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Studies of apoptosis in the neonatal cortex and hippocampus of mice exposed to the anesthetic agent Etomidate

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Summary

Etomidate is a widely used anesthetic drug in pediatric medicine. It works by binding to the β subunit of the $GABA_A$ receptor (γ-aminobutyric acid type A receptor) and thereby opening the receptor's chloride channel, which leads to its anesthetic effect. Studies of ketamine, which is an anesthetic drug that works by blocking NMDA receptors (N-methyl-d-aspartate receptor) and propfol, which is another $GABA_A$ receptor enhancing anesthetic, have shown to induce neurodegenerative apoptotic effects in the brain during the brain growth spurt. These findings have led to concerns about the effects of etomidate on the developing central nervous system.

In this study, the hypothesis is that if these agents induce apoptosis in the developing brain, this will be reflected by the Caspase-3 activity in the brain of test animals that have been exposed to the agents during the brain growth spurt. Caspase-3 is an executor caspase that belongs to the family of cysteine aspartate proteases. Caspases are critical in the two main apoptotic pathways, and Caspase-3 is involved in the finishing step of both pathways. Its activity is therefore a good reflection of the apoptosis going on in the cells of the brain.

In this experiment the Caspase-3 concentration was measured with a caspase-3 ELISA in cortex and hippocampus tissue homogenates of neonatal mice 24 hours after a single subcutaneous dose of etomidate 0.3 mg/kg bw, 3.0 mg/kg bw, 10 mg/kg bw, ketamine 50 mg/kg bw, or propofol 60 mg/kg bw on postnatal day 10.

The results of this analysis indicated that exposure to etomidate, ketamine and propofol did not increase apoptosis in the neonatal cortex and hippocampus. The Caspase-3 concentrations in the exposed groups did not differ significantly from the concentration in the control group.

Further studies on this matter are necessary, in order to state if etomidate ketamine and propofol can be used in clinical practice on children without any consequences on the developing brain.

Introduction

Brain growth spurt

The brain and the central nervous system is the most complicated organ in the human body. The development of the brain is therefore extremely importantand complex. During the development of the brain there is a period when the brain is increasing rapidly in weight (Dobbing *et al.* 1974). This period is called the brain growth spurt and is characterized by synaptogenesis, outgrowth of axons and dendrites, formation of neural connections and other developmental processes in the brain (Robertsson 2010). In rodents the peak of brain growth is around postnatal day 10. In humans this period goes on from the third trimester into the first two years of life (Dobbing *et al.* 1979).

Several studies in animals, during this phase of life, have shown that the brain is especially vulnerable to substances that affect the brain during the brain growth spurt (Jevtovic-Todorovic *et al.* 2003, Eriksson *et al.* 2002). The use of anesthetic agents for example, which potentiate $GABA_A$ receptors have shown to cause apoptotic neurodegeneration in the developing brain (Jevtovic-Todorovic *et al.* 2003). This can lead to persistent damage in the brain and may also lead to learning impairments (Jevtovic-Todorovic *et al.* 2003).

Apoptosis

Apoptosis is the vital and complicated natural process by which cells undergo a programmed cell death to maintain homeostasis and a stabile cell population in tissues during development, and aging. Apoptosis also occurs as a defense mechanism when cells are damaged by toxic agents or diseases (Elmore 2007). Some chemicals are thought to induce apoptosis in the developing brain, during the brain growth spurt (Jevtovic-Todorovic *et al* 2003).

Unlike necrosis, which is an accidental and uncontrolled process that does not require energy, apoptosis is a controlled process with no release of cellular content to the neighboring cells and without an inflammatory response (Bonfoco *et al* 1995). Apoptosis occurs through two main pathways, the extrinsic pathway and the intrinsic pathway. The extrinsic pathway, also called the death receptor pathway, involves binding of ligands to death receptors in the transmembrane, which leads to activation of Caspase-8 which in turn activates Caspase-3. The intrinsic pathway or the mitochondrial pathway is initiated by positive or negative

stimuli resulting in different responses by the mitochondria, including the release of two main groups of pro-apoptotic proteins into the cytosol. This then leads to the activation of Caspase-9 which then activates Caspase-3 (Elmore 2007).

Both pathways end in the same execution pathway. This process is initiated by the cleavage of Caspase-3, which then leads to DNA fragmentation, degradation of nuclear and cytoskeletal proteins and other processes until the cell is finally phagocytized (Elmore 2007).

The family of cysteine aspartate proteases or caspases, are critical in both apoptotic pathways. Caspases are expressed in catalytically inactive forms and each pro-caspase has three domains, a pro-domain, a large subunit (20 kDa) and a small subunit (10 kDa). Caspases are present in the cytosol of the cell (Robertson *et al* 2000, Cryns and Yuan 1998). Activation of caspases occurs when the three domains are separated. The pro-domain is removed and the large and small subunit heterodimerize, resulting in an active caspase, which can cleave other caspases and thereby activate them (Cryns and Yuan 1998). Ten major caspases have been identified. Each caspase is classified according to its actions in the cell. Caspase-8 and -9 are classified as initiators, and Caspase-3 is classified as an executioner caspase, because of its involvement in cleaving substrates involved in regulating nuclear changes during apoptosis (Elmore 2007, Robertson *et al* 2000).

In this study the apoptotic pathway is not of major importance. The goal is to find out whether the anesthetic agent etomidate, which the test animals have been exposed to, has a neurotoxic effect on the brains of the animals by causing apoptosis. In order to do so, the activity of Caspase-3, in which both pathways end with, as mentioned earlier, is measured. If the Caspase-3 concentration found in the brains of the exposed animals differs significantly from the concentrations in the brains of control animals, it can be concluded that this agent has a toxic effect on the brain by causing apoptosis.

Etomidate

Etomidate (R-1-(1-ethylphenyl) imidazole-5-ethyl ester) (Forman 2011) is a carboxylated imidazole that is widely used as an anesthetic agent (Yang *et al.* 1996). The R (+) isomer of etomidate is almost 5 times as potent as the S (+) isomer, and is primarily the one that gives the anesthetic effect (Tomlin *et al.* 1998). When administered intravenously the induction dosage is 0,2-0,4 mg/kg body weight for children up to 15 years (Forman 2011, Schou 2004). Maintenance of anesthesia is achieved by constant dosing at 30-100 µg/kg/min (Forman

2011). In pediatric patients etomidate can be administered rectally (Linton *et al.* 1983). The LD 50/ED 50 ratio (therapeutic index) for R (+) etomidate is 26 (Forman 2011).

Although the mechanism of action for etomidate is not completely understood it is thought that the agent works as an anesthetic by binding to the β subunit of the $GABA_A$ receptor (γ-aminobutyric acid type A receptor) (Belelli *et al.* 1997). The $GABA_A$ receptor is a ligand-gated ion channel in the cell membrane in the central nervous system. Binding of GABA, which is an inhibitory neurotransmitter (Hill-Venning *et al.* 1997) causes influx of chloride ions to the post synaptic cell causing hyperpolarization, which gives an inhibitory effect by preventing a successful action potential (Tanelian *et al.* 1993). It is thought that etomidate works as a positive allosteric modulator by binding to the β subunit and thereby enhancing the $GABA_A$ activity, leading to anesthesia (Belelli *et al.* 1997, Tomlin *et al.* 1998).

Ketamine

Ketamine (2-(O-chloro-phenyl)-2-methylamino cyclohexanone) (White *et al.* 1982) is a phencyclidine hydrochloride derivative that is used in human and veterinary medicine as an anesthetic (Krystal *et al.* 1994). Ketamine is administered as a racemic mixture of its two optical isomers S(+) and R(-) (Hirota and Lambert 1996). Ketamine is an N-methyl-d-aspartate receptor (NMDAR) blocking drug (Ikonomidou *et al* 1999). The NMDA receptor is a subtype of ionotropic excitatory amino acid receptors that facilitates the transfer of electrical signals between neurons in the brain and in the spinal cord (Monyer *et al.* 1992). For signals to pass glutamate and glycine must bind to the receptor and keep it activated and open. Blocking of the receptor keeps the Ca^{2+} channel of the NMDA receptor closed and stops signals from passing (Kemp *et al.* 2002). Ketamine is a non-competitive antagonist of the NMDA receptor Ca^{2+} channel pore. Ketamine also interacts with the phencyclidine (PCP) binding site at clinically relevant concentrations. This leads to significant inhibition of the NMDA receptor activity (Hirota and Lambert 1996).

Propofol

Propofol (2,6-diisopropylphenol) is a short-acting, widely used anesthetic drug in clinical practice. Propofol is a liphophilic compound and is therefore concentrated in lipid-rich tissues such as the brain (Pontén *et al.* 2011). Even though the use of propofol in pediatric patients is restricted because of the risk for myocardial failure after long-term infusion, the anesthetic is still used on children for short-term application (Bercker *et al.* 2009). Like etomidate,

propofol gives an anesthetic effect by modulating the activity of the $GABA_A$ receptor and directly activate its chloride channel, thereby enhancing the $GABA_A$ receptor activity (Peduto *et al.* 1991). As mentioned earlier, the influx of chloride ions to the post synaptic cell gives an inhibitory effect by preventing a successful action potential (Tanelian *et al.*1993).

Animal studies of use of anesthetic agents that act as NMDA receptor antagonists (N-meyl-D-aspartate), such as ketamine, and GABA-mimetics, such as propofol, indicate that these agents can induce neurodegradation and apoptosis in the developing brain of neonates (Mellon *et al.* 2007, Ikonomidou *et al.*1999, Jevtovic-Todorovic *et al.* 2003, Bercker *et al.* 2009). The mechanism causing apoptosis by ketamine is still unknown. A study by Slikker and co-workers suggests an up-regulation of the NMDA-receptor NR1, which leads to the production of reactive oxygen, which might be the cause of induction of apoptosis in the brain (Slikker *et al.* 2007).

These observations have raised concerns about other anesthetic agents such as etomidate. Although there are very few studies on this matter, there is reason to believe that etomidate, which have a similar mechanism of action via $GABA_A$ receptors, and is used on pediatric patients, could have similar effects on the developing brain.

Aim

In this study it will be investigated if etomidate has an adverse effect on the neonatal, developing central nervous system, by measuring the Caspase-3 concentration in the cortex and hippocampus from neonatal mice which have been exposed to etomidate, ketamine or propofol on postnatal day 10.

Material and methods

Animals

Pregnant Naval Medical Research Institute (NMRI) mice were purchased from B&K, Sollentuna, Sweden. The mice were kept individually in plastic cages in a room where the temperature was 22°C and a 12/12-h cycle of light and dark. They had free access to standard pellet food (Lactamin, Stockholm, Sweden) and tap water *ad libitum*. The day of birth was assigned postnatal day (PND) 0. Each group of offspring were kept with their mothers in the cages. The cages were adjusted to contain equal numbers of male and female pups within the first 48 h after birth.

Animal experiments were done in accordance with and after approval from the local ethical committee (Uppsala University and Agricultural Research Council) and by the Swedish Animal Welfare Agency (license C185/9).

Chemicals

Etomidate (Etomidate-Lipuro 2 mg/ml Emulsion for injection B. Braun Melsungen AG) was purchased from Apoteksbolaget. Propofol (Diprivan 10 mg/ml Astra. Södertälje, Sweden) was purchased from Apoteksbolaget. Ketamine (Ketalar® 50 mg/ml Pfizer Inc. New York, USA) was purchased from Pfizer Inc.

Procedure

The Caspase-3 concentration of each sample of neonatal mouse cortex and hippocampus was analyzed by doing a three step experiment. The first step was to homogenize each sample, in the next step the total protein concentration of each sample was measured with the BCA assay (bicinchoninic acid assay), and in the last step an ELISA (enzyme-linked immunosorbent assay) was carried out to detect and quantify the Caspase-3 concentration in each sample.

The cortex and hippocampus samples used in this experiment came from neonatal mice. These neonatal mice had been divided into 6 groups, and each group had been treated with a specific dose of saline (0.9% NaCl), Etomidate (0.3 mg/kg bw, 3.0 mg/kg bw and 10 mg/kg bw), a dose of Propofol (60 mg/kg bw) or Ketamine (50 mg/kg bw) subcutaneously at postnatal day 10 (Table 1). 24 h after exposure the mice were killed and the brains were then

dissected. The cortex and hippocampus of each mouse were frozen at -80˚C until they were homogenized.

Table 1. List of groups of mice and what individuals in each group were treated with.

Group	**Substance**
1	NaCl 0.9 %
2	Etomidate low dose 0,3 mg/kg bw
3	Etomidate medium dose 3,0 mg/kg bw
4	Etomidate high dose 10 mg/kg bw
5	Propofol 60 mg/kg bw
6	Ketamine 50 mg/kg bw

Homogenization

A total of 36 samples of both cortices and hippocampi, 6 samples from each group, were randomly selected for the experiment and homogenized according to protocol (Homogenisering av hjärnvävnad av möss). The samples were first weighed in ice cold PBS, in order to keep the samples cold , in a volume of PBS giving a cortex homogenate with the concentration 40 mg tissue/ml and a hippocampus homogenate with the concentration 20 mg tissue/ml. The PBS and tissue were then transferred into a Potter-Evehjelm tube and homogenized with a homogenizer. The homogenate from each sample was decanted into centrifuge tubes and then the solutions were sonicated with an ultrasound sonicator to further break binding between the cells and the membranes of the cells in the samples and release the content. Each homogenate was then centrifuged for 5 min at 500 x g, and the supernatant was decanted into Eppendorf tubes and stored at -80˚C until the second part of the experiment was carried out.

BCA Assay

In order to relate the Caspase-3 concentration in the samples to the total protein concentration of the samples a BCA Assay was done. The detection reagent was made by mixing 20 ml reagent A with 0.4 ml of reagent B (www.thermoscientific.com/pierce). A 96 well microplate was then filled with the protein standards (for standard curve) homogenized samples, PBS buffer and distilled water (10 µl) according to the protocol (BCA Protein Assay SOP). 200 µl detection reagent was added to each well and the plate was incubated at 37°C for 30 minutes.

After incubation the plate was read for absorbance at 562 nm in a microplate reader (Victor) and a standard curve was obtained. The total protein concentrations in the samples were calculated.

Caspase-3 ELISA

The last part of the experiment was carried out by using an ELISA kit, which contained all the reagents for the experiment except our samples, PBS buffer and distilled water (www.antibodies-online.com). 8 standard concentrations (40 ng/ml, 20ng/ml, 10ng/ml, 5ng/ml, 2.5ng/ml, 1.25ng/ml, 0.625ng/ml and 0 ng/ml) were diluted in a series according to the protocol and the detection reagents A and B were mixed (ELISA protokoll 140204). 100 µl of each standard concentration and samples were added to a pre-coated 96 well strip plate containing the antibody and the plate was incubated at 37°C for 2 hours. After incubation the liquid in each well was removed and 100 µl detection reagent A was added to each well and the plate was once again incubated for 1 hour at 37°C. The liquid was then removed and the wells were washed 3 times with a previously made wash buffer. 100 µl of detection reagent B was added to each well. The plate was incubated at 37°C for 30 minutes. After incubation the liquid was once again removed from each well and the plate was washed 5 times. 90 µl of substrate solution (TMB) was then added to each well, in order for the colorimetric reaction to occur in the wells. The plate was incubated for 15-20 minutes and taken out depending on when a clear color change to blue could be observed. At that point 50 µl of stop solution was added to each well and the liquid in the wells turned yellow. The absorbance, at 450 nm, was measured in a microplate reader (Victor) and a standard curve was obtained from the absorbance values. The Caspase-3 concentrations in the samples were calculated. The calculated concentrations were analyzed statistically with a one way ANOVA (Prism 5.01) in order to compare each group to each other and find out if there were any differences between the different groups.

Results

Capase-3 concentration in cortex of mouse pups neonatally exposed etomidate, propofol or ketamine

The total protein concentrations, obtained after the BCA Protein Assay, were used to relate the detected Caspase-3 concentration to the total protein content in the cortices of each group.

The one-way ANOVA test, where the caspase-3 concentrations were compared between the different groups, showed that there were no significant differences (p>0. 05) between the exposure groups and the control group (Figure 1).

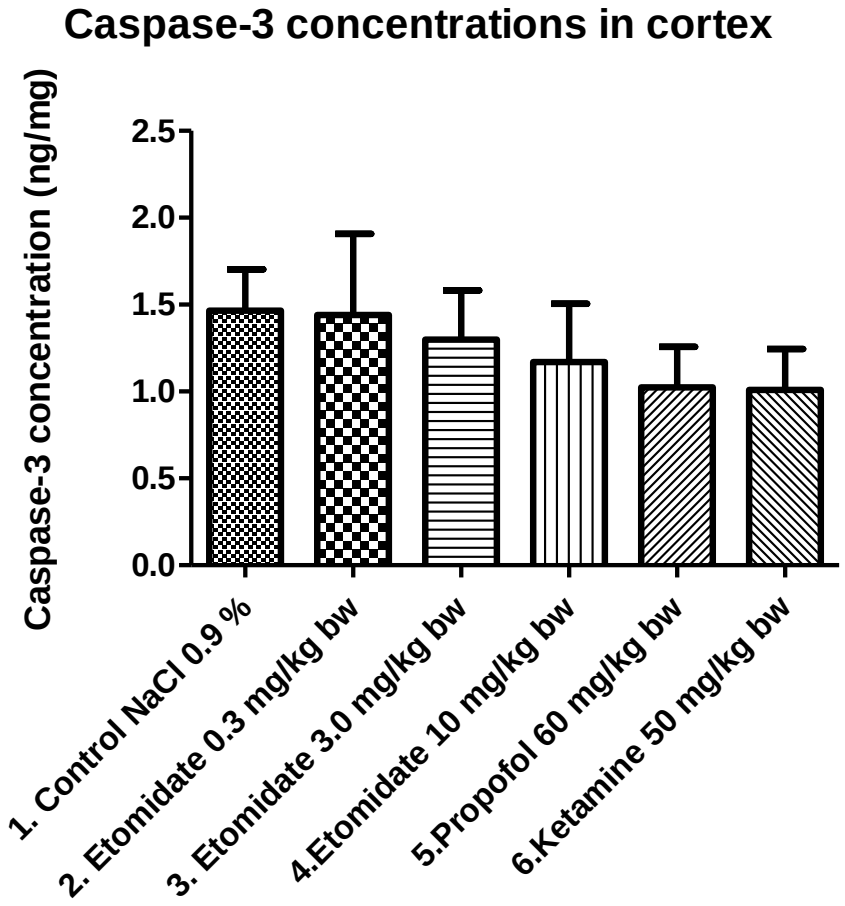

Figure 1. Caspase-3 concentrations in mouse cortices 24 hours after a single subcutaneous dose of either 0.9 % NaCl, etomidate 0.3 mg/kg bw, etomidate 3.0 mg/kg bw, etomidate 10 mg/kg bw, propofol 60 mg/kg bw, or ketamine 50 mg/kg bw on postnatal day 10. The results are presented as the mean value ± SD from 6 animals in each group. The statistical evaluations were made using one-way ANOVA and there were no significant differences between control and exposed groups (p>0.05).

Capase-3 concentration in hippocampus of mouse pups neonatally exposedto etomidate, propofol or ketamine.

The total protein concentrations, obtained after the BCA Protein Assay, were used to relate the detected Caspase-3 concentration to the total protein content in the hippocampi of each group.

The one-way ANOVA test, where the caspase-3 concentrations were compared between the different groups, showed that there were no significant differences (p>0. 05) between the exposure groups and the control group (Figure 2).

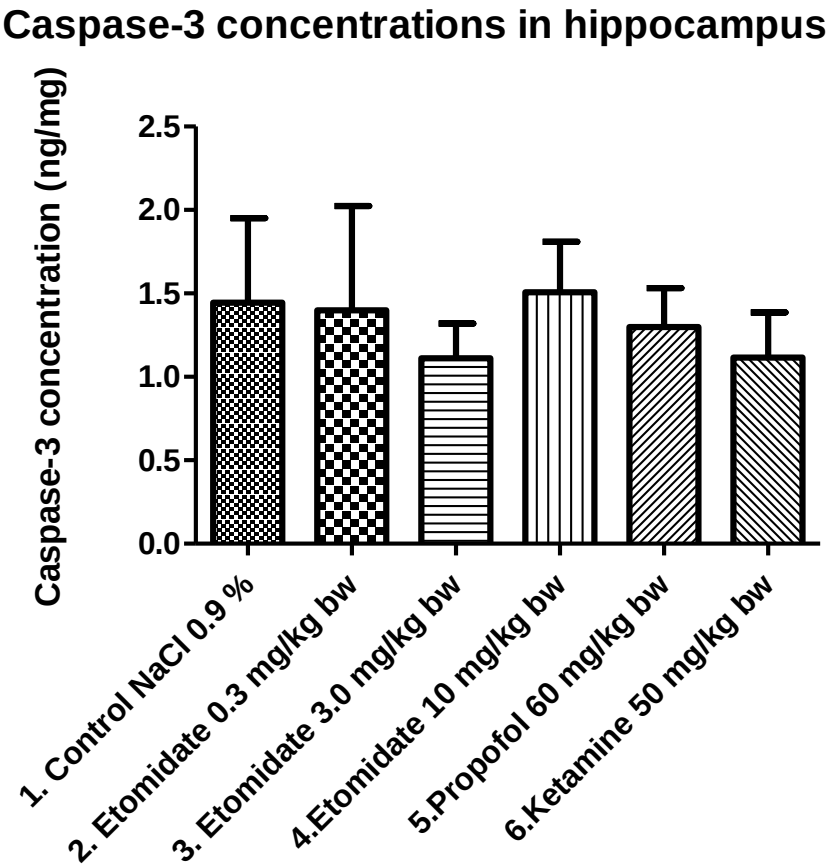

Figure 2. Caspase-3 concentrations in mouse hippocampi 24 hours after a single subcutaneous dose of either 0.9 % NaCl, etomidate 0.3 mg/kg bw, etomidate 3.0 mg/kg bw, etomidate 10 mg/kg bw, propofol 60 mg/kg bw, or ketamine 50 mg/kg bw on postnatal day 10. The results are presented as the mean value ± SD from 6 animals in each group. The statistical evaluations were made using one-way ANOVA and there were no significant difference between control and exposed groups (p>0.05).

Discussion

In order to invesigate if the anesthetic agent etomidate has any apoptotic effects on the developing brain of neonatal mice, 10 day old mice were given three different doses (0.3 mg/kg bw, 3.0 mg/kg bw, 10 mg/kg bw) of etomidate subcutaneously and 24 hours after exposure the Caspase-3 concentrations were analyzed in homogenates of the animals cortices and hippocampi. Another two groups of animals, were exposed to ketamine 50 mg/kg bw or propofol 60 mg/kg bw, to see if these agents had any apoptotic effects on the developing brain.

The Caspase-3 concentrations that were analyzed in both the cortices and the hippocampi of the exposed groups did not differ significantly from the Caspase-3 concentration in the control group, indicating that the exposure to etomidate, propofol and ketamine did not induce apoptosis in the neonatal brain. As mentioned before, there is a natural apoptosis occurring in the brain during brain development, when cells that are formed incorrectly or in overabundance must be eliminated. During development almost half of all the initially formed neurons are eliminated via apoptosis (Blomgren *et al.* 2007). The low levels of Caspase-3 that were detected in the cortices and hippocampi of all groups were probably due to the normal apoptosis that occurs during the development of the central nervous system, since the test animals in this study were analyzed during the brain growth spurt, on postnatal day 11 (Elmore 2007).

Activation of the executor Caspase-3 is a late event in the apoptotic pathway (Elmore 2007). In order for Caspase-3 to be activated other caspases must be activated first. The animals were killed 24 hours after exposure, and 24 hours might be too short to be able to observe increased Caspase-3 activity, induced by xenobiotics. On the other hand, it is possible that apoptosis might have occurred already before the analysis was done, and that the Caspase-3 concentration had declined once the analysis was done.

There were no significantly increased levels of Caspase-3 in the group exposed to ketamine in this study. Ketamine has in previous studies been shown to induce wide spread apoptosis and/or neurodegeneration in the brain (Jevtovic-Todorovic and Olney 2008, Olney *et al.* 2002, Fredriksson *et al.* 2004). In those studies, where ketamine has shown to induce apoptosis (Jevtovic-Todorovic and Olney 2008, Olney *et al.* 2002) the animals have been exposed on postnatal day 7. In this study the mice were exposed on postnatal day 10.

Postnatal day 7 might be a sensitive part of the brain development and thus the damage on the brain might be more detectible if animals are exposed on this day.

In some of these studies (Slikker *et al.* 2007, Jevtovic-Todorovic *et al.* 2003) repeated dosing has been used as opposed to the present study where the mice have received one single dose. It is possible that higher concentration of the drug in the target organ may affect the apoptosis process more.

There is also a possibility that the analyzing method was not sensitive enough to detect neuroapoptosis by ketamine. In the present study tissue homogenates were used to detect apoptosis in the cortices and hippocampi of the treated mice. Earlier studies, where ketamine has shown to induce apoptosis, sections of the brain have been used to detect apoptosis, by immuno-histo chemistry (Fredriksson *et al.* 2004, Jevtovic-Todorovic and Olney 2008). It is possible that using tissue sections with antibodies is a more sensitive approach than to use a caspase-3 ELISA on tissue homogenates.

Since there are very few earlier studies of this kind on etomidate, there is not enough evidence from just the present study, to state if etomidate is a safe anesthetic agent that can be used in pediatric patients without any negative consequences. Many studies suggest that $GABA_A$ receptor enhancing agents, which is what etomidate is as mentioned earlier, can cause apoptosis in the brain of neonates, leading to memory and learning deficiencies (Jevtovic-Todorovic *et al.* 2003). This is why it is important to try other analyzing methods and redo the experiment and change some of its circumstances.

In conclusion the results from this study indicate that etomidate does not induce apoptotic effects on the neonatal brain, although further studies are required to assure safe use the anesthetic in pediatric patients, Furthermore, there were no indications in this study that ketamine and propofol has apoptotic effects in the developing brain, which in previous studies (Jevtovic-Todorovic and Olney 2008, Olney *et al*. 2002, Bercker *et al.* 2009) have shown to give neurodegenerative and/or apoptotic effects.

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