After surgery, the wounds were infiltrated with a local anaesthetic, and the pups were allowed to recover for 1–1.5 h. The litters were placed in a chamber perfused with a humidified gas mixture (10% oxygen in nitrogen) for 65 min (P5), 60 min (P9), 50 min (P21) or 40 min (P60) to produce a similar extent of severe brain injury at the different ages (Zhu *et al*. 2005). Moderate injury was induced by shortening the hypoxia times to 40 min (P5 and P9), 30 min (P21) and 25 min (P60). The temperature in the incubator and the water used to humidify the gas mixture was kept at 36°C. After hypoxic exposure, the pups (P5, P9 and P21) were returned to their biological dams and P60 adult mice were returned to their cages. Animals were allowed to recover for 3, 8, 24 or 72 h for P9 mice, or for 72 h only for P5, P21 and P60 animals (Zhu *et al*. 2005). The injury was evaluated at 72 h after HI by neuropathological scoring and measurement of tissue volume loss. Control pups, subjected to neither ligation nor hypoxia, were killed on P5, P9, P21 or P60. All animal experimentation was approved by the Ethics Committee of Göteborg (94-2003).

Sample preparation for immunoblotting and activity assay

Animals from the P9 group were killed by decapitation at 3, 8, 24 or 72 h after HI ($n = 12$ per group)(Wang *et al*. 2001; Zhu *et al*. 2005). Control animals were killed on P9 ($n = 6$ per group). The brains were rapidly dissected on a bed of ice. Parietal cortex was dissected out by first removing the frontal and occipital poles (approximately 2–3 mm) of the brain and, second, after positioning the brain with the occipital pole face down on the dissection tray, removing the medial cortex and the ventrolateral (piriform) cortex, leaving an approximately 50 mg piece of parietal cortex. The striatum was dissected out from the thalamus. Nine volumes of ice-cold homogenization buffer [15 mM Tris-HCl, pH 7.6, 320 mM sucrose, 1 mM dithiothreitol, 1 mM $MgCl_2$, 0.5% protease inhibitor cocktail (Sigma, Stockholm, Sweden) and 3 mM potassium EDTA] was added to each tissue piece. Homogenization was performed gently by hand in a 2-mL glass–glass homogenizer. Half of the homogenate was sonicated and used for immunoblotting. The other half of was centrifuged at 800 *g* for 10 min at 4°C. The supernatant was then centrifuged at 9200 *g* for 15 min at 4°C, producing a crude cytosolic fraction in the supernatant (S2), subsequently used for the caspase 3 activity assay. The pellet (P2), enriched in mitochondria, was washed, recentrifuged and used for immunoblotting.

Sex identification by PCR

The sex of the immature mice (P5, P9) was identified by PCR. Genomic DNA was isolated from tail samples. The tail was digested with 400 μL lysis buffer (50 mM Tris-HCl, pH 8.0, 100 mM EDTA, 100 mM NaCl, 1% sodium dodecyl sulphate) containing 1 mg/mL proteinase K (Roche, Indianapolis, IN, USA). Following incubation at 60°C overnight, 200 μL 5 M potassium acetate was added to the lysate, which was thoroughly mixed and centrifuged at 10 000 *g* for 20 min. The supernatant was transferred into a clean tube and 800 μL 100% ethanol was added and mixed. After incubation at −20°C for 30 min, the DNA was pelleted by centrifugation at 10 000 *g* for 25 min at 4°C. The pellet was washed once with 500 μL 75% ethanol. It was then dried and dissolved in 50 μL sterile water, and the DNA concentration was determined. The reaction mixture for PCR contained 100 ng mouse tail DNA, 0.5 μM mouse sex-determining region Y gene (*SRY*) gene primers (forward 5′-ATCTTAGAGAGACAGGAGAGCAG-3′; reverse 5′-TGAC-TAATCACCACCTGGTAGCT-3′), 200 μM each dNTP, 1 U Taq polymerase and 1.5 mM $MgCl_2$ in a final volume of 25 μL. Reactions were carried out for 40 cycles of 1 min at 94°C, 1 min at 61°C, 2 min at 72°C and a final extension step at 72°C for 10 min. PCR products were separated on a 1.5% agarose gel containing 0.5 μg/mL ethidium bromide. A 100-bp ladder was used to verify the size of the PCR products. The gels were exposed in a LAS 1000 cooled charge-coupled device (CCD) camera (Fujifilm, Tokyo, Japan). Males were identified by the presence of a single 335-bp DNA band, which agreed with the visual sex definition for all pups.

Immunohistochemistry

Mice were deeply anaesthetized with 50 mg/mL phenobarbital and fixed by perfusion with 5% formaldehyde in 0.1 M phosphate buffer through the ascending aorta for 5 min. The brains were rapidly removed and fixed by immersion at 4°C for 24 h. The brains were dehydrated with xylene and graded ethanol, embedded in paraffin, cut into 5-μm sections and mounted on glass slides. Antigen retrieval was performed by boiling deparaffinized sections in 10 mM sodium citrate buffer (pH 6.0) for 10 min. Non-specific binding was blocked for 30 min with 4% horse serum [for microtubule-associated protein (MAP)-2, cytochrome *c* (Cyt *c*) and AIF) or goat serum (for active caspase 3 and nitrotyrosine] in phosphate-buffered saline (PBS). Anti-MAP-2 (clone HM-2; Sigma), diluted 1: 2000 (4 μg/mL) in PBS, anti-Cyt *c* (clone 7H8.2C12; Pharmingen, San Diego, CA, USA), diluted 1 : 500 (2 μg/mL) in PBS, anti-AIF

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(Santa Cruz Biotechnology, Santa Cruz, CA, USA), diluted 1 : 100 (2 μg/mL), anti-active caspase 3 (Pharmingen), diluted 1 : 50 (10 μg/mL), or anti-nitrotyrosine (Molecular Probes, Eugene, OR, USA), diluted 1 : 100 (10 μg/mL) in PBS, was incubated for 60 min at 21°C, followed by another 60 min with biotinylated horse anti-mouse IgG (2 μg/mL), horse anti-goat IgG (2 μg/mL) or goat anti-rabbit IgG (2 μg/mL) diluted in PBS. Endogenous peroxidase activity was blocked with 3% H_2O_2 in PBS for 5 min. Visualization was performed using Vectastain ABC Elite (Vector Laboratories, Burlingame, CA, USA) with 0.5 mg/mL 3,3′-diaminobenzidine enhanced with 15 mg/mL ammonium nickel sulphate, 2 mg/mL β-D-glucose, 0.4 mg/mL ammonium chloride and 0.01 mg/mL β-glucose oxidase (Sigma).

Immunofluorescence staining

Antigen retrieval, blocking and incubation with AIF antibody were performed as described above. After incubation with biotinylated horse anti-goat antibody, the sections were incubated with avidin-conjugated Alexa Fluor 594, diluted 1 : 100 in PBS for 60 min. After washing with PBS, anti-poly(ADP-ribose) (PAR) antibody (Kawamitsu *et al*. 1984) was applied, diluted 1 : 300 in PBS containing 0.2% Triton X-100, followed by rinsing in PBS. The slides were incubated with secondary FITC-conjugated horse anti-mouse antibody, diluted 1 : 100 in PBS, for 60 min. After washing, the sections were placed in 1 μg/mL Hoechst 33342 (Molecular Probes) in PBS for 10 min at room temperature with gentle agitation, and washed and mounted using Vectashield mounting medium.

Immunoblotting

Protein concentration was determined by a published method (Whitaker and Granum 1980), adapted for microplates, using a Spectramax Plus plate reader (Molecular Devices, Sunnyvale, CA, USA). The samples were mixed with an equal volume of concentrated (3 ×) sodium dodecyl sulphate–polyacrylamide gel electrophoresis buffer and heated to 96°C for 5 min. Homogenates (50 μg protein) were run on 4–20% or 14% Tris-Glycine gels (Novex, San Diego, CA, USA) and transferred to reinforced nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). The membranes were blocked in 30 mM Tris-HCl (pH 7.5), 100 mM NaCl and 0.1% Tween 20 (TBS-T) containing 5% fat-free milk powder for 60 min at room temperature. After washing in TBS-T, they were incubated with anti-AIF (1 : 1000, 0.2 μg/mL, goat polyclonal antibody; Santa Cruz Biotechnology), anti-caspase 3 (1 : 1000; Santa Cruz Biotechnology), anti-Cyt *c* (1 : 500, clone 7H8.2C12), anti-nitrotyrosine (1 : 1000, 1 μg/mL; Molecular Probes), anti-actin (1 : 200; Sigma) or anti-MAP-1 light chain 3 (LC3) (1 : 800; rabbit polyclonal antibody) (Yu *et al*. 2004) for 60 min at room temperature. The LC3 antibody detected 16- and 14-kDa bands. The 16-kDa band (LC3-I) was ubiquitously expressed, whereas the 14-kDa band (LC3-II) is an autophagy-specific marker (Yu *et al*. 2004). After washing, the membranes were incubated with a peroxidase-labelled secondary antibody for 30 min at room temperature (goat anti-rabbit, 1 : 2000; horse anti-goat, 1 : 2000 or horse anti-mouse 1 : 4000). Immunoreactive species were visualized using Super Signal West Dura substrate (Pierce, Rockford, IL, USA) and a LAS 1000 cooled CCD camera. Immunoreative bands were quantified using Image Gauge software (Fujifilm).

Caspase activity assays

Protein concentrations were determined as above. Samples (25 μL) of crude cytosolic fractions (S2) were mixed with 75 μL extraction buffer as described earlier (Wang *et al*. 2001). Cleavage of N-Acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (Ac-DEVD-AMC) (Peptide Institute, Osaka, Japan) was measured with an excitation wavelength of 380 nm and an emission wavelength of 460 nm, and expressed as pmoles AMC released per milligram protein per minute.

Injury evaluation

Neuropathological scoring

Brain injury in different regions of P5, P9, P21 or P60 animals was evaluated using a semiquantitative neuropathological scoring system as described earlier (Hagberg *et al*. 2004). Briefly, sections were stained for MAP-2 and scored by an observer blinded to the treatment of the animals. The cortical injury was graded from 0 to 4, 0 being no observable injury and 4 confluent infarction encompassing most of the cerebral cortex. Damage in the hippocampus, striatum and thalamus was assessed both with respect to hypotrophy (0–3) and observable cell injury/infarction (0–3), resulting in a neuropathological score for each brain region (0–6). The total score (0–22) was the sum of the scores for all four regions.

Tissue volume

The volume of tissue loss was measured at 72 h after HI by sectioning whole brains into 5-μm sections and staining every 100th section for MAP-2. The areas in the cortex, striatum, thalamus and hypothalamus displaying MAP-2 staining were measured in both hemispheres using Micro Image (Olympus, Tokyo, Japan) and the volumes calculated according to the Cavalieri principle using the formula $V = \sum A \times P \times T$, where V is total volume, $\sum A$ is the sum of the areas measured, P is the inverse of the sampling fraction, and T is the section thickness (Mallard *et al*. 1993). The ratio of tissue loss was calculated as (contralateral volume – ipsilateral volume)/contralateral volume.

Cell counting

Cell counting for AIF, Cyt *c* and active caspase 3 was performed in the cortex and striatum in P9 animals. Immunopositive cells were counted at 400 × magnification (one visual field = 0.196 mm^2). Three visual fields within an area displaying loss of MAP-2 (if any) were counted and expressed as average number (mean) per visual field. For double labelling of AIF and PAR, the images were obtained at 400 × magnification.

Statistical analysis

All data were expressed as mean ± SD. The Mann–Whitney *U*-test was used to compare injury scores, tissue loss and percentage of immunopositive cell co-localization between males and females. ANOVA with Fisher's *post hoc* test was used when comparing more than two groups. $p < 0.05$ was considered statistically significant.

Results

Sex difference brain injury after HI at different ages

Moderate or severe brain injury was induced by adjusting the duration of hypoxia in both males and females at P5, P9, P21

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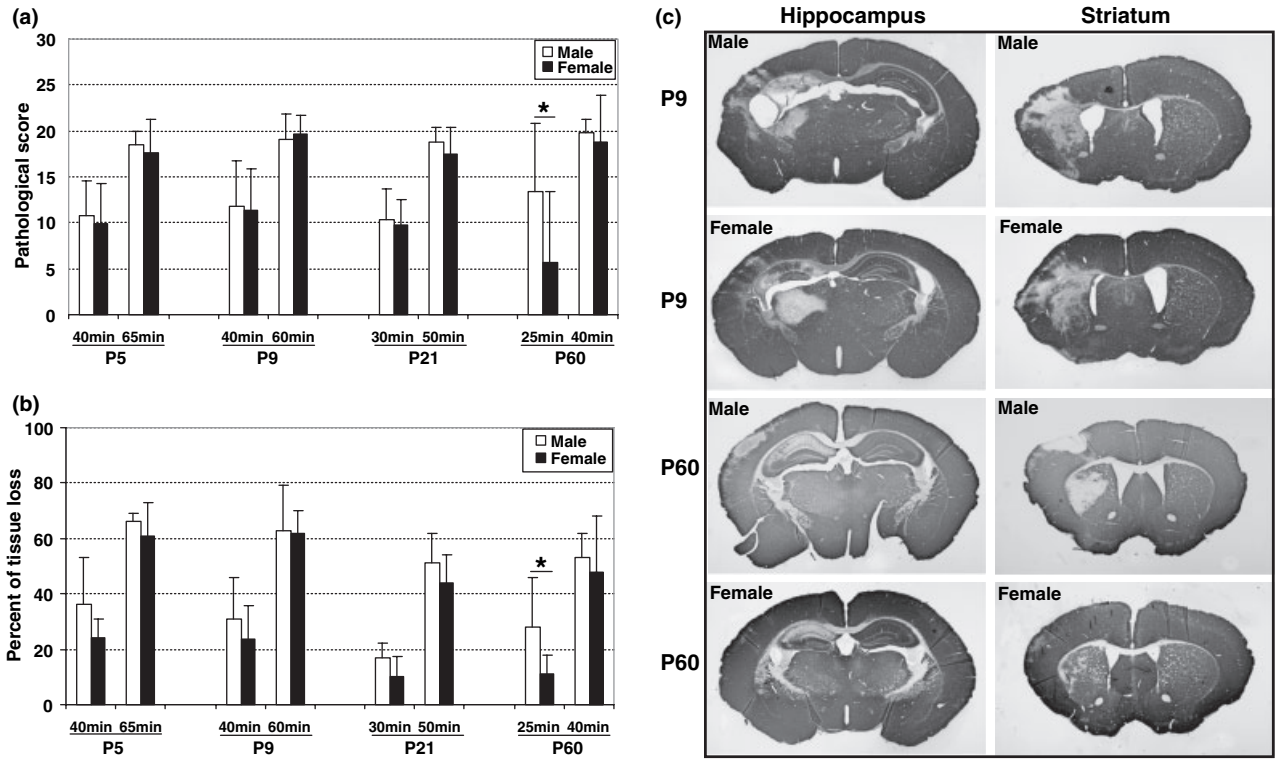

Fig. 1 Sex-related brain injury after moderate and severe HI at different ages. Severe and moderate brain injury was induced in both male and female mice by adjusting the hypoxia time at different ages. Bar graphs show mean ± SD pathological scores (a) and percentage tissue loss (b) in mice exposed to HI at P5 for 40 min (male $n = 6$, female $n = 5$) or 65 min (male $n = 6$, female $n = 6$), at P9 for 40 min (male $n = 10$, female $n = 11$) or 60 min (male $n = 6$, female $n = 6$), at P21 for 30 min (male $n = 6$, female $n = 6$) or 50 min (male $n = 6$, female $n = 6$), and at P60 for 25 min (male $n = 6$, female $n = 8$) or 40 min (male $n = 6$, female $n = 6$). In response to a moderate insult in P60 mice, brain injury was significantly less in female than in male mice (*$p < 0.05$). (c) Representative examples of MAP-2 staining at the level of the dorsal hippocampus and striatum 3 days after HI at P9 and P60 in male and female mice subjected to moderate HI (40 min hypoxia for P9, 25 min for P60). The injury was comparable in males and females at P9, whereas at P60 brain damage was more pronounced in males. The thalamus was resistant to HI and the cortical infarction was laminar at P60, compared with a more columnar pattern of cortical infarction at P9.

or P60 (Fig. 1). There were no sex differences in the extent of brain injury after severe HI, but in response to moderate HI there was more pronounced brain injury at P60 in males than in females, as assessed both by pathological score (Fig. 1a) and percentage tissue loss in the ipsilateral hemisphere (Fig. 1b). The sex-related difference in brain injury was substantial in the striatum (Fig. 1c). The thalamus was resistant to HI in P60 animals and cortical infarction was laminar, compared with a more columnar pattern found at P9.

PAR accumulation and mitochondrial translocation of AIF after HI

The total level of AIF protein in normal P9 mouse brain homogenates was similar in males and females (Fig. 2a). The percentage of AIF lost from mitochondria was significantly higher in male than in female striatum at 8 h after HI (Fig. 2d). No sex differences in AIF release were observed in cerebral cortex (Fig. 2d). The number of AIF-positive nuclei was higher in males at 3 h in cortex and at 8 h in the striatum after HI (Fig. 2e).

The increase in PAR immunoreactivity was used as a marker of PARP-1 activation for comparison with the cellular distribution of AIF translocation after HI. As shown previously, the number of PAR-positive cells after HI was similar in males and females (Hagberg *et al*. 2004). However, double labelling showed some PAR-positive cells without nuclear AIF staining and with normal nuclear morphology early after HI, followed by an increasing number of cells (8 h after HI) with apoptotic morphology exhibiting double AIF–PAR staining; this was most pronounced in males (Figs 3a and b).

Cyt *c* release from mitochondria and caspase 3 activation after HI

The total level of Cyt *c* in control P9 mouse brain did not differ between males and females (Fig. 4a). Cyt *c* was

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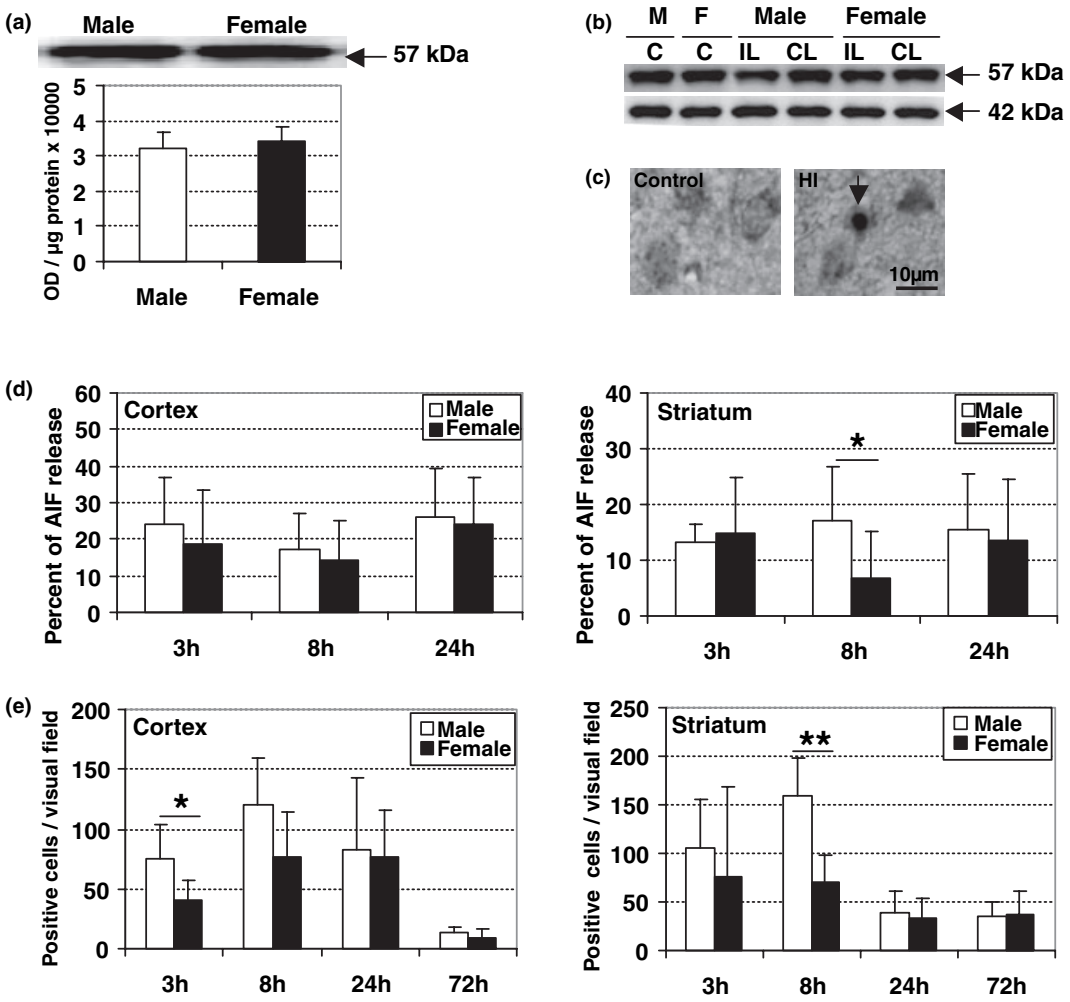

Fig. 2 Expression of AIF in P9 male and female mice. (a) AIF (57 kDa) immunoreactivity in cerebral cortex homogenates of control P9 male ($n = 6$) and female ($n = 6$) mice. Quantification of the 57-kDa band showed that the total amount of AIF was no different between the sexes (left panel). (b) Representative AIF immunoblots of mitochondrial fractions from the striatum from male and female control cortices and those after 8 h of HI. There were no sex differences in controls, but more AIF was lost from mitochondria in the male ipsilateral hemisphere samples after HI. The actin immunoblot confirmed equal protein loading (42 kDa; bottom panel). C, control; IL, ipsilateral hemisphere; CL, contralateral hemisphere. (c) Typical sections showing AIF immunostaining of control striatum and that after HI. Injured cells displaying nuclear AIF staining are indicated by an arrow. (d) Bar graph showing mean ± SD percentage AIF loss from mitochondria in males and females at 3, 8 and 24 h after HI ($n = 12$/group). More AIF was released from mitochondria in the male compared with female striatum at 8 h after HI (right panel). There were no significant differences between males and females in the loss of AIF in the cortex (left panel). (e) Mean ± SD number of AIF-positive nuclei in the cortex (left panel) and striatum (right panel) after severe HI ($n = 6$/group). There were more AIF-positive nuclei in males than in females in cortex at 3 h ($^*p < 0.05$) as well as in striatum at 8 h ($^{**}p < 0.01$) after HI.

released from mitochondria after HI (Fig. 4b), resulting in strong cytoplasmic immunoreactivity (Fig. 4c). There was a tendency towards more pronounced Cyt *c* release in females at most time points, as assessed by immunoblotting, but the difference was not statistically significant (Fig. 4d). The number of cells with Cyt *c* translocation, as visualized by immunohistochemistry, in cortex and striatum was not sex dependent (Fig. 4e).

There was no sex difference in immunoreactivity for the 32-kDa caspase 3 pro-form in cerebral cortex of P9 mice (Fig. 5a). After HI, the 32-kDa pro-form was cleaved, yielding a 29-kDa cleavage product, as previously reported (Blomgren *et al*. 2001), and this immunoreactivity was significantly stronger in females than in males after HI (Fig. 5b). DEVDase assays showed that the caspase 3-like activity increased and reached a peak at 24 h after HI both in cortex and striatum. The activity was significantly greater in the cortex and striatum of females than males at 24 h after HI (Fig. 5d). Active caspase 3 staining in tissue sections increased at 3 h and reached a peak at 24 h after HI in

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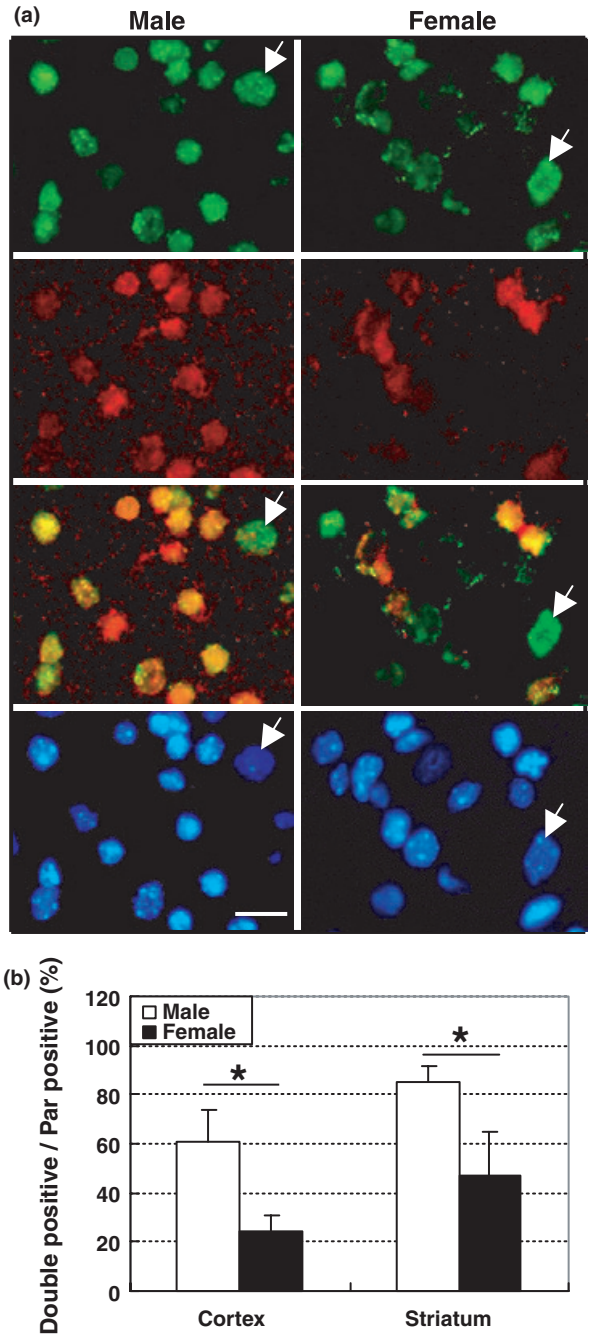

Fig. 3 Double labelling of AIF and PAR in P9 males and females at 8 h after HI. (a) Representative immunostaining for PAR (green), AIF (red) and overlay, plus Hoechst (blue) staining in the ipsilateral striatum at 8 h after HI, demonstrating a high degree of PAR–nuclear AIF co-localization in cells with condensed nuclei preferentially in males. PAR-positive but nuclear AIF-negative cells usually exhibited nuclei with normal morphology as indicated by Hoechst staining (indicated by arrow). (b) Bar graph shows mean ± SD percentage of PAR–AIF double labelled cells in cortex and striatum of the ipsilateral hemisphere in males and females ($n = 6$/group). There were more cells labelled by both PAR and nuclear AIF in males than in females ($^*p < 0.005$).

cerebral cortex and striatum. There were more active caspase 3-positive cells in the striatum of females than males 24 h after the insult (Fig. 5e).

Nitrotyrosine formation after HI

Several nitrotyrosine-immunoreactive protein bands were detected on immunoblots of brain samples(Figs 6a and b). The density of the 50-kDa band was the strongest and was therefore used for quantification of nitrotyrosine formation. There was no sex difference in nitrotyrosine immunoreactivity of control brains (Fig. 6c). Nitrotyrosine formation increased in the ipsilateral hemisphere after HI but there were no differences between males and females (Fig. 6c). The number of nitrotyrosine-positive cells was significantly higher in males compared with females at 72 h in the striatum, but there were no sex differences in any region at any other time point after HI (Fig. 6d).

Autophagy after HI

Immunoblots of homogenates using antibody against MAP-1 LC3 showed that the ubiquitously expressed LC3-I (16 kDa) was abundant in the brain. LC3-I is cytosolic, whereas LC3-II (14 kDa) is membrane bound, and the amount of LC3-II correlates with the extent of autophagosome formation (Kabeya *et al.* 2000). Both LC3-I and LC3-II could be detected in control brains and after HI (Figs 7a and b), but there were no sex differences (data not shown). After HI, LC3-II increased in the ipsilateral hemisphere, and a peak level was reached at 24–72 h after HI (Fig. 7c). The relative increase in LC3-II in the ipsilateral hemisphere was similar in males and females in both cortex and striatum (Fig. 7c).

Discussion

This study yielded several interesting findings. First, there were no sex differences with respect to brain injury after a severe hypoxic–ischaemic insult at any age. However, in response to moderate HI, more extensive brain injury was found in males than females at P60 but not at P5, P9 or P21. Second, in spite of a similar extent of brain injury at P9, AIF translocation and AIF–PAR double immunostaining after HI were more pronounced in males, whereas loss of pro-caspase 3, immunohistochemical detection of cells with active caspase 3 and caspase 3 (DEVDase) activity was more marked in the female CNS after HI, indicating important sex differences with regard to mode of induction of the apoptotic cascade after HI.

Age-related sex difference on hypoxic–ischaemic brain injury

Sex-related differences in neuroanatomy, neurochemistry and behaviour have been described (Ayoub *et al.* 1983; Byne 1998; Madeira *et al.* 1999). Increasing evidence has demonstrated striking sex differences in the pathophysiology of and

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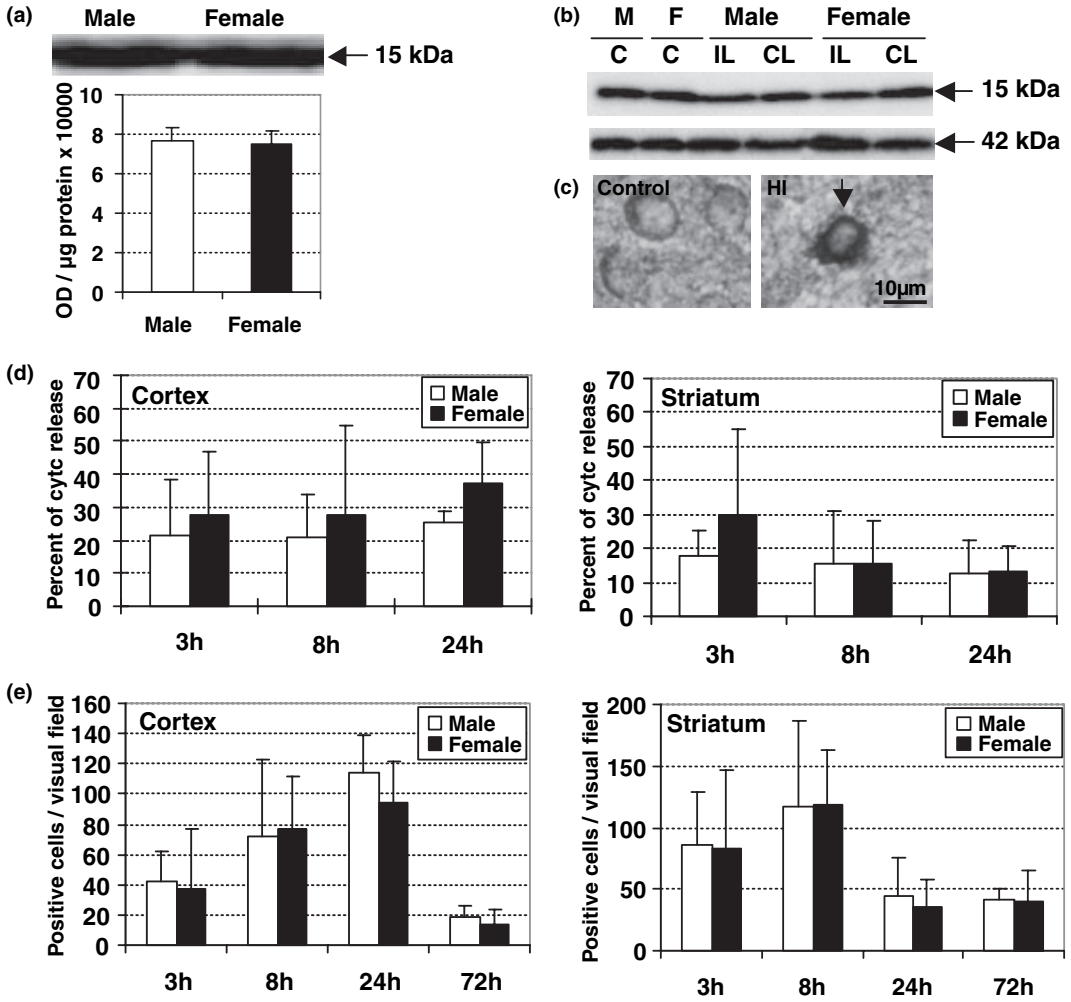

Fig. 4 Expression of Cyt *c* in P9 male and female mice. (a) Immunoblots of Cyt *c* in control P9 male ($n = 6$) and female ($n = 6$) mouse cortex homogenates. Quantification of the 15-kDa band showed that the total amount of Cyt *c* was similar in males and females (left panel). (b) Representative Cyt *c* immunoblots of the mitochondrial fraction from controls and at 8 h after HI in the striatum of males and females showed no sex differences. The immunoreactivity of actin (42 kDa) confirmed equal protein loading (right panel). (c) Typical example of Cyt *c* immunostaining in control and post-ischaemic striatum; arrow indicates a cell with strong Cyt *c* staining in the cytoplasm. (d) Bar graphs showing mean ± SD percentage of Cyt *c* released from mitochondria as analysed by western blotting in males and females at 3, 8 and 24 h after HI ($n = 12$/group). There was no significant difference in Cyt *c* release between males and females in cerebral cortex (left panel) or striatum (right panel). (e) Mean ± SD number of Cyt *c*-positive cells determined immunohistochemically in the cortex (left panel) and striatum (right panel) after HI ($n = 6$/group). There was no significant sex difference at any time point.

outcome after acute neurological injury (Roof and Hall 2000). Lesser susceptibility to post-ischaemic and post-traumatic brain injury in adult females has been observed in experimental models (Alkayed *et al*. 1998). Additional evidence suggests that these sex differences extend to humans (Hindmarsh *et al*. 2000). Studies in children have shown that girls have a more favourable neurological outcome after traumatic brain injury (Donders and Hoffman 2002) and respond more favourably to treatment (Weil *et al*. 1998). Pre-term males seem to be at greater risk of perinatal brain injury and later neurological cognitive impairment and learning difficulties (Wolke 1998).

The sex difference was age and insult related. The greater neuroprotection afforded to females is likely to at least partly relate to the effects of circulating oestrogens and progestins. In fact, exogenous administration of both hormones has been shown to improve outcome after cerebral ischaemia and trauma (Hurn and Macrae 2000). The fact that a sex difference with respect to tissue loss was seen only in adult mice (P60) indicates that circulating hormones play an important role when adult levels are reached in rodents (Hurn and Macrae 2000). Previous studies have shown that apoptotic cell death was more pronounced in immature brain than in juvenile and mature brain (Hu *et al*. 2000; Gill *et al*. 2002; Zhu *et al*.

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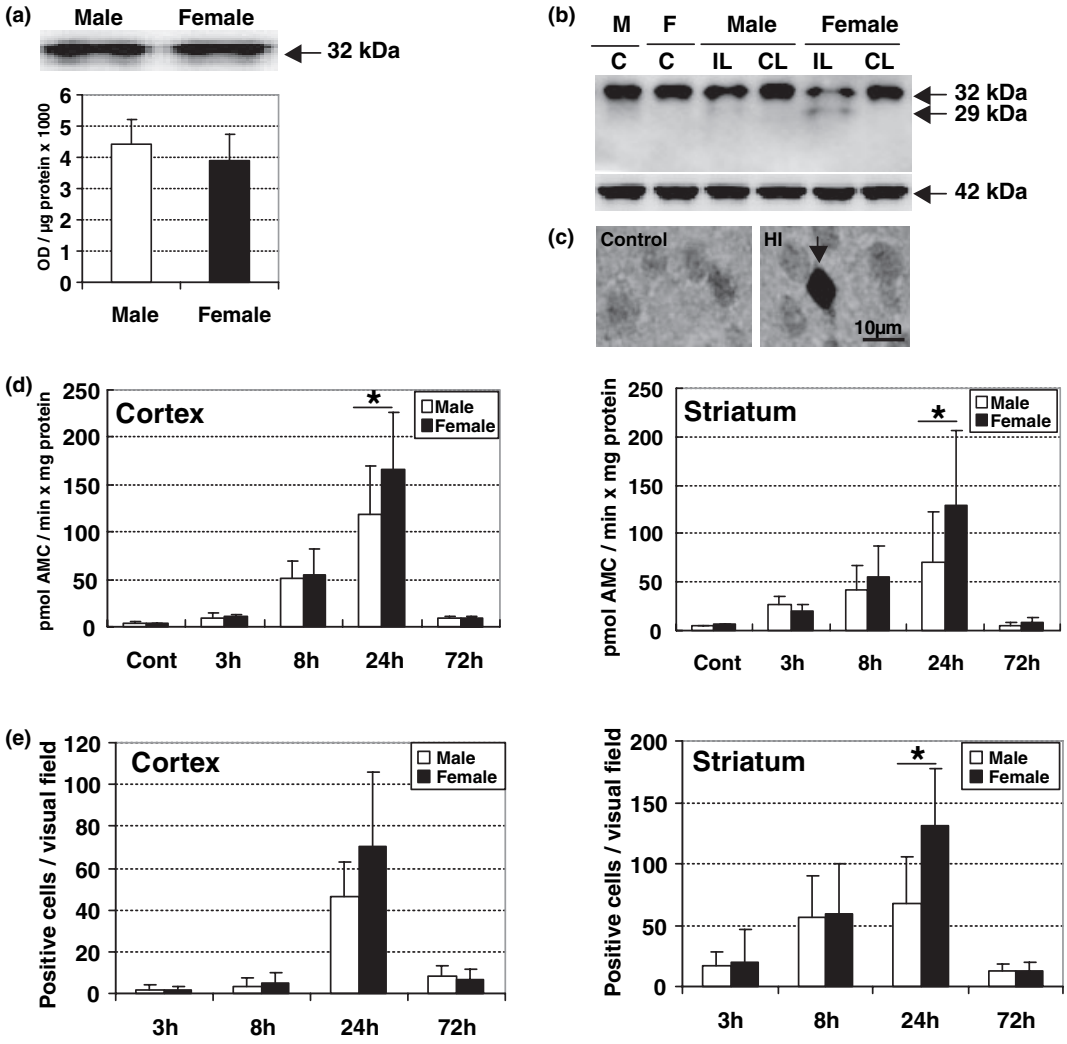

Fig. 5 Expression of caspase 3 in P9 male and female mice. (a) Immunoblots of pro-caspase 3 (32 kDa) in cortex homogenates of P9 controls showed no differences between male ($n = 6$) and female ($n = 6$) mice. (b) Representative caspase 3 immunoblots of crude cytosolic fractions from the striatum in controls and after HI. There was more cleavage of pro-caspase 3 in females than in males in the ipsilateral hemisphere 24 h after HI. Equal immunoreactivity of actin (42 kDa) indicated equal protein loading (right panel). (c) Micrograph showing strong cellular immunostaining of cleaved caspase 3 (arrow) after HI in striatum compared with weak staining in controls. (d) Bar graphs showing mean ± SD caspase 3-like (DEVDase) activity in crude cytosolic fractions at 3, 8 and 24 h ($n = 12$/group) and 72 h ($n = 8$/group) after HI. Caspase 3 activity was significantly higher in females than males at 24 h after HI in both cortex (left panel) and striatum (right panel) ($^*p < 0.05$). (e) Mean ± SD number of active caspase 3-positive cells in the cortex (left panel) and striatum (right panel) after HI ($n = 6$/group); there were more cells with cleaved caspase 3 immunoreactivity in the striatum of females than in males 24 h after HI ($^*p < 0.05$).

2005). In addition, sex-related differences in neuroprotective responses have been found in immature rodents (Bona *et al*. 1998; Hagberg *et al*. 2004). However, the sex-related changes in apoptotic and other pathways of neuronal cell death after HI in the immature brain have not been investigated. In this study mice at P9, an age corresponding developmentally to the term or near-term human brain (Hagberg *et al*. 2002), were chosen for comparison of cell death cascades between males and females after HI.

Influence of sex on cell death mechanisms in the immature brain

We have previously found in a neonatal rat model of HI that female pups were more protected after hypothermia (Bona *et al*. 1998). Furthermore, PARP-1 deficiency rendered immature mice partially resistant to HI. However, complete *PARP-1* gene disruption conferred protection in male but not female mice (Hagberg *et al*. 2004). These differences found in postnatal rats and mice appear independent of circulating

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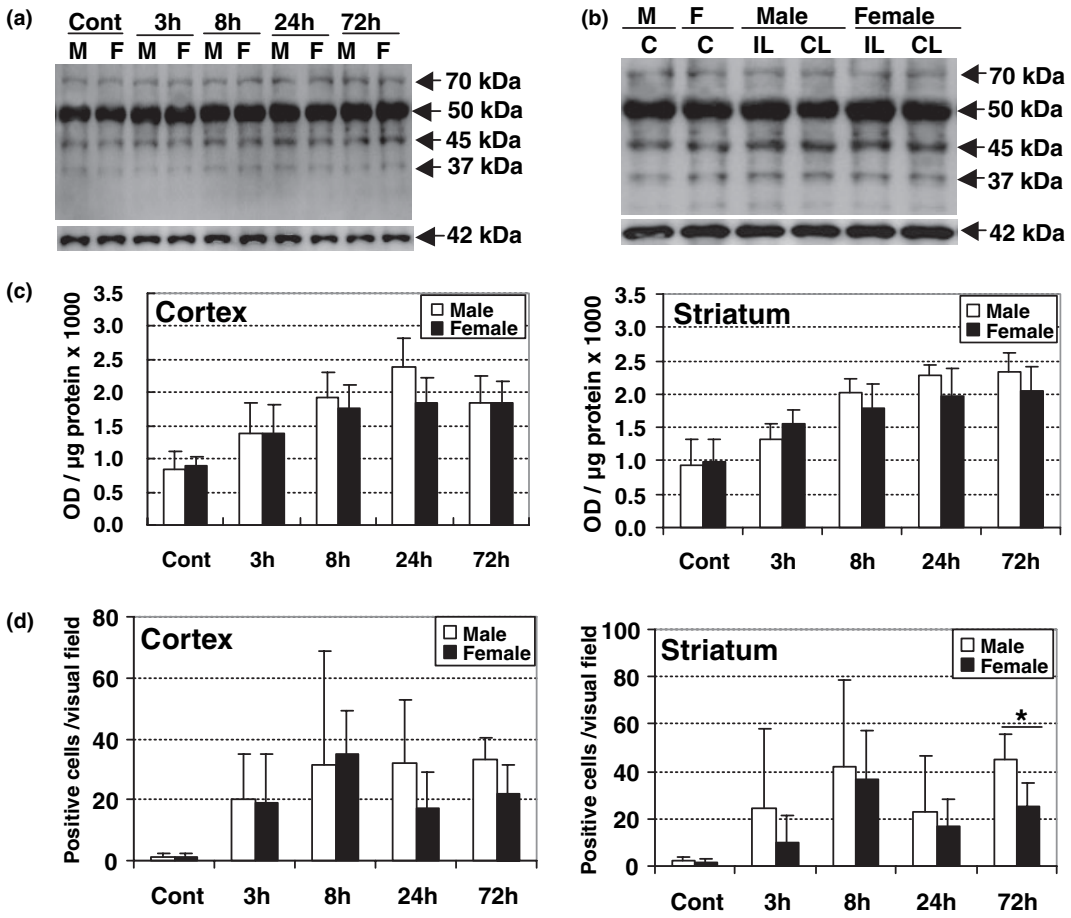

Fig. 6 Nitrotyrosine formation in P9 male and female mice. (a) Western blots of nitrotyrosine immunoreactivity in control P9 (n = 6/group; Cont), and 3, 8 and 24 h (n = 12/group) and 72 h (n = 8/group) after HI in the ipsilateral cortex homogenate of both males and females. Actin (42 kDa) was included to confirm equal loading (lower panel). (b) Representative nitrotyrosine immunoblots of striatal homogenates demonstrating higher immunoreactivity 24 h after HI compared with that in controls, but no sex differences. (c) Bar graphs showing quantification of the 50-kDa nitrotyrosine-positive band in cerebral cortex and striatal homogenates in controls and in the ipsilateral hemisphere at different time points after HI. Values are mean ± SD. Nitrotyrosine formation increased after HI in both brain regions but there were no sex differences. (d) Counting of cells with nitrotyrosine immunoreactivity in the cortex (left panel) and striatum (right panel) (n = 6/group) demonstrated no consistent sex difference. However, the number of cells with nitrotyrosine staining in the striatum was moderately higher in males than in females 72 h after HI (*$p < 0.05$). Values are mean ± SD.

sex steroids as they occurred before sexual maturity. These data suggest that cell death in the brain may follow different paths depending on sex (Nunez *et al*. 2001; Zhang *et al*. 2003; McCullough *et al*. 2005). It has also been shown that cultured XY and XX brain cells, independent of the influence of the gonads, differ in phenotype, suggesting that the sex of brain cells is also important for sexual differentiation of the CNS as a complement to the influence of sexual steroids (Carruth *et al*. 2002). The susceptibility to a variety of cytotoxic agents and responses to therapies were also found to be different between XY and XX neurones (Du *et al*. 2004).

AIF, a major player in caspase-independent cell death (Susin *et al*. 1999), has been demonstrated to be important in different brain injury models (Ferrand-Drake *et al*. 2003; Zhu *et al*. 2003; Plesnila *et al*. 2004). The total protein level of AIF did not change appreciably during brain development in rats or mice (Zhu *et al*. 2003; Zhu *et al*. 2005). We did not find any sex-related differences in AIF expression in P9 control mice, in agreement with results from embryonic rat XY and XX neuronal cultures (Du *et al*. 2004). In response to HI, AIF is released from mitochondria and translocates to the nucleus in this model (Zhu *et al*. 2003; Matsumori *et al*. 2005). There was a somewhat more pronounced AIF loss from mitochondria in males than in females (significant at 8 h in striatum but not in cortex), and quantification of AIF-positive nuclei demonstrated a more pronounced AIF translocation in cortex (3 h) and striatum (8 h) in males

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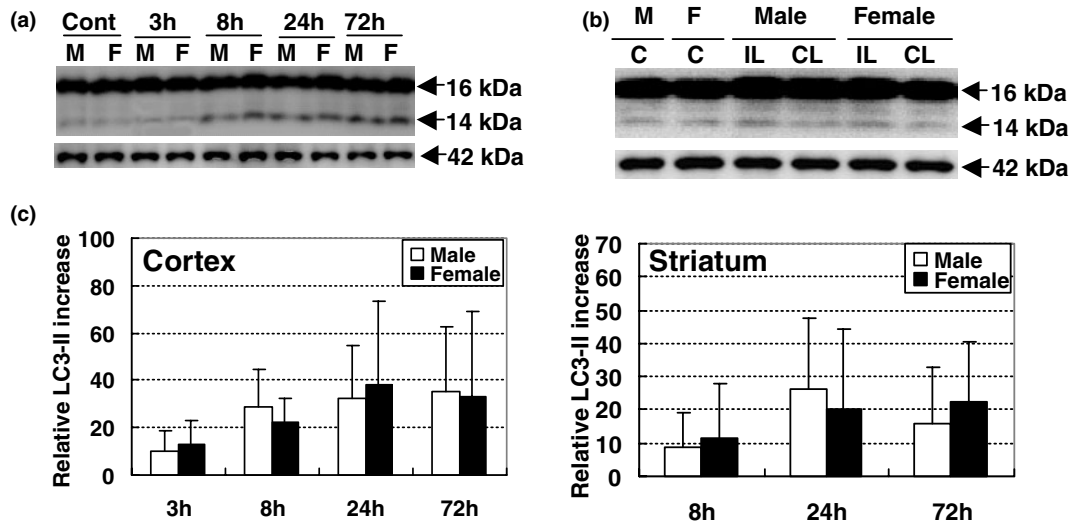

Fig. 7 Immunoblot analysis of MAP-1 LC3-II, a marker of autophagy, in P9 male and female mice. (a) Immunoblots of LC3-I (16 kDa) and LC3-II (14 kDa) in normal P9 mice ($n = 6$/group) and at 3, 8 and 24 h ($n = 12$/group) and 72 h ($n = 8$/group) after HI in ipsilateral cortex homogenates from males and females. LC3-II immunoreactivity was detected in all samples. The actin blot (42 kDa) confirmed equal loading (lower panel). (b) Representative LC3-I and -II immunoblots of striatal homogenates from control mice and 24 h after HI in males and females. (c) Bar graphs showing the mean ± SD relative increase in LC3-II in ipsilateral compared with contralateral hemisphere homogenates in males and females at 3 (cortex only), 8 and 24 h ($n = 12$/group) and 72 h ($n = 8$) after HI. There were no significant differences between males and females in cortex (left panel) or striatum (right panel).

compared with females. Our results principally agree with those of cell culture experiments demonstrating that AIF translocation was more pronounced in XY neurones than XX neurones exposed to nitrosative and excitotoxic stress (Du *et al*. 2004). Mitochondrial release of AIF has been suggested as a pathway for PARP-mediated chromatolysis and cell death (Yu *et al*. 2002). The fact that complete *PARP-1* gene disruption in female mice did not confer protection indicates that the AIF pathway does not predominate in female neuronal cell death (Hagberg *et al*. 2004). This was supported by a higher percentage of double AIF–PAR-positive cells in males than in females.

Cyt *c* is believed to represent an essential component of caspase-dependent cell death. Release of Cyt *c* from mitochondria as a consequence of HI has been detected previously by immunoblotting and immunohistochemistry (Zhu *et al*. 2003). The brain protein levels of Cyt *c* were similar in male and female controls. There was a tendency (non-significant) for more Cyt *c* to be released from mitochondria in female cortex compared with that of males after HI, but this could not be confirmed by immunohistochemistry.

Caspase 3 activation is a critical downstream event in caspase-dependent cell death. We found here that, in response to HI, the loss of pro-caspase 3, the increase in cells containing active caspase 3 and the increase in caspase-3 (DEVDase) activity were all more pronounced in female than in male neurones. These results strongly support a more powerful activation of the caspase-dependent pathway in XX versus XY neurones (Du *et al*. 2004). We cannot fully explain, however, why activation of caspase 3 but not Cyt *c* translocation was sex dependent in our experiments. There are, however, several other pathways, besides that involving Cyt *c* (Li *et al*. 1997), that could trigger activation of caspase 3; caspase 8 and caspase 12 might be involved (Nakagawa *et al*. 2000) and such pathways need to be investigated in future experiments. Furthermore, there are several reports of the effects of caspase inhibitors in neonatal HI, with conflicting results, but no information on the influence of sex (Cheng *et al*. 1998; Han *et al*. 2002; Zhu *et al*. 2003; Wang *et al*. 2004).

Nitrosylation of proteins is an important pathway of neurodestruction induced by nitric oxide and can be used as an indicator of peroxynitrite formation after HI (Ischiropoulos and Beckman 2003; Zhu *et al*. 2004). Combined inhibition of inducible nitric oxide synthase (iNOS) and neuronal nitric oxide synthase (nNOS) provided short- and long-term neuroprotection (Peeters-Scholte *et al*. 2002; van den Tweel *et al*. 2005). Studies in adult mice showed that *nNOS* knock-out or iNOS mutant females, unlike males, were not more resistant to ischaemic damage than wild-type controls (Loihl *et al*. 1999; McCullough *et al*. 2005). Furthermore, female neurones were strikingly less sensitive to peroxynitrite toxicity than male neurones (Du *et al*. 2004), offering additional support to the hypothesis that nitric oxide brain toxicity is sex dependent. In this study, we found that nitrotyrosine formation increased after HI but there were no consistent differences in the response between males and females. It is possible that developmental differences exist

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that might explain the discrepancy, or that peroxynitrite formation as measured here does not give a complete picture of the involvement of nitric oxide. It would be interesting to compare the response in male and female neonatal mice with and without gene disruption for nNOS and iNOS.

Autophagy is a process responsible for the bulk degradation of intracellular material in double- or multiple-membrane autophagic vesicles. Autophagy is part of the caspase-independent genetically controlled, physiological programmed cell death, which is more pronounced during embryonic development and tissue remodelling (Xue *et al*. 1999; Bursch 2001). We hypothesized that autophagy may represent an additional type of apoptosis-related cell death in the neonatal brain (Nixon *et al*. 2005; Zhu *et al*. 2005) and that autophagy, similar to caspase-dependent and -independent cell death, might be different in males and females. Autophagy increased in the ipsilateral hemisphere after HI as indicated by increased expression of LC3-II (Zhu *et al*. 2005). However, we found no sex dependence in the induction of autophagy after neonatal HI.

In summary, brain injury after moderate HI was more pronounced in males than in females at P60, but there were no sex differences in the extent of the lesion at P5, P9 or P21. However, the mechanisms of neuronal cell death after HI in P9 mice were sex dependent. The AIF pathway seemed to be activated more in males, whereas caspase activation was more pronounced in females. We could not detect any sex differences in the induction of autophagy or in peroxynitrite formation after neonatal HI.

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