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Mitochondrial protectant pramipexole prevents sex-specific long-term cognitive impairment from early anaesthesia exposure in rats

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Editor's key points

- Neonatal exposure to a combination of general anaesthetics results in developmental neurotoxicity and cognitive impairment in adult rats.
- The cognitive impairment could be prevented by co-treatment with the mitochondrial targeted anti-oxidant pramipexole.
- Female rats were more vulnerable to anaesthesia-induced cognitive impairment, and thus might benefit more by such protective therapy.

Background. Exposure to general anaesthesia during critical stages of brain development results in long-lasting cognitive impairment. Co-administration of protective agents could minimize the detrimental effects of anaesthesia. Co-administration of *R*(+)pramipexole (PPX), a synthetic aminobenzothiazol derivative that restores mitochondrial integrity, prevents anaesthesia-induced mitochondrial and neuronal damage and prevents early development of cognitive impairment. Here, we determine the protective effects of PPX into late adulthood in male and female rats.

Methods. Postnatal day 7 rats of both sexes were exposed to mock anaesthesia or combined midazolam, nitrous oxide, and isoflurane anaesthesia for 6 h with or without PPX. Cognitive abilities were assessed between 5 and 7 months of age using Morris water maze spatial navigation tasks.

Results. Examination of spatial reference memory revealed that female, but not male, neonatal rats exposed to anaesthesia showed slowing of acquisition rates, which was significantly improved with PPX treatment. Examination of memory retention revealed that both male and female anaesthesia-treated rats have impaired memory retention performance compared with sham controls. Co-treatment with PPX resulted in improvement in memory retention in both sexes.

Conclusion. PPX provides long-lasting protection against cognitive impairment known to occur when very young animals are exposed to anaesthesia during the peak of brain development. Anaesthesia-induced cognitive impairment appears to be sex-specific with females being more vulnerable than males, suggesting that they could benefit more from early prevention.

Keywords: anaesthetics volatile, isoflurane; anaesthetics gases, nitrous oxide; memory; neonates; oxygen, toxicity; recovery, cognitive

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Both animal and human data suggest that exposure to general anaesthesia (GA) during critical stages of brain development results in long-lasting cognitive impairment.[1–7] This problem begs the question of how GA can be used safely in the very young. One possibility would be co-administration of protective agents to minimize the detrimental effects of GA.

In an effort to address this possibility, we previously examined the effects of early exposure to anaesthesia on mitochondria as their proper functioning and morphogenesis are crucial for timely neuronal development. We found that mitochondria are very vulnerable to GA-induced toxicity and are one of the first intracellular organelles targeted by anaesthetics.[7] Injured mitochondria cause substantial up-regulation of free oxygen radicals, which, in turn, lead to lipid peroxidation in cellular membranes and neuronal loss in vulnerable brain regions.[7] As neurones have high oxygen requirements and relative deficiency in oxidative defences, they are highly sensitive to excessive free oxygen radical production, suggesting that GA-induced mitochondrial damage and ensuing oxidative stress could be one of the key mechanisms leading to neuronal damage during early stages of brain development.

Motivated by this finding we examined the free oxygen scavenger, *R*(+)pramipexole (PPX), a synthetic aminobenzothiazol

derivative that restores mitochondrial integrity,[8] and found that when administered at the time of GA exposure, PPX prevents GA-induced mitochondrial and neuronal damage and prevents the development of cognitive impairment in young adult rats.[7]

To assess whether the protective effect of PPX is long-lasting and sex-specific we followed animals into late adulthood (5–7 months of age) and compared benefits of PPX on cognitive performance of male and female rats exposed to GA at the peak of brain development (7 days of age).

Methods

We exposed postnatal day 7 (P7) Sprague-Dawley rats of both sexes to one of four treatment protocols: (i) sham controls (mock GA-vehicle, 0.1% dimethyl sulfoxide+21% oxygen for 6 h); (ii) GA-treated (midazolam, 9 mg kg^{-1}, i.p.; single injection immediately before administration of 0.75% isoflurane+75% nitrous oxide+24% oxygen for 6 h); (iii) GA+PPX-treated (PPX, 1 mg kg^{-1} i.p.; four doses—at 9 h before, immediately before, immediately after, and 9 h after 6 h of GA); and (iv) PPX alone (same dosing regimen but mock GA). At the end of the treatments, rat pups were reunited with their mothers. Rats were housed using standard housing on a 12 h light/dark cycle with *ad libitum* access to food and water. All experiments were approved by the Animal Care and Use Committee of the University of Virginia Health System and were done in accordance with the Public Health Service's Policy on Human Care and Use of Laboratory Animals. All efforts were made to minimize the number of animals used.

R(+)PPX doses and the dosing regimen were selected based on results from our prior work[7] and the half-life of *R*(+)PPX which is estimated to be 8–12 h[9] [i.e. *R*(+)PPX was dosed ~every half-life around the time of anaesthesia exposure]. An adult rodent dose of 1 mg kg^{-1} is equivalent to a human dose of 0.2 mg kg^{-1}.[9 10] Thus, these doses are very small and clinically feasible. Based on high-pressure liquid chromatography assays, *R*(+)PPX was >99.9% chemically and >99% enantiomerically pure.[11]

We found no differences among the groups in general appearance or body weight over the next 7 months (data not shown). Cognitive abilities were assessed between 5 and 7 months of age using the Morris water maze test with the adult size pool (180 cm inner diameter).[1] To examine their ability to swim, animals were tested in cued trials using a visible platform that was switched to a new location for each trial. During the place trials, rats were tested on their ability to learn the location of a platform (submerged, not visible), which remained in the same location during all trials. Two acquisition place trials were performed, each four blocks long (2 days per block). Probe trials were performed after each acquisition place trial (after blocks four and nine). During these trials, the platform was removed and times and patterns of swimming were analysed with special attention focused on time spent in the target quadrant.

Data were analysed by analysis of variance using treatment and sex as between-subject variables and blocks of trials as within-subject variables. Pairwise comparisons were done after analysis of significant treatment effects and *P*-values exceeding Bonferroni corrected levels were noted. We considered $P<0.05$ to be statistically significant.

Results

To assess whether GA-induced impairment in performance could be attributable to confounding visual or motor impairments, we performed a visual platform trial. The data shown in Figure 1 confirm that there were no differences in the time it took male or female rats from each group to reach a visible platform raised 1 cm above the water level ($n=4$ trials).

The examination of spatial reference memory capabilities in the same animals during place trials (submerged platform, fixed location different from that used in the visual platform trials), revealed that female, but not male, rats exposed to

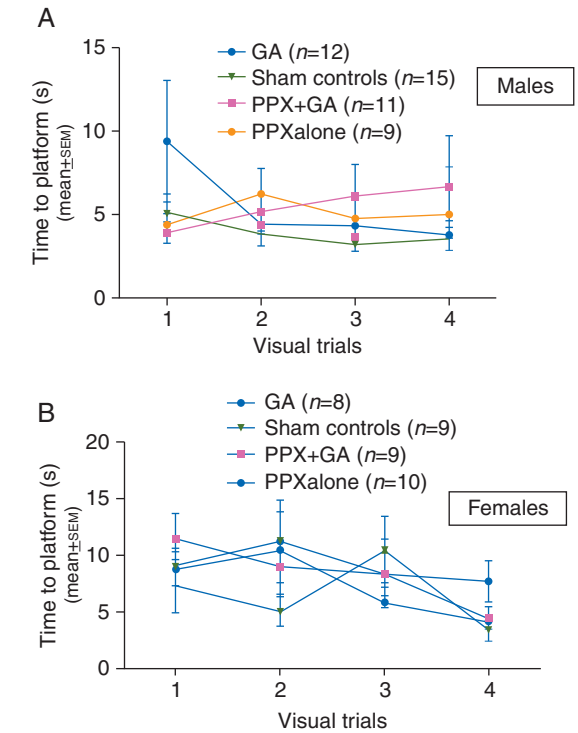

Fig 1 Neonatal anaesthesia exposure has no effect on visual and motor performance in Morris water maze. (A) There was no difference in the time it took male rats from each group (sham controls, anaesthesia—GA, GA+PPX, and PPX alone) to reach a visible platform raised above the water level (visual platform trial) in any of the four trials. (B) There was no difference in the time it took female rats from each group (sham controls, anaesthesia—GA, GA+PPX, and PPX alone) to reach a visible platform raised above the water level in any of the four trials. The number of animals in each experimental group and for each sex is indicated in the graphs.

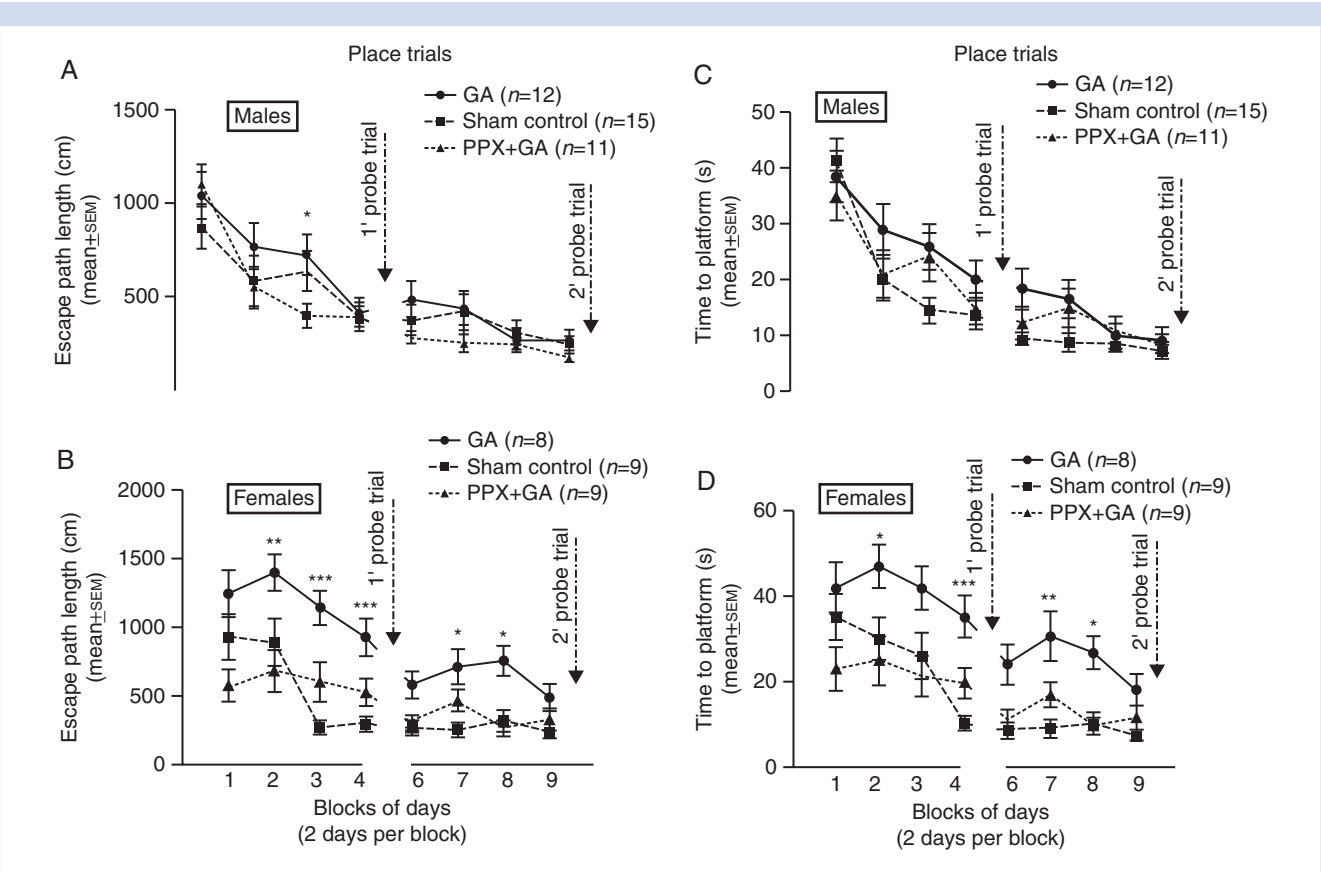

Fig 2 Neonatal anaesthesia exposure has a long-lasting effect on spatial reference learning that is sex-specific and preventable by timely treatment with PPX. Male (A and C) and female (B and D) rats were tested at 5–7 months of age for their ability to learn the location of a submerged (not visible) platform during place trials. We used escape path length (A and B) and time to escape to the platform (C and D) as two main measures of spatial reference learning. Male rats exposed to GA showed no signs of impaired acquisition rates in terms of the escape path length (except during the third block of trials, $*P<0.05$) (A) or the time to escape to the platform (C). Spatial learning of PPX+GA male rats was indistinguishable from controls. Female rats exposed to GA showed significant slowing of acquisition rates in terms of the escape path length (B) and the time to the platform (D) ($*P<0.05$; $**P<0.01$; and $***P<0.001$). It took GA-treated animals until the end of training (ninth block of trials) to perform similarly to controls. Females treated with PPX+GA performed much like sham controls throughout the testing period ($P>0.05$) (B and D). The timing of the probe trials after the last place trials is indicated in blocks 5 and 10. (The number of animals in each experimental group and for each sex is indicated in the graphs.) PPX only animals behaved much like controls ($n=9$ males and 10 females; data not shown).

GA showed significant slowing of acquisition rates in terms of the escape path length (Fig. 2A and B) and the time to the platform (Fig. 2C and D); it was not until the very end of training (Day 9) that GA-treated females (B and D) improved and performed similarly to controls. PPX treatment offered significant protection of cognitive function; females treated with PPX+GA performed much like sham controls throughout the testing period ($P>0.05$) (B and D). As GA-treated males did not show significantly impaired performance (A and C) (except on escape path length during the third block of trials), the protective effect of PPX on place trials in males was less impressive. PPX only animals behaved much like controls ($n=9$ males and 10 females; data not shown).

To assess memory retention, we used probe trials wherein the submerged platform was removed from the pool (after the last place trials—after blocks four and nine as indicated in Fig. 2). Hence, the animals were forced to rely on learned visual cues to find the quadrant where the platform had been (target quadrant). Both male and female GA-treated rats demonstrated impaired memory retention performance compared with sham controls (Fig. 3). Control male (A) and female rats (B) spent significantly more time during the second probe trial in the target quadrant ($P<0.01$) and significantly more time in the target quadrant compared with other three quadrants ($P<0.001$), which suggests a significant level of spatial memory retention. GA-treated male (C) and female (D) animals had no preference for any quadrant in the first or second probe trials despite previous training. Their behaviour could be described best as aimless swimming from one quadrant to another, suggesting a complete lack of retention and learning. In male (E) and female (F) animals exposed to GA+PPX

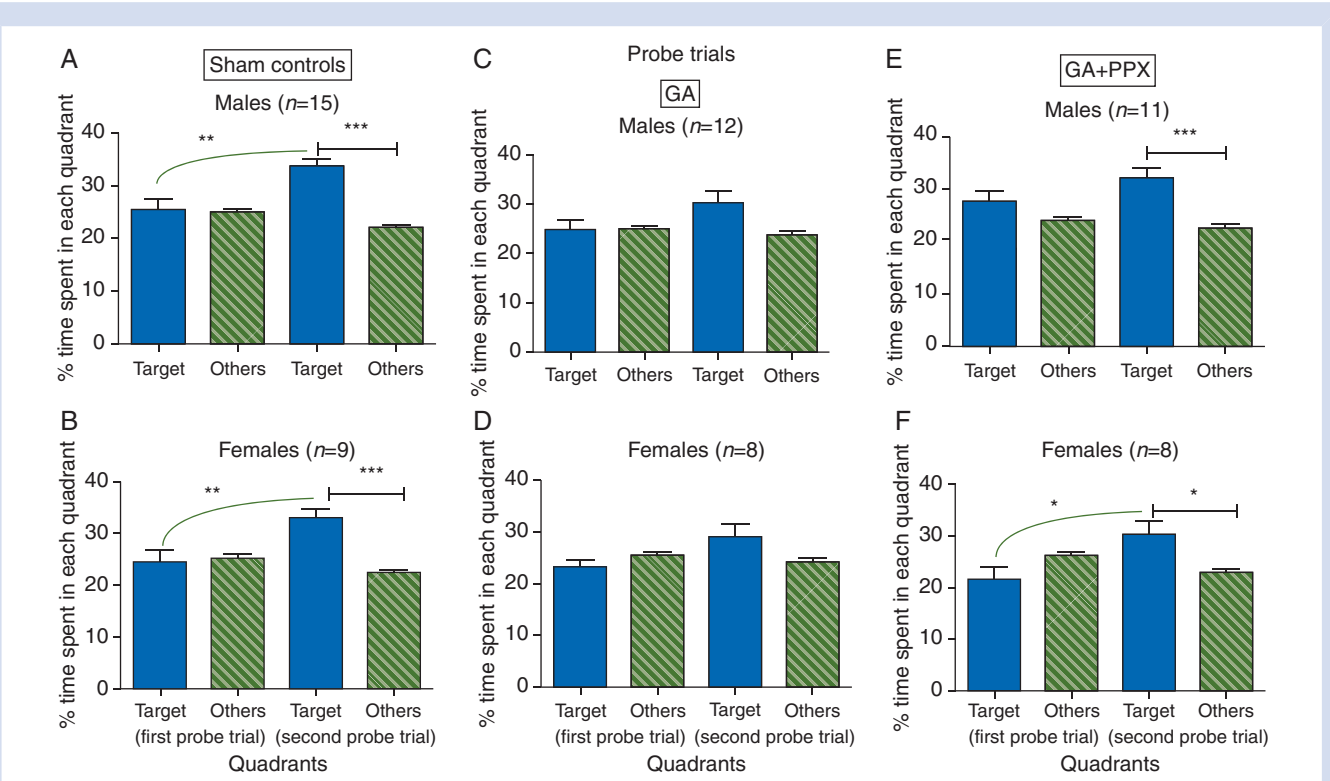

Fig 3 Neonatal anaesthesia exposure has long-lasting effect on retention in both sexes and was preventable by timely treatment with PPX. Retention was examined using probe trials where animals were forced to rely on learned visual cues to find the quadrant where the platform had been (target quadrant). Control male (A) and female rats (B) spent significantly more time during the second probe trial in the target quadrant (**$P<0.01$) and significantly more time in the target quadrant compared with the other three quadrants (***$P<0.001$). GA-treated male (C) and female (D) animals had no preference for any of the quadrants in the first or second probe trials despite previous training. GA+PPX-treated male (E) and female (F) animals spent significantly more time in the target quadrant during the second probe trial (females, *$P<0.05$) and significantly more time in the target quadrant compared with the other three quadrants [(E) males, ***$P<0.001$; (F) females, *$P<0.05$]. This behaviour was very similar to that of sham controls. The number of animals in each experimental group and for each gender is indicated in the graphs. PPX only animals behaved much like controls ($n=9$ males and 10 females; data not shown).

treatment, there was an improvement in the memory retention shown as significantly more time spent in the target quadrant during the second probe trial (females, $P<0.05$) and significantly more time in the target quadrant compared with the other three quadrants (males, $P<0.001$; females, $P<0.05$), thus displaying learning behaviour that resembled sham control animals. PPX only animals behaved much like controls ($n=9$ males and 10 females; data not shown).

Swimming speeds also were analysed during cued and place trials. No differences were observed (data not shown), further arguing that swimming performance deficits were not responsible for place-learning impairment in GA-treated rats.

Discussion

Cognitive impairment is known to occur when very young animals are exposed to anaesthesia during the peak of brain development.[1–7] Our previous work showed that GA impairs mitochondrial integrity and function resulting in increased oxygen free radicals, lipid peroxidation, and neuronal deletion.[7 12] PPX was tested as a potential protective agent because it scavenges free oxygen radicals inside mitochondria and protects mitochondrial integrity.[13 14] PPX readily crosses the blood–brain barrier and is concentrated in the brain where it is taken up and most highly concentrated in mitochondria.[15] Previously, we showed that PPX decreases production of free oxygen radicals and protects neuronal survival while protecting against GA-induced cognitive impairment in rats when assessed in early adolescence.[7] Here, we show that this protection is long lasting because it can be detected in late adulthood as well.

Retention memory was impaired by GA administration at P7 (the peak of synaptogenesis) in both sexes, but administration of PPX around the time of anaesthesia exposure completely prevented the development of this deficit, as demonstrated by normal cognitive performance in adulthood (5–7 months of age). GA exposure at P7 caused more detrimental long-term effects on spatial learning in female than in male rats compared with sex-specific controls; this too was prevented by PPX.

Available evidence regarding sex differences in Morris water maze performance is conflicting. Some studies have shown that males have an advantage in spatial learning,[16 17] whereas others suggest that this advantage disappears when females undergo additional training aimed at alleviating the stress response.[18 19] However, newer evidence shows no baseline sex difference in Morris water maze performance.[20] Similarly, we observed no difference in spatial learning between sham control males and females.

The effect of sex on GA-induced developmental neurotoxicity remains poorly understood. An earlier study by Rothstein and colleagues[21] suggests that although exposure to GA during very early stages of synaptogenesis (postnatal day 0) resulted in significant impairment in cognitive abilities in both sexes, male rats were more affected than females when assessed in very young adulthood (1.5 months of age). The reasons for sex differences on GA-induced cognitive impairments over the course of adolescence and adulthood are yet to be deciphered, although a direct correlation between a decrease in performance in the Morris water maze and a decrease in hippocampal volume has been reported.[21]

The fact that spatial reference memory performance can be confounded by stress[18 19] suggests the possibility that female-specific long-term impairment of spatial reference memory could be attributable to an anaesthesia-modified stress response that is female-specific. It is reasonable to propose that this could be caused by complex influences on hormonal cycling and mitochondrial development as a result of an early exposure to anaesthesia, although this notion needs to be examined critically.

We conclude that early exposure to GA causes long-term cognitive impairment that can be prevented by timely administration of PPX. As females appear to be more affected than males when studied in later adulthood, we suggest that they might benefit more from early prevention.

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Declaration of interest

J.B. holds multiple patents protecting the use of *R*(+)PPX and receives royalty payments from those patents. Patents protecting use of *R*(+)PPX have been licensed to Knopp Biosciences and Biogen-Idec. Neither company provided support for this study or was involved in data collection, data analysis nor manuscript preparation.

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Authors' role

The roles of each author in the study and preparation of the manuscript:

A.B.—data collection, data analysis, data interpretation, study design, drafting the article, providing intellectual content, final approval of the manuscript;

C.O.—providing intellectual content, final approval of the manuscript;

J.B.—providing PPX for the study, providing intellectual content, assistance with revisions, final approval of the manuscript;

B.W.—providing Morris water maze facility, study design, data analysis and interpretation, providing intellectual content, final approval of the manuscript;

V.J.-T.—conception and design of the study, data interpretation and analysis, drafting the article, revising it, providing intellectual content, final approval of the manuscript.

References

1 Jevtovic-Todorovic V, Hartman RE, Izumi Y, *et al.* Early exposure to common anaesthetic agents causes widespread neurodegeneration in the developing rat brain and persistent learning deficits. *J Neurosci* 2003; **23**: 876–82

2 Frederickson A, Archer T, Alm H, Gordh T, Eriksson P. Neurofunctional deficits and potentiated apoptosis by neonatal NMDA antagonist administration. *Behav Brain Res* 2004; **153**: 367–76

3 Frederickson A, Pontén E, Gordh T, Eriksson P. Neonatal exposure to a combination of N-methyl-D-aspartate and gamma-aminobutyric acid type A receptor *anaesthetic ag*ents potentiates apoptotic neurodegeneration and persistent behavioural deficits. *Anesthesiology* 2007; **107**: 427–36

4 Paule MG, Li M, Allen RR, *et al.* Ketamine anaesthesia during the first week of life can cause long-lasting cognitive deficits in rhesus monkeys. *Neurotoxicol Teratol* 2011; **33**: 220–30

5 Block RI, Thomas JJ, Bayman EO, Choi JY, Kimble KK, Todd MM. Are anaesthesia and surgery during infancy associated with altered academic performance during childhood? *Anesthesiology* 2012; **117**: 494–503

6 Wilder RT, Flick RP, Sprung J, *et al.* Early exposure to anaesthesia and learning disabilities in a population-based birth cohort. *Anesthesiology* 2009; **110**: 796–804

7 Boscolo A, Starr JA, Sanchez V, *et al.* The abolishment of anaesthesia-induced cognitive impairment by timely protection of mitochondria in the developing rat brain: the importance of free oxygen radicals and mitochondrial integrity. *Neurobiol Dis* 2012; **45**: 1031–41

8 Sayeed I, Parvez S, Winkler-Stuck K, *et al.* Patch clamp reveals powerful blockade of the mitochondrial permeability transition pore by the D2-receptor agonist pramipexole. *FASEB J* 2006; **20**: 556–8

9 Hubble JP. Pre-clinical studies of pramipexole: clinical relevance. *Eur J Neurol* 2000; **7** Suppl 1: 15–20

10 Zou L, Xu J, Jankovic J, *et al.* Pramipexole inhibits lipid peroxidation and reduces injury in the substantia nigra induced by the dopaminergic neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine in C57BL/6 mice. *Neurosci Lett* 2000; **281**: 167–70

11 Alavian KN, Dworetzky SI, Bonanni L, *et al.* Effects of dexpramipexole on brain mitochondrial conductances and cellular bioenergetic efficiency. *Brain Res* 2012; **1446**: 1–11

12 Sanchez V, Feinstein SD, Lunardi N, *et al.* General anaesthesia causes long-term impairment of mitochondrial morphogenesis and synaptic transmission in developing rat brain. *Anesthesiology* 2011; **115**: 992–1002

13 Cassarino DS, Fall CP, Smith TS, Bennett JP Jr. Pramipexole reduces reactive oxygen species production in vivo and in vitro and inhibits the mitochondrial permeability transition produced by the parkinsonian neurotoxin methylpyridinium ion. *J Neurochem* 1998; **71**: 295–301

14 Zou L, Xu J, Jankovic J, He Y, Appel SH, Le W. Pramipexole inhibits lipid peroxidation and reduces injury in the substantia nigra induced by the dopaminergic neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine in C57BL/6 mice. *Neurosci Lett* 2000; **281**: 167–70

15 Danzeisen R, Schwalenstoecker B, Gillardon F, *et al.* Targeted antioxidative and neuroprotective properties of the dopamine agonist pramipexole and its nondopaminergic enantiomer SND919CL2x [(+)2-amino-4,5,6,7-tetrahydro-6-propylamino-benzathiazole dihydrochloride]. *J Pharmacol Exp Ther* 2006; **316**: 189–99

16 Brandeis R, Brandys Y, Yehuda S. The use of the Morris water maze in the study of memory and learning. *Int J Neurosci* 1989; **48**: 29–69

17 Jonasson Z. Meta-analysis of sex differences in rodent models of learning and memory: a review of behavioural and biological data. *Neurosci Biobehav Rev* 2005; **28**: 811–25

18 Perrot-Sinal TS, Kostenuik MA, Ossenkopp KP, Kavaliers M. Sex differences in performance in the Morris water maze and the effects of initial nonstationary hidden platform training. *Behav Neurosci* 1996; **110**: 1309–20

19 Simpson J, Kelly JP. An investigation of whether there are sex differences in certain behavioural and neurochemical parameters in the rat. *Behav Brain Res* 2012; **229**: 289–300

20 Mazor A, Matar MA, Kaplan Z, Kozlovsky N, Zohar J, Cohen H. Gender-related qualitative differences in baseline and post-stress anxiety responses are not reflected in the incidence of criterion-based PTSD-like behaviour patterns. *World J Biol Psychiatry* 2009; **10**: 856–69

21 Rothstein S, Simkins T, Nuñez JL. Response to neonatal anaesthesia: effect of sex on anatomical and behavioural outcome. *Neuroscience* 2008; **152**: 959–69

Handling editor: H. C. Hemmings