Apopotic neurodegeneration and spatial memory are not affected by sedative and anaesthetics doses of ketamine/medetomidine combinations in adult mice

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Editor’s key points
- The combined effect of ketamine and medetomidine on cognition and apoptosis was studied in mice.
- No differences were seen between groups in terms of spatial memory or apoptosis.
- Hyperlocomotive activity in mice receiving ketamine was attenuated by medetomidine.
- Co-administration of ketamine and medetomidine may be clinically useful.

Background. Ketamine is increasingly popular in clinical practice and its combination with α2-agonists can provide good anaesthetic stability. Little is known about the effects of this combination in the brain. Therefore, we investigated the effects of different concentrations of ketamine combined with medetomidine on cognition and its potential apoptotic neurodegenerative effect in adult mice.

Methods. Seventy-eight C57BL/6 adult mice were divided into six different groups (saline solution, 1 mg kg⁻¹ medetomidine, 25 mg kg⁻¹ ketamine + 1 mg kg⁻¹ medetomidine, 75 mg kg⁻¹ ketamine + 1 mg kg⁻¹ medetomidine, 25 mg kg⁻¹ ketamine, and 75 mg kg⁻¹ ketamine). Eight animals per group were tested in the T-maze, vertical pole, and open-field test. Five animals per group were used for histopathological [haematoxylin and eosin (HE) staining] and immunohistochemical analyses [caspase-3 activation and expression of neurotrophin brain-derived neurotrophic factor (BDNF)]. Cells showing clear HE staining and positive immunoreactions for caspase-3 and BDNF in the retrosplenial cortex, visual cortex, pyramidal cell layer of the cornu Ammonis 1 and cornu Ammonis 3 areas of the hippocampus, and in the granular layer of the dentate gyrus were counted.

Results. There were no differences between groups regarding the number of dead cells and cells showing positive immunoreactions in the different areas of the brain studied. Similarly, no differences were detected in the number of trials to complete the T-maze task. Nevertheless, α2-agonist decreased hyperlocomotion caused by ketamine in the open field.

Conclusions. Neither apoptotic neurodegeneration nor alterations in spatial memory were observed with different concentrations of ketamine combined with medetomidine in adult mice.

Keywords: anaesthetics; apoptosis; cognition; ketamine; medetomidine

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Ketamine, a non-competitive glutamate N-methyl-D-aspartate acid receptor antagonist, is gaining popularity in adult human medicine mainly thanks to its analgesic properties1–3 and its importance for emergency procedures.4–5 However, ketamine impairs brain activity inducing post-anaesthetic delirium in humans6–7 and hyperlocomotion in rodents.8

Ketamine may be combined with α2-agonists such as dexmedetomidine9–10 in humans or medetomidine in animals.11–12 Levanen and colleagues13 showed in humans that the combination of ketamine with dexmedetomidine provides stable anaesthesia with a reduction in adverse effects caused by ketamine, such as post-anaesthetic delirium. However, in Europe, dexmedetomidine has not been approved for human clinical use and was only very recently introduced in veterinary medicine where medetomidine is used routinely.

Dexmedetomidine is the dextro enantiomer of medetomidine and has the pharmacological activity of medetomidine.14 Ketamine/medetomidine and ketamine/dexmedetomidine have similar effects in induction of anaesthesia, heart rate, respiratory rate, temperature, blood gas values, and recovery.15

The use of α2-agonists reduce anaesthetic requirements and improve perioperative haemodynamic stability.16–17 In addition, ketamine/(dex)medetomidine anaesthesia has the advantage of a rapid recovery with the administration of atipamezole, a specific α2-antagonist available for the use of animals, which immediately reverses (dex)medetomidine

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effects. However, there is a lack of information about potential secondary effects of this combination on the brain, such as effects on memory and neurodegeneration.

The purpose of this study was to assess the influence of two different concentrations of ketamine combined with $\alpha_2$-agonists on performance of a simple spatial cognitive task and the potential neurodegenerative effects in adult mice.

**Methods**

This study was reviewed and ethically approved by the Portuguese competent authority for animal protection, Direção Geral de Veterinária (Lisbon, Portugal).

**Animals**

Seventy-eight 28-week-old, male C57BL/6 mice bred in the animal facility of the institute (F1-F2 offspring of animals bought from Charles River, Barcelona, Spain) were used. The mice were housed with controlled temperature (21°C) and a relative humidity of 55%.

Animals received a commercial pellet diet (4RF25-GLP Mucedola, SRL, Settimo Milanese, Italy) and water ad libitum. A food restriction schedule 1 week before the T-maze habituation and during the task was applied to animals used in behavioural tests. A limited amount of food administered once daily was adjusted to a level that kept the mice at 85–95% of free-feeding weight.

**Anaesthesia**

Ketamine (Imalgène® Merial, Portugal; 100 mg ml$^{-1}$), medetomidine (Domitor® Phizer, Portugal; 1 mg ml$^{-1}$), or both were used for anaesthesia. Standard physiological saline 0.9% (Soro Fisiologico, Braun Vet, Portugal) was used in the control group and for diluting the drugs to ease handling of small volumes.

Mice were randomly assigned into six treatment groups, described in Table 1; all groups consisted of 13 animals (eight mice for behavioural tests and five for histology).

The mice were weighed using an electronic scale and volumes to be injected were calculated for each animal. Ketamine and medetomidine were mixed and administered as a single intraperitoneal (i.p.) injection. After administration, each animal was placed alone in a cage until it lost its righting reflex (RR), thereafter the animal moved to a homeothermic blanket connected to a rectal thermal probe (50-7061-F, Harvard Apparatus Ltd, Kent, UK) maintaining the temperature at 36–37.5°C throughout anaesthesia. One hundred per cent of oxygen was delivered to the animals and ophthalmic gel was placed in the eyes of the animals (LACRYVISC, Alcon, Paço D’Arcos, Portugal).

To avoid isolation stress, the animals that did not lose consciousness were returned to their home cage after the i.p. injection and heating was provided. Animals in which consciousness was achieved after the i.p. injection, the time to loss of RR, loss of response to tail pinch, and loss of pedal withdrawal reflex were recorded. In addition, the respiratory rate, depth of anaesthesia, pulse, and systolic pressure were recorded at 10 min intervals. Systolic pressure and heart rate values were obtained with pressure meter (LE 5001, Panlab, Spain). A pulse oximeter (S&W 9040, Athena, Germany), placed on the upper right hind leg of each mouse, was used for monitoring oxygen saturation ($S_aO_2$).

Animals were anaesthetized for 1 h and were observed throughout the period of anaesthesia. After 1 h, the $\alpha_2$-antagonist, atipamezole (Antisedan® Phizer, Portugal; 5 mg ml$^{-1}$) was administered (1 mg kg$^{-1}$ i.p.) to reverse anaesthesia induced by medetomidine. This drug was administered to the animals from the 25Ket./Med.; 75Ket./Med., and Med. groups.

**Behavioural tests**

The timing of behavioural tests in relation to anaesthesia is illustrated in Figure 1.

**T-maze**

The T-maze test was used to access spatial memory. The procedure used in this study was based on a protocol used in our laboratory with the exception that more time points were studied. The test ended when the learning criterion was achieved, that is, when the mouse entered the correct arm nine times out of 10 consecutive tests trials (excluding the three learning trials); hence, every mouse had to perform a minimum of 10 trials. The number of trials to complete the task was measured.

**Vertical pole test**

The vertical pole test was used to assess post-anaesthetic recovery. Deficits in motor coordination and balance were detected by the mouse falling off the pole. This test was performed as previously described by Bellum and colleagues with minor modifications.

Each mouse was placed in the centre of a round rough-surfaced pole (50 cm long, 2 cm diameter). The pole was initially positioned horizontally and then slowly inclined to 90° (1° s$^{-1}$); the animal faced the end that was lifted up. Performance was determined by the latency (s) for the mouse to turn downwards and completely descend the pole (s).

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<tbody>
<tr>
<td>Dose of drugs administered</td>
<td>Saline solution</td>
<td>1 mg kg$^{-1}$ medetomidine</td>
<td>25 mg kg$^{-1}$ ketamine</td>
<td>75 mg kg$^{-1}$ ketamine</td>
<td>25 mg kg$^{-1}$ ketamine + 1 mg kg$^{-1}$ medetomidine</td>
<td>75 mg kg$^{-1}$ ketamine + 1 mg kg$^{-1}$ medetomidine</td>
</tr>
</tbody>
</table>
Animals had a maximal time of 120 s to complete the test. This maximal time was recorded for animals that were not able to turn downwards and instead dropped from the pole.

**Open field**

The open-field test was used to measure the locomotor and exploratory activity as an indicator of any difference in anxiety.

The apparatus consisted of a circular arena (1 m of diameter) made of grey polypropylene and surrounded by a wall of 30 cm height. Each animal was released in the centre of the arena and allowed to explore it for 20 min.

The number of fecal boli was counted and the arena was cleaned with alcohol at 70% to avoid the presence of olfactory cues. The test was recorded with a camera placed above the apparatus and collected into a computer with a multi-camera vigilance system GeoVision (GV-800/8, Taipei, Taiwan).

The video analysis was carried out using the software VideoMot 2 (TSE-systems, Bad Homburg, Germany) which measured several parameters: distance walked, time spent, and the number of visits on each region. The latency to exit the centre, the total distance walked, and the total speed were also calculated. Three areas were defined in the open-field arena: periphery, middle, and centre.

**Histopathological (haematoxylin–eosin staining) and immunohistochemical (caspase-3 activation and neurotrophin brain-derived neurotrophic factor) studies**

Three hours after anaesthesia, the mice were killed by decapitation and the brain was removed. Brains were fixed for 48 h in 4% buffered paraformaldehyde (PBS, pH 7.4, 0.1 M), processed, and embedded in paraplast (SHANDON, Hypercentre XP and Histocentre 2, Burlingame, CA, USA). For each block, serial sections of 4 μm thick were made making coronal cuts from Bregma –2.06 mm until Bregma –2.70 mm at 30 μm intervals. Slices were used for haematoxylin and eosin (HE) staining (observation of cellular death) and for caspase-3 activation (apoptosis) and neurotrophin brain-derived neurotrophic factor (BDNF) detection. Anticaspase-3-antibody (CPP32 Ab-4, Rabbit Polyclonal Antibody, Thermo Scientific, Waltham, USA) and anti-BDNF-antibody [BDNF: (H-117): sc-20981, Santa Cruz Biotechnology, Inc., Heidelberg, Germany] were used. Details regarding immunohistochemical protocols and image capture are found in our previous work.25

Cellular death was based on morphological definitions from previous studies.26 27 Cells with abnormal morphologies (shrinkage, hypereosinophilic cytoplasm, condensed and hyperchromatic nuclei, marginated chromatin, and apoptotic bodies) were considered positive for cell death. Dead cells and cells showing clear positive immunoreactions were manually counted per square millimetre in the pyramidal cell layer of the cornu Ammonis 1 (CA1) and cornu Ammonis 3 (CA3) areas of the hippocampus, the granular layer of the dentate gyrus, retrosplenial cortex, and visual cortex. For BDNF expression, a relationship between positive cells and negative cells was established. The average of the neuronal counts from three coronal sections selected at 30 μm intervals from each brain was calculated.

**Statistical analysis**

Student’s t-test or one-way ANOVA with Bonferroni post hoc tests were used to analyse parametric data which are expressed as mean (SD), while non-parametric data were evaluated with the Kruskal–Wallis U-test followed by the Mann–Whitney U-test and are expressed as median (range). A value of P≤0.05 was considered statistically significant. All results were analysed by using Microsoft Office Excel 2003 for data management and SPSS 16.0 for Windows (Apache Software Foundation, Forest Hill, MD, USA) for statistical analysis.

**Results**

All animals from 25Ket./Med. and 75Ket./Med. groups lost consciousness. Animals treated with ketamine only did not lose consciousness. Mice from the 25Ket. group walked quickly with some motor inco-ordination and mice from the 75Ket. group walked slowly with motor inco-ordination.
Animals from the medetomidine group were less active and generally remained in a corner of the box.

**Anaesthesia**

Results for anaesthesia are based on anaesthetized groups (25Ket./Med. and 75Ket./Med.). No significant differences were detected between groups for time needed to induce anaesthesia [2.25 (0.71) and 1.87 (0.83) min, respectively], temperature, heart rate, systolic pressure, and oxygen saturation. Unconscious animals were anaesthetized for 1 h; afterwards, atipamezole was administered for reversal. The 75Ket./Med. group had a higher respiratory rate compared with the mice receiving lower concentrations of ketamine/medetomidine, at 10 (P=0.017), 30 (P=0.019), and 60 (P<0.045) min after loss of consciousness. The same group also had a greater depth of anaesthesia and took longer to recover from anaesthesia. Table 2 shows the haemodynamic and oxygen saturation data.

**Behavioural tests**

No differences were observed between groups regarding the number of trials needed to complete the T-maze test at 28 h, 1 week, and 2 weeks after anaesthesia (Fig. 2). During the vertical pole test, animals from the 75Ket/Med. group took longer than the others to completely descend the pole and also showed increased latency to turn downwards (Fig. 3). The high values of the 75Ket./Med. group were due to the fact that six animals fell from the pole.

The open-field test showed differences between groups in terms of speed and total distance. The 75Ket. group moved faster and covered a greater distance than the control group. Since the time to explore the arena is the same for all the animals, the speed only depends on the distance walked; hence, only speed is represented graphically (Fig. 4). Animals anaesthetized with 75 mg kg\(^{-1}\) ketamine walked a larger distance in the peripheral area compared with the control group (P=0.002), the Med. group (P=0.002), and the 25Ket./Med. group (P=0.003). There were no differences detected between groups in the other parameters measured in the open field.

**Brain analyses**

HE staining revealed no differences in the number of dead cells between groups in the different brain regions studied. Immunohistochemical analysis showed a similar number of cells with clear positive staining for caspase-3 and BDNF expression (Fig. 5).

**Discussion**

The results from this work showed no apoptotic neurodegeneration after different sedative and anaesthetic doses of ketamine/medetomidine combinations in adult mice. No effect on cell death or on spatial memory and no alterations in BDNF expression were observed. While ketamine induced hyperlocomotion in the open field when administered alone, this effect was reduced by the \(\alpha_2\)-agonist medetomidine. The ketamine/medetomidine combination seemed to result in physiologically anaesthetic stable anaesthesia since there were no significant differences between groups regarding heart rate and arterial pressure.

The haemodynamic stability observed in ketamine/medetomidine combinations is in accordance with previous studies, which reported that the action of ketamine helps to oppose the depressant actions of \(\alpha_2\)-agonists on the circulatory system, improving perioperative haemodynamic stability.\(^{16}\) Our observation that the higher concentration of ketamine/medetomidine increased respiratory rate is supported by reports of the respiratory-stimulating properties of ketamine.\(^{28}\)

High concentrations of ketamine/medetomidine also increased anaesthetic depth, with longer latency to recover equilibrium as indicated by the worst performance in the vertical pole test compared with lower concentrations.

### Table 2  Haemodynamic and oxygen saturation data (n=10). Data are expressed as mean (SD)

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Group</th>
<th>Heart rate (beats min(^{-1}))</th>
<th>Respiratory rate (respiratory movements per minute)</th>
<th>Systolic pressure (mm Hg)</th>
<th>(pO_2) (%)</th>
<th>Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>25Ket./Med.</td>
<td>484.1 (80.6)</td>
<td>128.0 (17.8)</td>
<td>126.4 (26.2)</td>
<td>99.0 (0.8)</td>
<td>37.2 (0.3)</td>
</tr>
<tr>
<td>75Ket./Med.</td>
<td>498.1 (69.1)</td>
<td>139.5 (28.2)</td>
<td>152.5 (31.0)</td>
<td>98.9 (0.8)</td>
<td>37.0 (0.5)</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>25Ket./Med.</td>
<td>474.1 (74.6)</td>
<td>131.0 (14.1)</td>
<td>125.8 (25.5)</td>
<td>98.6 (0.9)</td>
<td>36.9 (0.4)</td>
</tr>
<tr>
<td>75Ket./Med.</td>
<td>493.8 (70.5)</td>
<td>156.0 (22.1)</td>
<td>124.4 (22.9)</td>
<td>99.0 (0.7)</td>
<td>36.5 (0.6)</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>25Ket./Med.</td>
<td>507.0 (62.8)</td>
<td>147.5 (17.3)</td>
<td>123.8 (24.5)</td>
<td>98.8 (0.7)</td>
<td>36.7 (0.5)</td>
</tr>
<tr>
<td>75Ket./Med.</td>
<td>518.1 (50.2)</td>
<td>162.5 (20.6)</td>
<td>150.1 (29.4)</td>
<td>98.9 (0.8)</td>
<td>36.9 (0.8)</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>25Ket./Med.</td>
<td>513.1 (68.9)</td>
<td>137.8 (21.8)</td>
<td>129.9 (25.4)</td>
<td>98.8 (0.7)</td>
<td>37.0 (0.2)</td>
</tr>
<tr>
<td>75Ket./Med.</td>
<td>488.5 (41.5)</td>
<td>168.0 (23.6)</td>
<td>130.8 (24.5)</td>
<td>98.9 (1.0)</td>
<td>36.2 (0.8)</td>
<td></td>
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<tr>
<td>40</td>
<td>25Ket./Med.</td>
<td>505.1 (54.3)</td>
<td>147.0 (21.1)</td>
<td>134.0 (26.7)</td>
<td>98.4 (1.2)</td>
<td>36.7 (0.6)</td>
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<tr>
<td>75Ket./Med.</td>
<td>524.6 (79.7)</td>
<td>162.5 (20.8)</td>
<td>136.6 (24.4)</td>
<td>98.6 (1.5)</td>
<td>36.5 (0.3)</td>
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<tr>
<td>50</td>
<td>25Ket./Med.</td>
<td>518.1 (41.4)</td>
<td>143.0 (18.5)</td>
<td>129.4 (26.8)</td>
<td>99.3 (0.7)</td>
<td>36.8 (0.5)</td>
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<tr>
<td>75Ket./Med.</td>
<td>506.8 (58.1)</td>
<td>153.5 (24.9)</td>
<td>135.8 (20.6)</td>
<td>98.9 (1.1)</td>
<td>36.5 (0.5)</td>
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<tr>
<td>60</td>
<td>25Ket./Med.</td>
<td>509.6 (50.7)</td>
<td>144.5 (16.3)</td>
<td>129.5 (17.6)</td>
<td>99.3 (0.7)</td>
<td>36.8 (0.7)</td>
</tr>
<tr>
<td>75Ket./Med.</td>
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<td>132.9 (15.6)</td>
<td>99.0 (0.8)</td>
<td>36.9 (0.4)</td>
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In the open field, all animals showed a species-specific response to the aversive condition of the brightly lit arena, spending more time near the wall than in the central region, with most measures being unaffected by the different sedative and anaesthetic combinations. However, animals from the 75Ket group showed increased distance walked in

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**Fig 2** Number of trials necessary to complete the T-maze task at 28 h, 1 week, and 2 weeks after i.p. injection in different groups (n=8). Data are presented as box and whisker plots showing the median, inter-quartile range, and 5th and 95th percentiles. O=outlier and *=extreme value.

**Fig 3** Latency time to turn (latency) and completely descend the vertical pole (end pole) in seconds, 4 h after anesthesia (n=8); 120 s was attributed to the animals that fell. *P≤0.021 and **P≤0.028. Data are presented as box and whisker plots showing the median, inter-quartile range, and 5th and 95th percentiles. O=outlier.
the periphery and total distance; consequently a higher speed was observed. This suggests a hyperlocomotion effect of ketamine, in agreement with previous observations that this drug induces hyperlocomotion by the alteration of pre-synaptic components of dopamine neurones in the nucleus accumbens of mice. When the $\alpha_2$-agonist medetomidine was administered, hyperlocomotion was reduced; perhaps because this drug reduces dopamine turnover. To our knowledge, this is the first study showing that the hyperlocomotion induced by ketamine may be inhibited by $\alpha_2$-agonists in mice.

In the T-maze test, used to assess spatial memory, no differences between groups were seen. All groups learned the task at 28 h after anaesthesia and recalled it 1 and 2 weeks post-anaesthesia, as indicated by the decreasing number of trials to complete the task with time. No data were found concerning the effects of the combined administration of ketamine/medetomidine in spatial memory or neurodegeneration. However, it has been consistently reported that ketamine alone causes memory impairment and neurodegeneration when administered in neonates. Nevertheless, the adult brain is very different from neonates and there is conflicting evidence on the effects of a single dose of ketamine on cognition in the adult brain, with effects depending on the type of memory studied, acute or chronic use, dose, and temperature. Chronic administration of ketamine resulted in disrupted spatial working memory after 10 days of treatment. However, this did not happen with less days of treatment which is in accordance with our results.

The dosages used in our study (25 and 75 mg kg$^{-1}$ ketamine) were subanaesthetic when administered alone. The addition of the $\alpha_2$-agonist medetomidine allowed anaesthesia, reducing the amount of ketamine required and consequently reducing the probability of inducing adverse effects caused by high ketamine concentrations.

The $\alpha_2$-agonist medetomidine and the drug used to reverse it, atipamezole, did not affect performance in the t-test. This is in agreement with the previous work carried out by Carlson and colleagues that reported that

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**Fig 4** Speed in the open-field test induced by the different treatment groups (n=8) during 20 min, 24 h post-anaesthesia. *P*=0.033 compared with the control group. Mean (±SD) shown.

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**Fig 5** (A) The number of dead cells (HE), (B) number of caspase-3 activation positive profiles (apoptosis), and (C) ratio of the number of positive BDNF expression/negative BDNF expression (mm$^2$) in the retrosplenial cortex, visual cortex, pyramidal cell layer of the CA1 and CA3 areas of the hippocampus, and in the granular layer of the dentate gyrus, 3 h after anaesthesia in different groups (n=5). Mean (±SD) shown. No significant difference between groups was observed.
medetomidine had no effect on spatial memory in the adult rats and that atipamezole (1 mg kg⁻¹) had no effect on spatial cognitive performance after a single dose in rats.³⁶

Cellular death in this study was evaluated mainly by measuring apoptosis, in which no difference between groups was observed. Other works showed that a single dose of ketamine induced age- and sex-dependent cell death. Adults were more sensitive than immature rats and females were more sensitive than males; males remained insensitive to ketamine-induced vacuolization of neurones until they reached full adulthood.³⁷ In this work, we used adult males and we found no negative impact of ketamine with medetomidine in the brain. However, we studied neurodegeneration as apoptosis and not by excitotoxicity (vacuolization of neurones). Furthermore, to study general toxicity, it would be necessary to evaluate cell death in more time points. The histopathological analyses were performed only at 3 h after anaesthesia because it was reported that this is a good time point to study apoptosis. ²⁵ ³⁸

BDNF is an important indicator of brain exposure to insults. This was not altered with ketamine/medetomidine combinations and is in accordance with the results obtained with caspase-3. Usually, BDNF increases after insults,²⁹ but we found no evidence in the literature of the effects of this combination on BDNF expression. Acute administration of low doses of ketamine has been reported to increase BDNF expression.⁴⁰ However, we used higher doses of ketamine. Interestingly, pro-apoptotic anaesthetic drugs modulate BDNF protein levels in the developing brain,⁴³ resulting in an increase in caspase-3 and caspase-9 activation and, consequently, in apoptotic neurodegeneration.⁴⁴ In our study, no differences were detected between groups regarding the BDNF level and in caspase-3 activation, reinforcing the suggestion that a single dose of ketamine/medetomidine was not an insult to the adult brain.

In conclusion, our work showed that a single administration of sedative and anaesthetic concentrations of ketamine/medetomidine combinations in adult mice did not affect spatial memory, BDNF expression, or neurodegeneration by apoptosis in the hippocampus, retrosplenial cortex, and visual cortex. Simultaneously, this study showed that medetomidine prevented ketamine-induced hyperlocomotion, suggesting that α₂-agonists may possibly attenuate post-anaesthetic delirium and agitation produced by ketamine.

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Declaration of interest

None declared.

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