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Authors

Schilling, JM
Kassan, A
Mandyam, C
et al.

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Inhibition of p75 neurotrophin receptor does not rescue cognitive impairment in adulthood after isoflurane exposure in neonatal mice

J. M. Schilling[1,2], A. Kassan[1,2], C. Mandyam[1,3], M. L. Pearn[1,2], A. Voong[1,2], G. G. Grogman[1,2], V. B. Risbrough[4], I. R. Niesman[5], H. H. Patel[1,2], P. M. Patel[1,2] and B. P. Head[1,2]*

[1] Department of Anesthesiology, Veterans Affairs San Diego Healthcare System, 3350 La Jolla Village Drive, San Diego, CA 92161, USA, [2]Department of Anesthesiology, School of Medicine, University of California, San Diego, 9500 Gilman Dr, La Jolla, CA 92093, USA, [3]Skaggs School of Pharmacy and Pharmaceutical Sciences, School of Medicine, University of California, San Diego, La Jolla, CA, USA, [4]Departments of Psychiatry and Anesthesiology, University of California, San Diego, La Jolla, CA, USA and [5]Department of Cellular & Molecular Medicine—Sanford Consortium for Regenerative Medicine, University of California, San Diego, La Jolla, CA, USA

*Corresponding author. E-mail: bhead@ucsd.edu

Abstract

Background: Isoflurane is widely used for anaesthesia in humans. Isoflurane exposure of rodents prior to post-natal day 7 (PND7) leads to widespread neurodegeneration in laboratory animals. Previous data from our laboratory suggest an attenuation of apoptosis with the p75 neurotrophin receptor (p75NTR) inhibitor TAT-Pep5. We hypothesized that isoflurane toxicity leads to behavioural and cognitive abnormalities and can be rescued with pre-anaesthesia administration of TAT-Pep5.
Methods: Neonatal mouse pups were pretreated with either TAT-Pep5 (25 μl, 10 μM i.p.) or a scrambled control peptide (TAT-ctrl; 25 μl, 10 μM i.p.) prior to isoflurane exposure (1.4%; 4 h) or control ($n = 15$–26/group). Three to 5 months after exposure, behavioural testing and endpoint assays [brain volume (stereology) and immunoblotting] were performed.
Results: No significant difference was observed in open field, T-maze, balance beam or wire-hanging testing. The Barnes maze revealed a significant effect of isoflurane ($P = 0.019$) in errors to find the escape tunnel during the day 5 probe trial, a finding indicative of impaired short-term spatial memory. No difference was found for brain volumes or protein expression. TAT-Pep5 treatment did not reverse the effects of isoflurane on neurocognitive behaviour.
Conclusion: A single isoflurane exposure to early post-natal mice caused a hippocampal-dependent memory deficit that was not prevented by pre-administration of TAT-Pep5, although TAT-Pep5, an inhibitor of p75NTR, has been shown to reduce isoflurane-induced apoptosis. These findings suggest that neuronal apoptosis is not requisite for the development of cognitive deficits in the adults attendant with neonatal anaesthetic exposure.

Key words: behaviour; isoflurane; mouse; neurotoxicity; pharmacology

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Editor's key points

- Exposure of neonatal mammals to general anaesthetics can produce widespread neurodegeneration, but its contribution to subsequent cognitive dysfunction is unclear.
- Treatment of neonatal male mice with an inhibitor that can prevent neuronal death did not prevent delayed cognitive dysfunction following exposure to isoflurane.
- Isoflurane-induced cognitive dysfunction can be dissociated from its apoptotic effects, which suggests that other mechanisms are involved.

Isoflurane is a volatile agent that is widely used for anaesthesia in humans. Recent experimental studies indicate that exposure to isoflurane and other anaesthetics such as midazolam, nitrous oxide, sevoflurane, propofol, thiopental, and ketamine during post-natal day 7 (PND 7) leads to widespread neurotoxicity.[1–2] This neurotoxicity appears to be limited to this period of development since isoflurane does not produce neurotoxic effects at PND 15, yet does alter synaptic plasticity that persists up to 4 weeks after exposure[3].

It is postulated that neonatal exposure to anaesthetics results in neurocognitive and behavioural abnormalities during adolescence and adulthood.[2] Previous data from our laboratory show apoptosis in developing primary neurones and in the hippocampus from neonatal mice (PND 5–7) upon exposure to isoflurane.[4] This injury was mediated by preferential signalling of proBDNF via p75 neurotrophin receptor (p75NTR). Importantly, proBDNF–p75NTR signalling also plays a key role in propofol-induced neuronal degeneration.[5] Neurodegeneration induced by isoflurane and propofol was almost completely prevented by administration of the p75NTR inhibitor TAT-Pep5. Moreover, neurones from p75NTR knockout mice were not vulnerable to propofol neurotoxicity. Expression of p75NTR in neurones from p75NTR$^{-/-}$ mice recapitulated vulnerability to propofol toxicity.[5] These data suggested that a common mechanism of toxicity, which links proBDNF–p75NTR signalling to downstream RhoA activation and actin depolymerization, might be relevant to both volatile and i.v. anaesthetic developmental neurotoxicity.[6]

Given the efficacy of p75NTR inhibition in preventing anaesthetic-induced neurodegeneration, we hypothesized that isoflurane-mediated behavioural and cognitive abnormalities in mice during adulthood could be ameliorated with prophylactic administration of TAT-Pep5 prior to isoflurane exposure. To address this question we investigated the effects of a single isoflurane exposure at PND 5–7 on cognitive and behavioural function in C57Bl/6J mice 3–5 months after exposure.[7] We utilized measures of general behaviour to complement the Barnes maze platform test to assess learning and memory in the context of anaesthetic neurotoxicity.[8–14]

Methods

All studies performed on animals were approved by the Veterans Affairs San Diego Institutional Animal Care and Use Committee (IACUC) and conform to relevant National Institutes of Health guidelines. Mice were handled and habituated as described.[12]

Mice

Male and female C57Bl/6J mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA) and bred in a monogamous breeding scheme to produce offspring. On a daily basis, the presence of new offspring was assessed and the gender of the pups determined.[13] On the day of the experiment, mouse pups received a toe tattoo for future identification and were randomized (by use of a random number generator) to one of four groups. PND 5–7 male pups were exposed to either isoflurane (1.4%; 4 h continuous exposure) in a 40% oxygen/air mix with a 30-min pretreatment with either a p75NTR inhibitor (TAT-Pep5; 25 μL of 10 μM, Calbiochem) or corresponding scrambled control peptide (TAT-ctrl; 25 μL of 10 μM, Calbiochem) administered i.p., or a 40% oxygen/air mix alone with either TAT-Pep5 or TAT-ctrl ($n = 16–27$/group). The temperature in the incubator was maintained at 37 °C. Behavioural testing took place at 3–5 months of age by observers without knowledge of the group allocation. Upon conclusion of behavioural testing, group allocation, which was kept by an individual not associated with the study, was revealed to the group of investigators who had conceptualized and conducted the study.

Open field activity test

Open field activity is a useful and simple test first described by Hall[14] that assesses basic activity and general behaviour/anxiety. Locomotion was recorded and analysed by computerized video-tracking system software (XT 7.1, Noldus, Wageningen, The Netherlands) software. Animals were habituated to the testing room, then spontaneous locomotion was assessed in a white Plexiglas open field box (41 × 41 × 34 cm) for 10 min. Recorded parameters were velocity (cm sec^{-1}), time spent in the centre of the arena (represented by 50% of the total arena; sec), and zone transitions.

Wire-hanging test

The wire-hanging test measures the ability of mice to hang on a metal wire.[15] The metal wire is elevated 40 cm above a soft surface to prevent physical trauma to the mice. Latency to fall was timed and the test was repeated three times with an intertrial interval of 30 s.

Balance beam test

In the beam-walking test, mice traverse an elevated, narrow beam to reach a platform.[16] The protocol measures foot slips while crossing the beam.

Continuous alternating T-maze (T-CAT) test

The continuous alternating T-maze test was used to assess cognitive ability; this enclosed apparatus is in the form of a T placed horizontally. Mice are started from the base of the T and allowed to choose one of the goal arms abutting the other end of the stem. Two trials are given in quick succession; on the second trial the mouse tends to choose the arm not visited before, reflecting memory of the first choice, termed 'spontaneous alternation'. We assessed this tendency in a test with 14 possible alternations as previously described .[12 17 18]

Barnes maze test

The Barnes maze (BM)[19] was designed for testing spatial learning and memory.[20] The BM is preferred over the Morris water maze to assess spatial memory in mice, taking advantage of their superior abilities to find and escape through small holes while minimizing the motor-dependent component in the task.

With the BM, animals receive reinforcement to escape from an open platform surface through a tunnel into a small, dark, recessed chamber ('target box') located under the platform. We adopted our test protocol according to a previously published model.[21] Mice were trained for four trials per day for 4 days. Trials were separated by 15–30 min. After each trial, the entire maze was cleaned with 70% ethanol and the maze was rotated to eliminate the use of intramaze cues. Trials were recorded using a computerized tracking/image analyser system (Ethovision XT 7.1, Noldus). Errors were defined as nose pokes and head deflections over any hole that did not have the tunnel. One day after the acquisition phase, mice received a probe trial for 90 s to check short-retention memory. During probe trial the tunnel leading to the target box was closed.

Stereology

Stereological assessment of hippocampal and prefrontal three-dimensional cortical volume was performed according to the Cavalieri principle.[22] Briefly, bilateral volume measurements were estimated from two pairs of prefrontal cortical sections (3.24 and 2.76 mm from the bregma) and 4 pairs of hippocampal sections (−1.3, −2.0, −2.3, −3.5 mm from the bregma) on the basis of surface area measurements made from two-dimensional coronal brain sections. Measurements were obtained with a Zeiss Axio Imager microscope equipped with Stereo Investigator software (MicroBrightField, Colchester, VT, USA), a three-axis Mac 5000 motorized stage (Ludl Electronics Products, Hawthorne, NY, USA), a Zeiss MRc digital video camera, peripheral component interface (PCI) colour frame grabber, and computer workstation.

Immunoblotting

Hippocampal tissue (50–100 mg) was homogenized using a carbonate lysis buffer (500 mM sodium carbonate, pH 11.0) containing protease and phosphatase inhibitors.[23] Lysates were sonicated on ice three times for 15 s each. Protein was quantified and normalized to the Bradford assay. Samples were run on 4–12% Bis-Tris gels (Life Technologies). After transfer to polyvinylidene fluoride membranes, samples were incubated overnight with primary antibodies for glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Cell Signaling, #2118S, 1:1000), PSD-95 (Cell Signaling, #2507S, 1:1000), and synaptobrevin (Abcam, #ab18013, 1:1000) and conjugated to species-specific secondary antibodies and horseradish peroxidase. Densitometric analysis (arbitrary units) was conducted as previously described.[23]

Statistical analysis

Statistical analysis was performed using Prism 6 (GraphPad Software, La Jolla, CA, USA) and SPSS Statistics version 22 (IBM, Armonk, NY, USA). Column statistics were used to assess the distribution of the data, and outliers were identified with Rout's method (Q = 1%). Data were then analysed with either a two-way analysis of variance (ANOVA) or repeated measure two-way ANOVA and Tukey's test for *post hoc* comparisons. A power analysis for the behavioural experiments was performed with G*Power software (Heinrich Heine University, Dusseldorf, Germany)[24] for a two-way ANOVA with an effect size $f = 0.4$, $\alpha = 0.05$, $1 - \beta = 0.8$, and $dfn = 1$ for four groups. This calculation resulted in a suggested minimal total sample size of 52 mice (13 mice per group). Data are presented as mean (SD). Significance was assumed when $\alpha < 0.05$ and $1 - \beta = 0.8$.

Results

Open field activity

Two-way ANOVA was conducted to compare the effects of p75NTR inhibition (TAT-Pep5 *vs* TAT-ctrl) treatment with anaesthetic exposure (isoflurane *vs* ctrl) (all groups $n = 16$–27). No difference between treatments was observed in the open field paradigm. Specifically, no difference was found for p75NTR inhibitor (p75NTR-inh), isoflurane, or p75NTR-inh × isoflurane interaction for velocity (cm s^{-1}) (Fig. 1b) [TAT-Pep5: $F(1,87) = 0.77$, $P = 0.63$; isoflurane: $F(1,87) = 3.37$, $P = 0.07$; interaction: $F(1,87) = 0.23$, $P = 0.63$], time spent in the centre of the arena (s) (Fig. 1c) [TAT-Pep5: $F(1,87) = 2.55$, $P = 0.11$; isoflurane: $F(1,87) = 0.43$, $P = 0.51$; interaction: $F(1,87) = 0.01$, $P = 0.92$], and zone transitions (Fig. 1d) between the periphery and centre [TAT-Pep5: $F(1,87) = 0.024$, $P = 0.878$; isoflurane: $F(1,87) = 0.05$, $P = 0.83$; interaction: $F(1,87) = 0.04$, $P = 0.84$].

Continuous alternating T-maze

Two-way ANOVA was conducted to compare the effects of p75NTR-inh (TAT-Pep5 *vs* TAT-ctrl) treatment with anaesthetic exposure (isoflurane *vs* ctrl) (all groups $n = 16$–27). No difference between treatments was observed in the T-CAT for p75NTR-inh, isoflurane, or p75NTR-inh × isoflurane interaction (Fig. 1e)

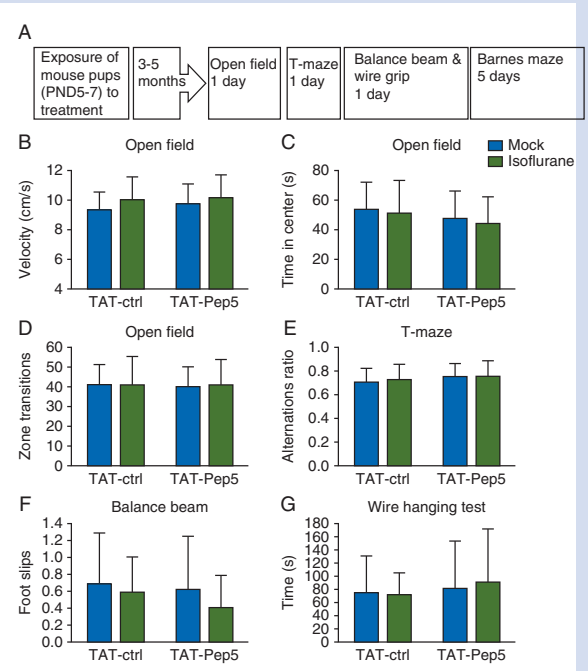

Fig 1 No baseline motor deficit or potential lack of movement and spatial exploratory performance measured by the balance beam, wire-hanging test, open field, and continuous alternating T-maze paradigm were observed. (A) Timeline of experiments, (B–D) no difference between treatments was observed in the open field paradigm [(B) time (s) spent in the centre of the arena; (C) time (s) spent in the centre of the arena; (D) zone transitions between centre and periphery], (E) continuous alternating T-maze (T-CAT) (alternation:no alternation ratio; >0.6 is considered as no hippocampal deficit, <0.6 is considered as a chance level or hippocampal deficit), (F) balance beam (number of foot slips), and (G) wire-hanging test (s). Data presented as mean (SD); $n = 16$–27 per group; *$P < 0.05$.

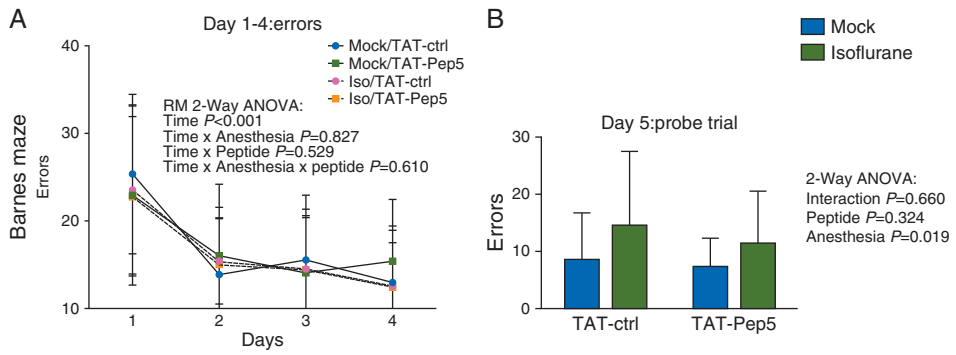

Fig 2 Decrement in contextual fear learning and spatial learning measured by the Barnes maze. This deficit could not be ameliorated with the administration of TAT-Pep5. Hippocampal-dependent memory was assessed by the Barnes maze. (A) Day 1–4, errors over time; (B) day 5, errors during the probe trial. Data are presented as mean (SD); $n = 15–25$ per group; $^*P < 0.05$.

[TAT-Pep5: F (1,69) = 1.59, $P = 0.22$; isoflurane: F (1,69) = 0.29, $P = 0.59$; interaction: F (1,69) = 0.08, $P = 0.77$].

Balance beam and wire-hanging test

Two-way ANOVA was conducted to compare the effects of p75NTR-inh (TAT-Pep5 *vs* TAT-ctrl) treatment with anaesthetic exposure (isoflurane *vs* ctrl) (all groups $n = 16–27$). No difference between treatments was observed in the balance beam or wire-hanging test. Specifically, no difference was found for p75NTR-inh, isoflurane, or p75NTR-inh × isoflurane interaction for foot slips in the balance beam (Fig. 1f) [TAT-Pep5: F (1,87) = 1.35, $P = 0.25$; isoflurane: F (1,87) = 1.98, $P = 0.16$; interaction: F (1,87) = 0.29, $P = 0.59$] or time hanging (s) in the wire-hanging test (Fig. 1g) [TAT-Pep5: F (1,76) = 0.79, $P = 0.38$; isoflurane: F (1,76) = 0.08, $P = 0.79$; interaction: F (1,76) = 0.22, $P = 0.64$].

Barnes maze

Hippocampal-dependent memory was assessed with the Barnes maze. Repeated measure two-way ANOVA was conducted to compare the effects of p75NTR-inh (TAT-Pep5 *vs* TAT-ctrl) treatment with isoflurane exposure (isoflurane *vs* ctrl) over time during the learning phase of the experiment on day 1–4 (Fig. 2a all groups $n = 15–25$). Mauchly's test indicated that the assumption of sphericity had been violated ($\chi^2 = 23.028$, $P < 0.001$), therefore degrees of freedom were corrected using Huynh–Feldt estimates of sphericity ($\epsilon = 0.91$). We found a significant effect of time, with no different interactions of time × isoflurane, time × TAT-Pep5, or time × isoflurane × TAT-Pep5 [time: F (2.73,202.03) = 37.69, $P < 0.001$; time × TAT-Pep5: F (2.73,202.03) = 0.719, $P = 0.53$; time × isoflurane: F (2.73,202.03) = 0.27, $P = 0.83$; time × isoflurane × TAT-Pep5: F (2.73,202.03) = 0.58, $P = 0.61$]. The probe trial on day 5 revealed a significant effect of isoflurane (Fig. 2b), with no significant effect of TAT-Pep5 alone or TAT-Pep5 plus isoflurane [TAT-Pep5: F (1,72) = 0.99, $P = 0.32$; isoflurane: F (1,72) = 5.80, $P = 0.02$; interaction: F (1,72) = 0.2, $P = 0.66$]. Tukey's *post hoc* test did not reveal further significant comparisons.

Stereology

Two-way ANOVA was conducted to compare the effects of p75NTR-inh (TAT-Pep5 *vs* TAT-ctrl) treatment with isoflurane exposure (isoflurane *vs* ctrl) (all groups $n = 3–4$). No difference between treatments was observed with TAT-Pep5, isoflurane, or TAT-Pep5 plus isoflurane after measuring volumes of the prefrontal cortex (Fig. 3a) [TAT-Pep5: F (1,11) = 0.42, $P = 0.53$; isoflurane: F (1,11) = 0.50, $P = 0.50$; interaction: F (1,11) = 0.575, $P = 0.464$] and the hippocampus (Fig. 3b) [TAT-Pep5: F (1,11) = 0.070, $P = 0.796$; isoflurane: F (1.11) = 0.220, $P = 0.648$; interaction: F (1.11) = 0.105, $P = 752$].

Immunoblotting

Two-way ANOVA was conducted to compare the effects of p75NTR-inh (TAT-Pep5 *vs* TAT-ctrl) treatment with isoflurane exposure (isoflurane *vs* ctrl) (all groups $n = 4–5$). No difference between treatments was observed with TAT-Pep5, isoflurane, or TAT-Pep5-inh plus isoflurane on protein expression of PSD-95 (Fig. 3c) [TAT-Pep5: F (1,15) = 1.042, $P = 0.323$; isoflurane: F (1,15) = 0.060, $P = 0.810$; interaction: F (1,15) = 3.964, $P = 0.065$] or synaptobrevin (Fig. 3d) [TAT-Pep5: F (1,15) < 0.001, $P = 0.987$; isoflurane: F (1,15) = 0.427, $P = 0.523$; interaction: F (1,15) = 1.150, $P = 0.300$]. Protein expression was normalized to GAPDH.

Discussion

Anaesthetics have been shown to induce neurotoxicity, especially when administered during neurodevelopment.[25–28] Even though many mechanisms have been proposed, it is still not completely understood how anaesthetic toxicity is mediated.[29] Our group has shown that administration of the p75NTR-inh TAT-Pep-5 mitigated acute isoflurane- and propofol-mediated neurotoxicity.[4,5] We conducted the present study to investigate the long-term cognitive effects of isoflurane and whether they can be reversed by pretreatment with TAT-Pep5. We show that male PND 5–7 mouse pups, when exposed to isoflurane, exhibit memory deficits in adulthood, an effect that was not mitigated by TAT-Pep5 pretreatment.

Previous work from others has suggested that TAT-Pep5 could be used as a promising agent for preventing neuroregeneration.[30] Parallel administration of a p75NTR-inh reduced injury in an *in vivo* model of ischaemic injury (oxygen glucose deprivation) and in an *in vivo* ischaemia–reperfusion model.[31] Regarding the pharmacokinetics of TAT-Pep5, as a proof of concept, Schwarze and colleagues[32] reported a TAT-FITC signal in

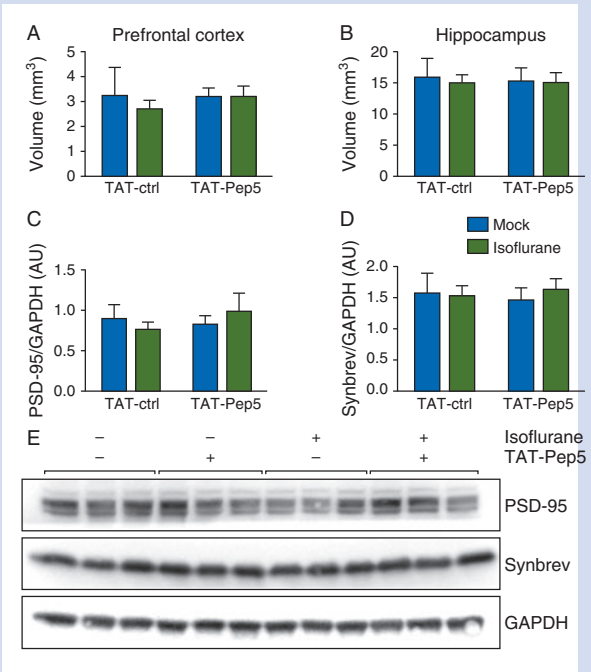

Fig 3 No gross differences in brain region volumes and synaptic marker expression were found. No difference between treatments was observed in stereological assessment of the (A) prefrontal cortex and (B) hippocampus, and immunoblotting of the synaptic markers (C) PSD-95 and (D) synaptobrevin. (E). Immunoblot image for PSD-95, Synbrev, and GAPDH. Data are presented as mean (SD); $n = 3$–5 per group; $^*P < 0.05$.

the brain after 20 min. Furthermore, they reported enzyme activity of TAT-β-Gal in the brain after 8 h. Moreover, TAT-conjugated Pep5 inhibited p75NTR interaction with Rho-GDI.[33] We therefore chose to expose mice to isoflurane 30 min after injection, at a time when the TAT-fused peptide has reached the brain.

Abnormalities in motor function and an increase in anxiety can potentially confound tests of cognition; that no differences among the groups were detected indicates that the differences in cognitive function detected were not due to abnormalities in motor function or to excessive anxiety. Our results show an internally consistent decrease in memory due to isoflurane as assessed by the Barnes maze, which detects hippocampal-dependent memory. In contrast, we found no reversal effect with TAT-Pep5 against anaesthetic-mediated cognitive deficits. No stereological alterations (using the neuron-specific Nissl stain) were detected for isoflurane or TAT-Pep5, suggesting that the cognitive deficits were not due to persistent neuronal loss. We investigated the presynaptic vesicle marker synaptobrevin and the postsynaptic marker PSD-95, which is important for synaptic plasticity during long-term potentiation, as markers of synaptic integrity. Although no biochemical changes were detected, whether the behavioural changes can be attributed to ultrastructural abnormalities (e.g., dendritic arborization, dendritic spine numbers or population, postsynaptic morphology, mitochondrial dysfunction, deficits in mitochondrial transport) independent of cell number needs further investigation.

Our data are consistent with those recently reported by Lee and colleagues,[35] which showed that exposure of rodents to isoflurane at PND 7 led to neuronal apoptosis in both male and female rats. The extent of apoptosis was similar between the two sexes. However, in tests of object recognition and social memory, deficits were observed only in males. Moreover, Stratmann and colleagues[34] have shown that although hypercarbia *per se* leads to significant apoptosis (equivalent to that achieved with isoflurane exposure), it does not cause cognitive deficit. These data indicate that apoptosis *per se* might not be the only mechanism by which cognitive dysfunction in adulthood is produced by neonatal anaesthetic exposure.[35] A current study by Loepke and colleagues[9] found no cognitive deficit after exposing PND 7 mice to 1.5% isoflurane for 6 h. Mortality in that study was 18%, while ours was 7% for all isoflurane-exposed mice. A reason for the differences between these studies could be the mouse strain used [C57Bl/6J female and CD-1 male *vs* C57Bl/6J (female and male)], the behavioural test (Morris water maze *vs* Barnes maze),[19] length and concentration of isoflurane, or the experimental design (individual groups and one-way ANOVA *vs* two-factor design and two-way ANOVA).

Our study was restricted to evaluation of male mice only, and we are therefore unable to determine the applicability of our findings to female mice. Two other investigations in rats have indicated that there is a sex difference in vulnerability to cognitive deficits following neonatal anaesthetic exposure. Sevoflurane exposure in adult rats led to worse cognitive dysfunction in males than females.[36] In another study in rats, repeated propofol exposure led to sex-specific differences in spatial (female) and recognition (male) memory.[37] Future studies should therefore consider sex-specific differences in anaesthetic neurotoxicity.

Previous work from our group has demonstrated that isoflurane produces structural changes in dendritic morphology (i.e. loss of filopodial spines),[4–6] including an acute reduction of ~50% of synapses in the stratum lacunosum of the hippocampus. Propofol causes an acute reduction in synapse number by ~50% in the prefrontal cortex, with a persistent reduction in synapse number by ~10% 90 days after exposure.[38] Preliminary data from our laboratory indicate that propofol exposure at PND 7 dramatically alters the development of mossy fibres (from dentate granule cells to the CA3 sector of the hippocampus). These data are consistent with the notion that anaesthetic-induced structural changes to neuronal networks during brain development contribute to cognitive changes in adult animals.

In addition to modulation of neuronal networks, factors other than apoptosis might contribute to long-term cognitive deficits caused by anaesthetics.[34–40] Alternative mechanisms such as $GABA_A$ receptor activation,[41] mitochondrial metabolism and function,[42] prolonged subcellular cytoskeletal changes,[43] and changes in spine density[44, 45] might contribute to the neurotoxic effects of anaesthetics in the developing brain. For example, mitochondrial dysfunction following exposure to volatile anaesthetics has been proposed and is currently under investigation.[46] In a model of general anaesthesia in neonatal rats, R(+)-pramipexole restored mitochondrial integrity and mitigated cognitive dysfunction.[42] Epigenetic influences such as environmental enrichment might also play a role, as Shih and colleagues[47] have shown that environmental enrichment ameliorates the neurotoxic effects of sevoflurane.

Conclusion

A single long-term exposure to isoflurane significantly impaired spatial learning measured by the Barnes maze, indicative of hippocampal-dependent deficits. These deficits were not ameliorated by administration of TAT-Pep5, an inhibitor of p75NTR, shown

previously to abolish isoflurane-induced neuronal apoptosis. There was no baseline motor deficit or potential lack of movement due to anxiety measured by the balance beam, wire-hanging test, and open field paradigm. These results control for possible confounders and therefore strengthen our findings in the cognitive assessments. No gross differences in brain region volumes and biochemistry were found. These findings extend previous observations from our group that neonatal anaesthetic-mediated neurotoxicity contributes to cognitive alterations in the adult. Importantly, factors other than apoptosis play a significant role in cognitive dysfunction. We conclude that the prevention of apoptosis by a p75NTR-inh does not prevent cognitive dysfunction.

Authors' contributions

J.M.S., V.B.R., H.H.P., P.M.P., and B.P.H. co-designed and conceptualized the experiments. J.M.S., M.L.P., V.B.R., H.H.P., P.M.P., and B.P.H. interpreted the data. J.M.S., A.K., C.M., A.V., G.C.G., and I.R.N. performed the experiments. J.M.S, H.H.P., P.M.P, and B.P.H. drafted the manuscript. All authors were involved in the editing and revision process. All authors gave their final approval and agreed to be accountable for all aspects of the work.

Declaration of interest

None declared

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