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Etomidate's effect on neuroproteins in the developing brain of neonatally exposed mice

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Abbreviations

BGS – Brain growth spurt
PND – Postnatal day
PSD-95 – Postsynaptic density protein-95
GluR1 – Glutamate receptor subtype 1
SYP - Synaptophysin
GABA - γ-amino butyric acid
NMDA - N-Methyl-D-aspartate
CNS – Central nervous system
SV – Synaptic vesicle
LTP – Long-term potentiation
NMRI – Naval Medical Research Institute
ANOVA – Analysis of variance
SD – Standard deviation
FAS – Fetal Alcohol syndrome

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Abstract

In today's society children, newborns and late term fetuses are commonly exposed to anesthetics. The human brain growth spurt (BGS) starts in the last trimester of pregnancy and continues throughout the first few years of life. This fact makes studies on neuroprotein levels of exposed animals of increasing importance. This study focused on using the three neuroproteins synaptophysin (SYP), glutamate receptor subtype 1 (GluR1) and postsynaptic density protein-95 (PSD-95) as markers for brain damage during neonatal development. These proteins are all involved in, and important for, the development of the brain and the BGS. Neonatal mice received a single subcutaneous dose of etomidate (0.3, 3 or 3mg/kg BW), propofol (60 mg/kg BW) or ketamine (50 mg/kg BW) on postnatal day (PND) 10, which coincides with the peak of the BGS in mice. They were euthanized 24 hours later and their neuroprotein levels were measured by slot blot chemiluminescence of the hippocampus and cerebral cortex. The results did not show any significant results in a one-way ANOVA. However, there was a trend of lower protein levels of SYP and GluR1 in the cortex area of the exposed mice.

Introduction

The development of the human brain is both pre- and postnatal. It continues through birth and the first few years of life. This period is quite prolonged in comparison to other species (Davidson & Dobbing, 1968). Due to that fact, there are concerns about what substances, and how much of them, the developing brain can be exposed to without being harmed. Nowadays, it is not unusual for newborns and toddlers to be exposed to anesthetics during surgical procedures. Consequently, it is important to increase the knowledge on how the developing brain is affected by these substances.

This study of etomidate exposure in neonatal mice is a part of an ongoing project that uses biochemical indicators to characterize effects of different chemicals on neonatal brain development. In this experiment, we will use the neuroproteins synaptophysin (SYP), postsynaptic density protein 95 (PDS-95) and glutamate receptor subtype 1 (GluR1) to map changes in the developing brain of mice exposed to the pharmaceutical etomidate. The levels of these proteins are known to increase during the brain growth spurt (BGS). In this study we will analyze the protein levels in the exposed animals and compare them to protein levels in control animals. In connection to this experiment, behavioral studies determining the effect of etomidate have also been conducted on mice. No harmful behavioral effects could be detected (personal communication, Henrik Viberg). In that experiment, two groups have been exposed to either ketamine or propofol, which are then used as positive (ketamine) and negative (propofol) controls for behavioral disturbances in this experiment.

The desired traits of an anesthetic agent include inducing a reversible sleep, relaxing muscles and suppressing reflexes, but not suppressing cardiovascular or respiratory function. In addition, a fast acting substance, that is inexpensive and easy to administer, with a rapid clearance after ceased exposure is also favourable (Nicholson, 2014). All modern anesthetics have either γ-amino butyric acid (GABA) receptor enhancing or N-Methyl-D-aspartate (NMDA) receptor blocking properties (Jevtovic-Todorovic et al., 2003).

Brain growth spurt

Davidson and Dobbing (1968) described the brain growth spurt (BGS) as a period where brain weight increases rapidly due to glia cell proliferation, the acquirement of neuronal connections, axonal and dendritic outgrowth and myelination, see figure 1. During this process, the brain is more vulnerable and susceptible to xenobiotics present in the environment. An individual who suffers a deficiency of nutrients or is exposed to a toxicant during this period would not recover and regain a fully developed brain regardless of the time for rehabilitation given (Dobbing & Smart, 1974). Dobbing and Smart (1974) concluded that exposures to xenobiotics during the BGS does not delay brain development, they suppress it entirely. Therefore, the individual should desirably not be exposed to xenobiotics during this period.

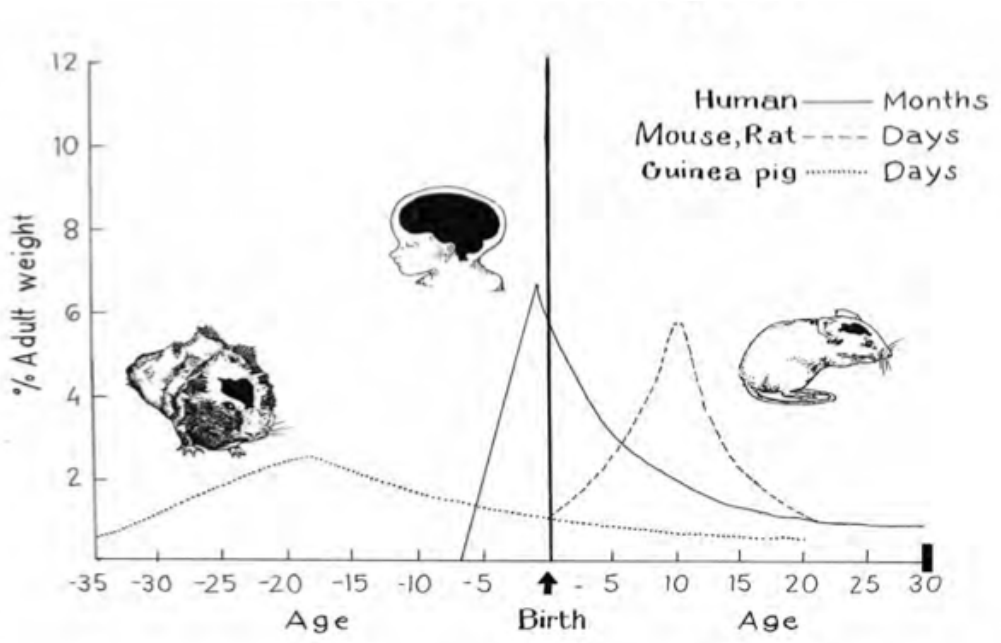


Figure 1. Timespan of the brain growth spurt (BGS) period in man (months), guinea pig (days), mouse and rat (days) in relation to birth (Davidson & Dobbing, 1968; Eriksson unpublished. Illustration by Ylva Stenlund).

Brain development differs between species. Mice have a BGS during the neonatal period, which extends from just after birth to the first few weeks of life, peaking around postnatal day (PND) ten (Dobbing & Sands, 1979). This study focuses on the hippocampus and the cortex regions of the brain; the neuroproteins tested here are involved in the BGS in different ways and have a distinct ontogeny (Viberg, 2009). In rodents, the hippocampus is involved in spatial learning as well as the recognition and working memory. It is also suggested to be involved in more recent memories. Test animals that have lesions in this area perform more poorly in certain spatial learning tasks (Lynch, 2004). The cortex is also involved in spatial learning and working memory. It is used for remote, or long term, memories. Lesions in the cortex area are considered to be more serious than lesion is the hippocampus since they can lead to severe impairments (Lynch, 2004).

Since damage to the hippocampus and cerebral cortex will affect learning and memory it will also affect the behavior of the exposed animal (Viberg et al., 2008), e.g. habituation is connected with these abilities (spatial learning) (Daenen et al., 2001; Giovannini et al., 2001).

The GABA receptor

The human BGS starts in the last trimester and continues during the first few years of life. During this time the GABA and NMDA receptor systems develop (Davidson and Dobbing, 1968; Dobbing & Sands, 1979; Ikonomidou et al., 1989; McDonald et al., 1988). The GABA receptor natural ligand is an amino acid named γ-Aminobutyric acid (GABA). GABA is an inhibitory neurotransmitter accountable for most synaptic inhibition of neurons (1/3 of all synapses) and is located in the central nervous system (CNS) of mammals (Bloom & Iversen, 1971; McCormick, 1989). An excessive activation of this ligand gated ion channel during the BGS can cause an inhibition of the communication, which induces a widespread apoptotic neurodegeneration (Ikonomidou et al., 2001).

The NMDA glutamate receptor

The ionotropic NMDA glutamate receptor is a ligand gated ion channel and therefore highly permeable to calcium ions. In addition, it is involved in many neuronal functions, e.g. spatial memory (Lynch, 2004; Wenthold et al., 2003). As the name suggests, a natural ligand of the NMDA receptor is glutamate, but also glycine (Guerrini et al., 1995). If the NMDA system is blocked, for a period of hours, during synaptogenesis in the developing brain that will cause an extensive apoptotic neurodegeneration in the brain due to the system's sensitivity to excitotoxic degeneration (Ikonomidou et al., 2001). In addition, the acquisition of spatial learning will be inhibited (Lynch, 2004).

Etomidate

The pharmaceutical etomidate (R-1-ethyl-1-(a-methylbenzyl)-imidazole-5-carboxylate) is a heterocyclic, non-barbital, imidazole derivative, which is used as an anesthetic agent. It affects the body by activating the GABA receptor (Uschida et al., 1995). This sedative was synthesized in the 1960's and introduced in Europe a decade later; its introduction to the U.S. was in the early 1980's (Ruth et al. 2001; Jackson, 2005). Today it is generally used on trauma patients, when performing endotracheal intubation (Jackson, 2005). Ever since the use of etomidate began, it was regarded as one of the more prominent anesthetic agents due to its rapid anesthetic properties (10 sec), minimal side effects and fast dissipation of clinical effects after ceased exposure. At first, these properties led to etomidate being used for prolonged sedation during surgical procedures (Fragen et al., 1984; Newberg Milde et al., 1985; Ruth et al., 2001; Streisand et al. 1998). However, it was soon discovered that etomidate increased mortality when used for a long-term sedation. Nowadays, etomidate is only used for short-term sedation (6-8 min) (Desborough, 2000; Jackson, 2005; Plewa et al., 1997).

Etomidate is mainly given intravenously, but can also be delivered through the oral mucosa (trans mucosal), which entails being absorbed through the cheek or under the tongue. This way is preferred to oral (swallowed) because the pharmaceutical reaches the blood stream faster and avoids hepatic metabolism (Ruth et al., 2001; Streisand et al., 1998). The average dose of etomidate in adults is 0.2-0.4 mg/kg body weight or 16-30 µg/kg body weight, depending on single or repetitive induction (de Jong et al., 1984; Fragen et al., 1984; Schenarts et al., 2001; Van Hamme et al., 1978).

Metabolism and excretion

The half-life of etomidate in humans is about 75 minutes. After 4-5 half-lives a substance is considered to be entirely eliminated from the body. The clearance of etomidate and its metabolites, which occurs through ester hydrolysis, should therefore be complete within 24 hours (Bahn et al., 2012). However, Fellows (et al., 1983) noted that adrenocortical suppression after induction of etomidate lasted for 4 days after the use was discontinued. It is not uncommon for the toxic effects of an exposure to outlast the presence of the substance in the body. These effects are also important for the desired traits of an anesthetic. Etomidate is ultimately metabolized in the liver and excreted by the kidneys (Banh et al., 2012). Only a small percentage of etomidate is excreted unchanged (2%) in the urine (Van Hamme et al., 1978).

Adverse effects

Despite the fact that etomidate is one of the most commonly used anesthetics for critically injured people it has some severe side effects. These entail adrenocortical suppression, myoclonus, vomiting and nausea (Fellows et al., 1983; Plewa et al., 1997; Streisand et al., 1998). The suppression of the adrenal cortex is an unusual side effect for an anesthetic, but a very important one for the continued use of etomidate, since the function of the adrenal glands involve secreting hormones that control body functions such as sexual maturation, metabolic processes, and the balance of salt and water (Plewa et al., 1997).

At first, scientists described a suppression of the adrenal gland that was thought to occur only during long-term induction (Fellows et al., 1983; Streisand et al., 1998; Varga et al., 1993). However, de Jong (et al., 1984) claim that short-term induction of etomidate also affects biosynthesis of adrenal steroids in an acute way. Varga (et al., 1993) indicated that the decrease in cortisol levels could be a result of etomidate blocking mitochondrial 11β-hydroxylation activity. This leads to a decreased conversion of 11-deoxycortisol to cortisol (Plewa et al., 1997; Schenarts et al., 2001).

Ketamine

Human use of the pharmaceutical ketamine (2-(2-chlorophenyl)-2-(methylamino)-cyclohexanone) can be either recreational, as an antidepressant, or it can be routinely used as an anesthetic during, for example, pediatric or obstetric medicine. Ketamine is an NMDA antagonist, which means that it has the ability to block these glutamate receptors. The mode of action of ketamine, beyond affecting the NMDA receptor is not fully understood (Ikonomidou et al., 2001).

The use of ketamine as an anesthetic first started in the mid 1960's (Domino et al., 1965). It was considered a safe and unique anesthetic since it induced dissociative anesthesia, which is a form of anesthesia that does not necessarily include unconsciousness.

Metabolism and excretion

To be able to excrete ketamine it has to be converted into a more hydrophilic compound. This is achieved by demethylation of its metabolites, a phase I reaction (oxidation). Once converted into phase I metabolites, they are excreted as glucoronide conjugates through the urine (Meyer & Maurer, 2011). Some metabolites can be detected in the urine within 24 hours and the parent compound has been identified in the urine two weeks after exposure. The concentration of the parent compound in the urine is relatively low compared to the concentration of the metabolites (Meyer & Maurer, 2011).

Adverse effects

As mentioned in the introduction, ketamine is used as a positive control for behavioral disturbances. Intoxication by ketamine can demonstrate cognitive difficulties following hippocampal dysfunction depending on the developmental stage of the exposed individual. The reversibility of detrimental effects caused by ketamine exposure, or use, also depends on age. Studies have indicated that apoptosis is common when the developing central nervous system (CNS), i.e. younger individuals, are exposed to ketamine, while other types of cell death affect more mature individuals (Hayashi et al., 2002; Ikonomidou et al., 1999; Majewski-Tiedeken et al., 2008).

Propofol

The general anesthetic propofol (2,6-diisopropylphenol) is a non-barbiturate used in clinical practice. Propofol is a GABA receptor agonist and NMDA glutamate receptor antagonist. The benefits of propofol are, like etomidate, its rapid onset and short duration. Its effect is exerted on the hippocampus.

Metabolism and excretion

Propofol has the shortest half-life of the pharmaceuticals tested in this experiment. It is metabolized either by direct glucoronidation or by hydroxylation followed by glucoronidation and about 50% is excreted as the parent compound (Meyer & Maurer, 2011).

Adverse effects

Propofol's effect on the NMDA and GABA receptors can cause irreversible neurologic dysfunctions (Cattano et al., 2008; Ikonomidou et al., 2001). Just as in ketamine exposure, the detrimental effects to the brain (by propofol) depend on the developmental stage of the exposed individual (Ikonomidou et al., 2001; Kozinn et al., 2006). This pharmaceutical might be a possible neurotoxicant in the developing human brain, and is therefore not recommended for children under the age of three. Even though, it is still used within pediatric or obstetric procedures (Wilder et al., 2009).

Neuro proteins

Three neuroproteins were analyzed and compared to protein levels in control animals. O'Callaghan (1988) determined that neuroproteins could be useful to help determine damage to the CNS during brain development. Therefore, these following proteins, which are all involved in (and important for) the BGS, are used in this experiment.

PSD-95

The Postsynaptic Density protein 95 (PSD-95) is the most abundant of the four known proteins within the PSD family. As the name suggests, it is present at the postsynaptic membrane in the glutamatergic synapses in the brain (Béïque et al., 2006; Kim & Sheng, 2004; Wenthold et al., 2003). The protein organizes the postsynaptic density (PSD) by binding glutamate receptors (NMDA), and other proteins present at the postsynaptic membrane, to the synapse. The protein also determines size and strength of the synapse (Kim & Sheng, 2004; Sheng & Sala, 2001; Wenthold et al., 2003). In addition, PSD-95 adds AMPA receptors to synapses, which increases synaptic transmission (Ehrlich & Malinow, 2004). Besides regulating synaptic transmission and plasticity it is also involved in synapse maturation and protein trafficking (Béïque & Andrade, 2003; El Husseini et al., 2000). Due to its clustering, PSD-95 can be used as a marker of excitatory synapses (Wenthold et al., 2003).

In vivo studies have presented PSD-95 as a protein that is involved in behavioral responses, learning and memory (Kim & Sheng, 2004). Therefore, a loss of this protein has shown to reduce synapses and affect plasticity and learning (Béïque et al., 2006). If PSD-95 is overexpressed it will promote dendritic spine growth in neurons and excitatory currents mediated by AMPA receptors (Kim & Sheng, 2004). Even though PSD-95 is involved in organizing NMDA receptors, their excitatory currents are not affected by up- or down regulation of the protein. The ontogeny of PSD-95 includes an increase at synapses during developmental AMPAfication (Sans et al., 2000). Thereafter, there is a deletion of PSD-95 (Béïque et al., 2006).

GluR1

Glutamate receptors are abundant in the vertebrate central nervous system (CNS) and glutamate, which is involved in synaptic plasticity, is a major neurotransmitter in the brain. The entry of calcium through glutamate receptor channels is considered important during development since it has been suggested that this receptor is connected to learning and memory (Hollmann & Heinemann, 1994; Dingledine et al., 1999).
The glutamate receptor subtype 1 (GluR1) is a subunit of an ionotropic glutamate receptor named α-amino-3-hydrozsay-5-methylisoxazole-4-propionic acid (AMPA). This receptor is a tetrameric ion channel located at postsynaptic membranes and mediates synaptic strength and fast excitatory synaptic transmission. The activation of AMPA results in depolarization of the synaptic terminal and imbalances in AMPA expression can be involved in mood disorders and psychosis. (Kerner, 2009; Lee et al., 2003).

If the AMPA receptor becomes increasingly permeable to Ca^{2+} early death of the organism can occur, which reveals detrimental effects (Rozov et al., 1998; Meyers et al., 1999). A reduction in GluR1 levels can block sensitization, for example to morphine by contributing to behavioral adaptations induced by drugs (Dingledine et al., 1999).

SYP

One of the first synaptic vesicle (SV) proteins to be identified and cloned was synaptophysin, a component in the membrane of SV at pre-synaptic nerve endings (Weidenmann & Franke, 1985; De Camilli et al., 1988). It is involved in formation and cycling of SV, which in turn is needed for communication between neurons, although its exact function in the SV life cycle is not yet fully understood (Evans & Cousin, 2005; Sarnat & Born, 1999). It is known that the function of this integral membrane protein includes neurotransmitter release, by exocytosis, and it can be used to determine neuronal density (Valtorta et al., 2004; Sarnat & Born, 1999). Kwon and Chapman (2011) demonstrated that the abundant membrane protein (8% of total SV protein) SYP was needed for endocytosis in hippocampal neurons. Recent studies have also shown that loss of SYP can lead to learning deficits and retardation due to effects on long-term potentiation (LTP) (Lynch, 2004; Schmitt et al., 2009; Tarpey et al., 2009). A decrease in SYP will also reduce long term synaptic plasticity, while an overexpression will increase the regularity of synaptic currents; however, it will not increase the amount (Janz et al., 1999; Valtorta et al., 2004).

The ontogeny of synaptophysin in mice involves a great, almost linear, increase of protein directly after birth and continuing for the first four weeks of life. The speed (of protein increase) in the cerebral cortex peaks around PND ten, in line with the BGS, and somewhat earlier in the hippocampus (Viberg, 2009).

Aim

The aim of this study is to determine how etomidate affects the developing brain. Neuroproteins, that are important for brain development, are used as markers to detect indications of neurotoxicity in an early state of life. The human brain develops in the pre- and postnatal state. Since late term fetuses, infants and toddlers are being increasingly exposed to toxic substances, it is important to map the vulnerability of the developing human brain.

Materials and methods

The method of this experiment was carried out according to Viberg (et al.) 2008 and 2009. Altogether, there were six groups with individuals from twenty different litters. This experiment and another experiment for behavioral testing had a total of 120 individuals combined.

In the experiment that resulted in this report five groups were exposed to one of the pharmaceuticals etomidate, ketamine or propofol and the sixth group was used as a control. The test animals were exposed subcutaneously to a control (0.9% saline solvent), a low dose (0.3 mg/kg BW), a medium dose (3 mg/kg BW) or a high dose (10 mg/kg BW) of etomidate. The last two groups were exposed to propofol (60 mg/kg BW) or ketamine (50 mg/kg BW).

In this study, half of all animals were used (60). In connection to this study, the other half was allowed to reach adulthood. After four months they were tested for behavioral disturbances (Viberg, personal communication). The groups exposed to ketamine and propofol were used as positive (ketamine) and negative (propofol) controls for behavioral disturbances.

Exposure

Etomidate (Etomidate-Lipuro 2 mg/ml Emulsion for injection B. Braun Melsungen AG) and Propofol (Diprivan 10 mg/ml Astra. Södertälje, Sweden) were purchased from Apoteksbolaget. Ketamine (Ketalar® 50 mg/ml Pfizer Inc. New York, USA) was purchased from Pfizer Inc. Pregnant Naval Medical Research Institute (NMRI) mice were purchased from Scanbur, Sollentuna, Sweden and housed individually in plastic cages in a room with an ambient temperature of 22 °C. They had 12/12 h cycle of light and dark and free access to standardized food pellets (Lactamin, Stockholm, Sweden) and tap water *ad libitum*.

The day of birth was assigned PND 0; the litters were culled to 10–14 pups within 48 h after birth. The experiment was conducted in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC), after approval from the local ethical committee (Uppsala University and Agricultural Research Council) and by the Swedish Committee for Ethical Experiments on Laboratory Animals, approval number C185/9.

On PND 10, the mice were given 0.3, 3 or 10 mg etomidate/kg BW; control mice received 10 mL/kg BW of a 0.9% saline solution. The mice exposed to propofol received a dose of 60 mg/kg BW and the mice exposed to ketamine received 50 mg/kg BW. All animals received the pharmaceuticals as a single subcutaneous injection to the neck. The animals were euthanized 24 hours after the exposure to etomidate, ketamine or propofol. The cerebral cortex and hippocampus brain regions were collected, frozen in liquid nitrogen and stored in –80 °C until protein analysis.

Homogenization

Cerebral cortex and hippocampus were homogenized in RIPA cell lysis buffer (50 mM Tris–HCl; pH 7.4; 150 mM NaCl; 1 mM EDTA; 1 mM EGTA; 1% Triton X-100; 20 mM sodium pyrophosphate; 2 mM sodium orthovanadate; 1% sodium deoxycholate and 0.1% SDS) with the addition of 5 μL protease inhibitor cocktail (Protease Inhibitor Cocktail Set III, Calbiochem) per mL of RIPA cell lysis buffer. The homogenate was then centrifuged at 14,000 × *g* for 10 min at 4 °C and the supernatant was collected and stored in –80 °C until use.

BCA

The total protein concentration (μg/μl) in the supernatant from the test animal's hippocampi and cortices was determined using the BCA assay method (Pierce).

Slot Blot

The total protein amount used for PSD-95 was 5 μg, for GluR1 and SYP 3 μg was used. The protein supernatants were diluted in sample buffer to a final volume of 200 μL containing the desired protein amount. The diluted supernatant was then applied in duplicates to a nitrocellulose membrane (0.45 mm, Bio-Rad) soaked in TBS buffer [NaCl (0.9%), Tris–HCl (42.1 mM) and Tris–Base (7.5 mM)], using a Bio-Dot SF microfiltration apparatus (Bio-Rad). The membranes were dried at 60 °C for 5 min, fixed in a 25% isopropanol and 10% acetic acid solution, washed in 1% TBS, and blocked for 1 h at room temperature in 5% non-fat dry milk containing 0.03% Tween-20. The membranes were then incubated overnight at 4 °C with either a mouse monoclonal PSD-95 (Millipore, MABN68) antibody (0.1 μL/mL), a rabbit polyclonal GluR1 (Millipore, AB-1504) antibody (1:1000), or a mouse monoclonal synaptophysin (Calbiochem, 573822) antibody (1:5000). Immunoreactivity was detected using a horseradish peroxidase-conjugated secondary antibody against mouse (KPL 074-1806, 1:20,000) or rabbit (KPL 074-1506, 1:20,000). Immunoreactive bands were detected using an enhanced chemiluminescent substrate (Pierce, Super Signal West Dura) with imaging on a LAS-1000 (Fuji Film, Tokyo, Japan). The intensity of bands was quantified using IR-LAS 1000 Pro (Fuji Film). The protein levels were expressed as a percentage of controls.

The specificity of the primary antibodies; PSD-95, GluR1 and synaptophysin was previously evaluated by Western Blot analysis (Viberg, 2009; Åberg, 2011). The antibodies were concluded to be specific for the respective proteins, as the analysis showed only the presence of one band at the appropriate molecular weight. Therefore, the antibodies were considered suitable for use in Slot Blot analysis. In the Slot Blot analysis the chemiluminescence increased with increased protein load. The antibodies recognize both phosphorylated and nonphosphorylated forms of the proteins.

Statistical analysis

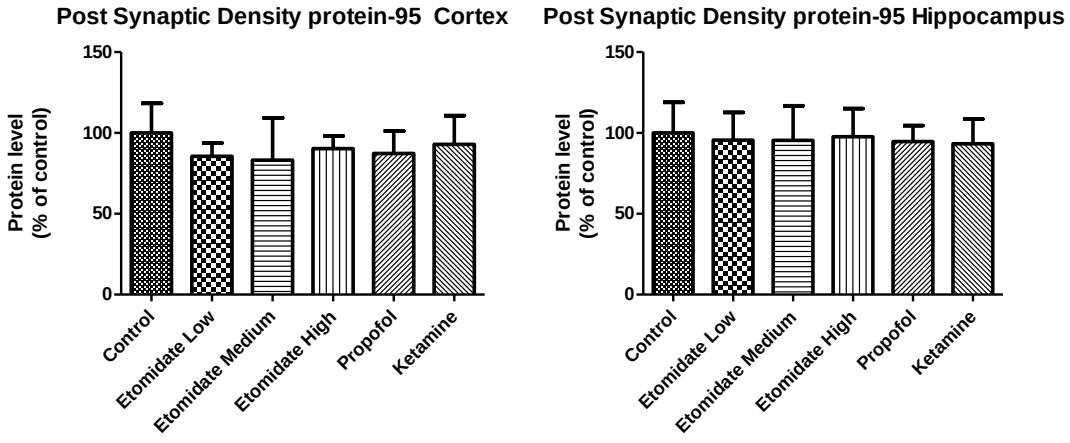
The statistical analysis compared animals from the different treatment groups, taken from multiple litters. The mean values of the chemiluminescent data from the animals were analyzed for all treatments by one-way ANOVA and Newman-Keuls Multiple Comparison Test (GraphPad Prism 5.01, San Diego, CA-USA). An ANOVA (a parametric statistical test) was chosen because our data was normally distributed. The ANOVA was used because it can compare all treatment groups against each other in addition to comparing each treatment group to the control group. A one way-ANOVA was chosen since only one variable (exposure) was of importance in this experiment.

Results

There were no visual signs of toxicity or significant deviations in body weight in the exposed animals compared to the controls throughout this experiment (data not shown).

Protein levels of PSD-95 in neonatally exposed mice

Below is a presentation of the expression of post synaptic density protein-95 (PSD-95) accompanied by the results of the statistical analysis (one-way ANOVA) that show neuroprotein expression in the cerebral cortex and hippocampus, see Figure 2.



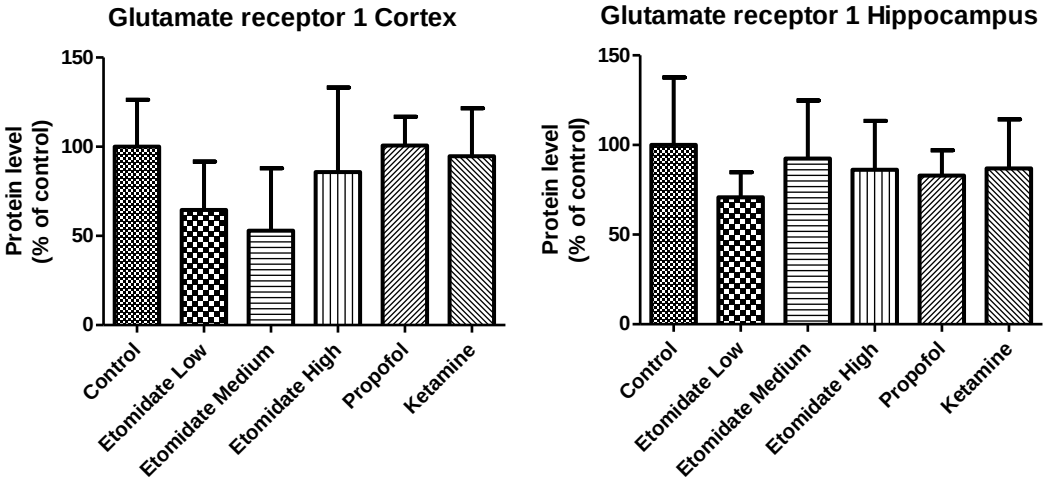
(A) **(B)**

Figure 2. PSD-95 level (mean + standard deviation) expressed in cortex (A) and hippocampus (B) as a percentage of controls in NMRI male mice exposed to control (saline solution 0.9%, n= 8), etomidate 0.3 (n=6), 3 (n=6) or 10 (n=6) mg/kg BW, ketamine (50 mg/kg BW, n=6) or propofol (60 mg/kg BW, n=6) on postnatal day ten and sacrificed 24 hours later.

After exposure to a single subcutaneous dose of etomidate (0.3, 3 or 10 mg/kg BW), propofol (60 mg/kg BW) or ketamine (50 mg/kg BW) on postnatal day ten, the mice were euthanized 24 hours later. The statistical analysis showed no significant differences in protein levels of PSD-95 in the test animals compared to control animals, neither in the cortex ($p = 0.5690$, $F=0.7844$) nor in the hippocampus ($p = 0.9829$, $F = 0.1356$). Furthermore, there were no significant differences between the different treatment groups.

Protein levels of GluR1 in neonatally exposed mice

The levels of GluR1 in cortex ($p = 0.0579$ $F = 2.431$) and hippocampus ($p = 0.5407$ $F = 0.8251$) did not differ significantly between the exposed mice and the controls. In addition, the different treatment groups were not significantly different from one another, see Figure 3.

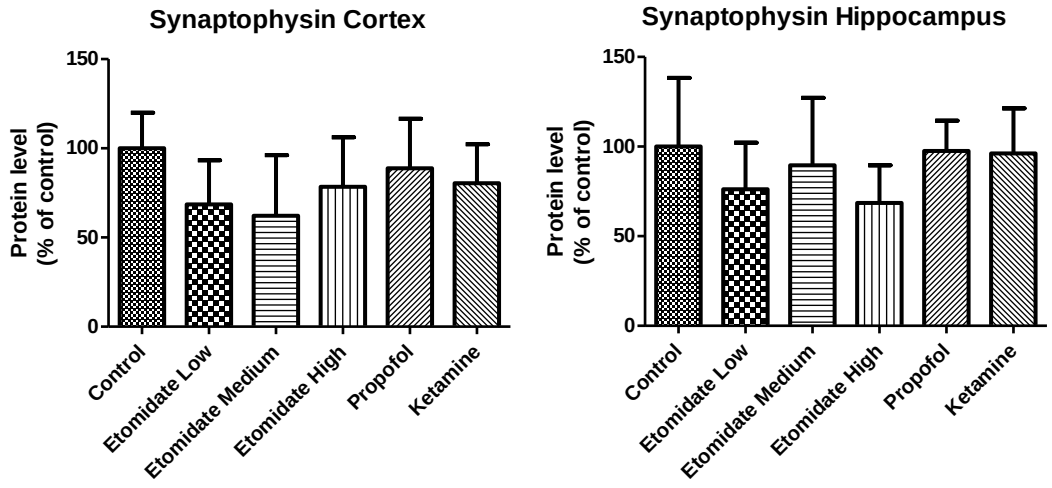


(A) **(B)**

Figure 3. GluR1 (mean + standard deviation) expressed in cortex (A) and hippocampus (B) as a percentage of controls in NMRI male mice exposed to control (saline solution 0.9%, n= 8), etomidate 0.3 (n=6), 3 (n=6) or 10 (n=6) mg/kg BW, ketamine (50 mg/kg BW, n=6) or propofol (60 mg/kg BW, n=6) on postnatal day ten and sacrificed 24 hours later.

Protein levels of SYP in neonatally exposed mice

Synaptophysin levels in the exposed animals were not significantly different from the controls in cortex ($p = 0.1973$ $F = 1.579$) or hippocampus ($p = 0.2718$ $F = 1.338$), nor were the results significantly different between the treatment groups, see Figure 4.



(A) **(B)**

Figure 4. SYP (mean + standard deviation) expressed in cortex (A) and hippocampus (B) as a percentage of controls in NMRI male mice exposed to control (saline solution 0.9%, n= 8), etomidate 0.3 (n=6), 3 (n=6) or 10 (n=6) mg/kg BW, ketamine (50 mg/kg BW, n=6) or propofol (60 mg/kg BW, n=6) on postnatal day ten and sacrificed 24 hours later.

Discussion

In this study, neonatal mice were exposed to a single subcutaneous dose of etomidate (0.3, 3 or 10 mg/kg BW), ketamine (50 mg/kg BW) or propofol (60 mg/kg BW) on PND ten. The animals were euthanized 24 hours later. The results did not show any significant differences in neuroprotein levels between the exposed animals and the controls, or between the different treatment groups.

The results of this study generate two main topics of discussion, besides discussing the three neuroproteins, which are cocktail the effects of mixed pharmaceuticals and differences in sensitivity/ susceptibility of humans compared to the animals used in exposure experiments.

Etomidate effect on PSD-95

The lack of significance in the results of PSD-95 protein levels can be due to an elevated variation in the individuals in this group. The standard deviation (SD) of the protein levels in the cortex region of the individuals who received the medium dose of etomidate (3 mg/ kg BW) can be considered to be somewhat higher than the rest of the treatment groups. This can most likely be due to inconsistencies in lab performance, e.g. when dissecting or homogenizing the brains. Another reason can be that the sample size is too low; if the sample size is increased, the SD will most likely decrease. If the variation of the individuals in the group was lower, i.e. if the individual samples were more similar, it might have resulted in a different effect since the slot blot results did indicate a decreasing trend in protein levels. However, one important thing to keep in mind is that populations will always vary because individuals will always be distinct

from one another (especially in these outbred mice), which in turn will govern the SD.

Etomidate effect on neuroproteins GluR1 and SYP

After each Slot Blot, a student's t-test was performed to get a quick estimate of differences between each treatment group and the control group. There was an evident trend that could be seen in the low (0.3 mg/kg BW) and medium (3 mg/kg BW) dose exposure of etomidate. The t-tests showed a significant decrease in neuroprotein level both in SYP and GluR1, see Table 1. These results were only seen in one of the brain regions analyzed, namely the cerebral cortex. An ANOVA performed only on etomidate exposure, compared against the control and each other, still generated significant results. However, when combined with the rest of the treatment groups (ketamine and propofol) in a one-way ANOVA, the mathematical algorithm of that analysis raised the bar for significance. This cancelled out the significant results from the t-tests (control vs. low and control vs. medium), and also the results from a previous one way-ANOVA where only the etomidate doses were compared.

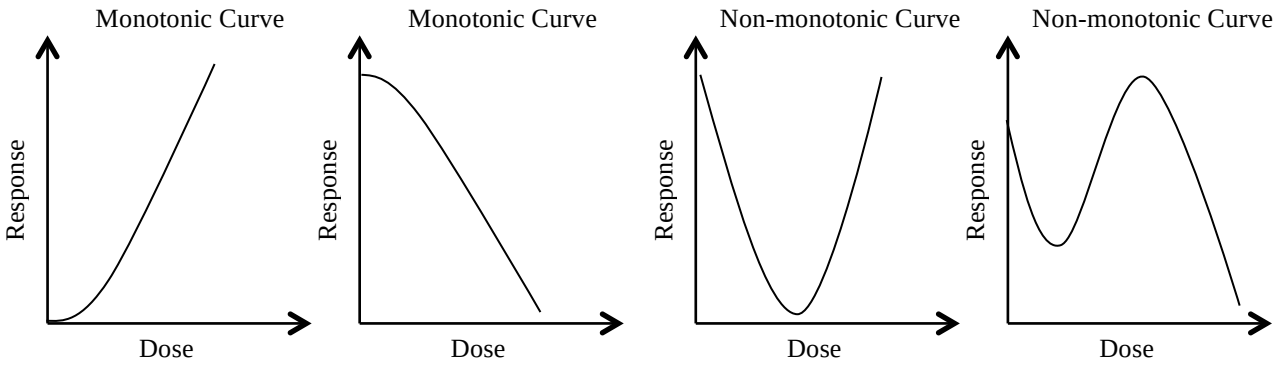
Table 1. p-values for Slot Blot results of GluR1 and SYP in the cortex and hippocampus of NMRI mice neonatally (on PND 10) exposed to 0.3 (n=6) or 3 mg etomidate/ kg BW (n=6) by a single subcutaneous injection to the neck. The animals were sacrificed 24 hours later.

Treatment group	Neuro protein	p-value	Brain region
Low (0.3 mg/ kg BW) and Medium (3 mg/ kg BW)	GluR1	> 0.1 < 0.05	Hippocampus Cortex
Low (0.3 mg/ kg BW) and Medium (3 mg/ kg BW)	SYP	> 0.1 < 0.05	Hippocampus Cortex

One puzzling thing about the results from an ANOVA comparing all the treatment groups is the disappearance of the significant value ($p = 0.0234$) of etomidate's low and medium dose compared to the control. When etomidate is used in a medical procedure, the patient, or child, is most likely to be exposed to the low dose (0.3 mg/ kg BW) of etomidate tested in this experiment, because this is the normal dose for humans (de Jong et al., 1984). Since the one-way ANOVA showed that there was a significant decrease of GluR1 and SYP protein levels in the cerebral cortex of the exposed animals, this should be of importance for future use of etomidate in the ER.

If we take a closer look at the one-way ANOVA, omitting the treatment groups with ketamine and propofol, etomidate exposure has a decreasing effect on protein levels in the two lower treatment groups (0.3 and 3 mg/kg BW) where the decrease is more pronounced the stronger the dose is. However, the decrease in the highest treatment group (10 mg/kg BW) is less than the decrease in the lower doses. This can be seen for both GluR1

and SYP protein levels in the exposed individual's cortex area, and it raises the question whether or not etomidate potentially has a non-monotonic dose-response curve, see Figure 5. Commonly, xenobiotics have a dose-response curve, which entails that the increase of a dose results in an increase or decrease in, for example, protein level. Nowadays, it is known that not all xenobiotics (e.g. endocrine disruptors) and/or endpoints follow a so-called monotonic response curve. However, I have not yet found any studies that have confirmed these suspicions



(A) **(B)**

Figure 5. (A) Standard monotonic dose-response curves follow a correlation between dose and effect. An increase in exposure increases the effect or contrariwise. (B) Non-monotonic dose response curves do not show a correlation in effect and dose, which can be seen in xenobiotics that affect the endocrine system.

Propofol and Ketamine effect on neuroproteins

In the introduction it is mentioned that the clearance of these pharmaceuticals is quite fast in humans. Despite this, effects (neuroapoptosis) of the exposure can be seen for an extended time in the individual (Ikonomidou et al., 2001). This is why studies like this are of importance.

As shown in the previous sections, propofol and ketamine did not produce significant changes in protein levels in this study. This is in agreement with Pontén (et al., 2011) where SYP was not affected by neonatal exposure to propofol. However, in the case of propofol, the lack of significant changes in protein levels might be explained by the fact that the dose used in this experiment was on the low range concerning effects, as seen in earlier studies (Cattano et al., 2008; Ikonomidou et al., 2001) The dose required for reaching a surgical plane of anesthesia in a young mouse is 200 mg/kg BW and the lowest dose that has yielded an effect (neuroapoptosis) is 50 mg/kg BW. Therefore, 60 mg/kg BW might be considered to be on the lower range of a relevant dose regarding rodent sedation by propofol (Cattano et al., 2008; Ikonomidou et al., 2001; Pontén et al., 2011). However, it might be relevant for neuroapoptosis even though our study did not see any visual signs of this. Moreover, the dose of propofol used in this study reflects the actual dose given to patients at hospitals (personal communication, Viberg).

An alarming discovery following Cattano's (et al., 2008) study on propofol showed neuroapoptotic effects in mice at 50 mg/kg BW. One major concern is that humans are given a slightly higher dose, while more sensitive. A translation of doses between species is needed so that suitable doses for humans can be administered, in order to ensure the safety of patients sedated with propofol. Nowadays, there is a difficulty in translating what a secure dose is equivalent to in a different species. Nevertheless, evidence of detrimental effects in rodents is accumulating and they bring grounds for concern (Fredriksson et al., 2007).

As mentioned in the results, there were no visible signs of toxicity in any of the exposed individuals in this study. However, animals exposed to a lower concentration of ketamine (10 and 25 mg/kg BW) at the same stage in life (PND 10) in another study showed an altered behavior after approximately two months (55 days). They became more hypoactive in the beginning and hyperactive in the end of behavioral tests compared to the controls (Viberg et al., 2008). Behavioral variables such as locomotion, rearing and total activity were analyzed. Viberg (et al., 2008) presented a clear dose-response curve for ketamine's irreversible effect on spontaneous behavior. The results of behavioral test performed on animals exposed to ketamine in connection to the present study also resulted in behavioral disturbances, which is in line with the results from previous, and other, studies (personal communication, Viberg).

Cocktail effects

Jevtovic-Todorovic (et al., 2003) claim it is common to use the two types of anesthetics (NMDA antagonists and GABA agonists) in combination to induce and keep a surgical plane of anesthesia when performing obstetric or pediatric surgical procedures. The combination of etomidate, propofol and ketamine is not examined in this study. However, improving the knowledge on how these pharmaceuticals (at different doses) affect the human body is important in order to determine their continued use in medical procedures. The effects of a dose that is harmless can be potentiated when combined with another pharmaceutical, depending on the mechanisms. Therefore, the ideal dose would be one that is strong enough to induce and keep a surgical plane of anesthesia, while not being high enough to produce detrimental effects when combined with a pharmaceutical that induces potentiation.

Since combinations of NMDA antagonists and GABA agonists are commonly used nowadays it is important to try and connect effects of early exposure with signs of toxicity later in life. There is seldom any morphological evidence. Fetal Alcohol Syndrome (FAS) was one of the first found by researchers, but the signs of toxicity from these pharmaceuticals are not gross enough to be visibly seen, even though they are of great importance. Time is progressing, and so are the methods used for determining damage during brain development. In experiments studying brain damage nowadays, visual effects (morphology) are not considered the most sensitive indicators. Therefore, a more suitable tool for determining detrimental effects during brain development is using a molecular marker, such as neuroprotein concentration (Jones et al., 1973; O'Callaghan, 1988).

Sensitivity of rodents and humans

There are some controversies regarding human and rodent sensitivity to these anesthetics. While Hayashi (et al., 2002) suggested humans are more resistant to a blockage of NMDA receptors than rodents due to our ability to compensate for neuronal damage, it is known that humans still are more sensitive to these anesthetics than rodents.

Ikonomidou (et al., 1999) determined that apoptotic neurodegeneration in the mammalian brain could be seen within 24 hours of NMDA blockage. The animals in this study were subjected to a single dose of ketamine and propofol, which both block the NMDA receptor, and euthanized 24 hours later. Consequently, there is a possibility that neuroapoptosis was induced. One important note that we also have to consider is the fraction of the BGS that 24 hours equals in the mouse, due to their much shorter life span compared to humans. Nevertheless, this does not dismiss grounds for concern on whether ketamine should be used on neonates or not. Blockage of NMDA receptors induces apoptotic effects that are most prominent at PND 5-7 (during the BGS), which coincides with the time the animals in this experiment received the ketamine dose.

Even though evidence from rat and mice are mounting, there is still an interspecies variability that has to be considered. We cannot know for sure that humans will react in the exact same way as the test animals. Nevertheless, the development and improvement of research methods nowadays has allowed researchers to find a test animal that is most alike humans in different areas when testing various substances. Studies revealing how to translate doses between species would form an essential tool for increasing the understanding of other studies that have already been conducted.

Concluding remarks

In conclusion, the effects seen at a single subcutaneous exposure of such low doses of etomidate (0.3 and 3 mg/kg BW) should be considered a contribution to the increasing knowledge on how these anesthetics affect the mammalian brain development. Furthermore, since lower doses of ketamine and propofol, than the ones used in this study, have showed detrimental effects in the developing rodent brain, there can be grounds for clinical significance concerning the future use of etomidate. This is mainly because the BGS of humans is more extended and the fact that we are more sensitive to these pharmaceuticals than rodents.

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Etomidate's effect on neuroproteins in the developing brain of neonatally exposed mice

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Table of contents

Abbreviations

BGS – Brain growth spurt
PND – Postnatal day
PSD-95 – Postsynaptic density protein-95
GluR1 – Glutamate receptor subtype 1
SYP - Synaptophysin
GABA - γ-amino butyric acid
NMDA - N-Methyl-D-aspartate
CNS – Central nervous system
SV – Synaptic vesicle
LTP – Long-term potentiation
NMRI – Naval Medical Research Institute
ANOVA – Analysis of variance
SD – Standard deviation
FAS – Fetal Alcohol syndrome

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Abstract

In today's society children, newborns and late term fetuses are commonly exposed to anesthetics. The human brain growth spurt (BGS) starts in the last trimester of pregnancy and continues throughout the first few years of life. This fact makes studies on neuroprotein levels of exposed animals of increasing importance. This study focused on using the three neuroproteins synaptophysin (SYP), glutamate receptor subtype 1 (GluR1) and postsynaptic density protein-95 (PSD-95) as markers for brain damage during neonatal development. These proteins are all involved in, and important for, the development of the brain and the BGS. Neonatal mice received a single subcutaneous dose of etomidate (0.3, 3 or 3mg/kg BW), propofol (60 mg/kg BW) or ketamine (50 mg/kg BW) on postnatal day (PND) 10, which coincides with the peak of the BGS in mice. They were euthanized 24 hours later and their neuroprotein levels were measured by slot blot chemiluminescence of the hippocampus and cerebral cortex. The results did not show any significant results in a one-way ANOVA. However, there was a trend of lower protein levels of SYP and GluR1 in the cortex area of the exposed mice.

Introduction

The development of the human brain is both pre- and postnatal. It continues through birth and the first few years of life. This period is quite prolonged in comparison to other species (Davidson & Dobbing, 1968). Due to that fact, there are concerns about what substances, and how much of them, the developing brain can be exposed to without being harmed. Nowadays, it is not unusual for newborns and toddlers to be exposed to anesthetics during surgical procedures. Consequently, it is important to increase the knowledge on how the developing brain is affected by these substances.

This study of etomidate exposure in neonatal mice is a part of an ongoing project that uses biochemical indicators to characterize effects of different chemicals on neonatal brain development. In this experiment, we will use the neuroproteins synaptophysin (SYP), postsynaptic density protein 95 (PDS-95) and glutamate receptor subtype 1 (GluR1) to map changes in the developing brain of mice exposed to the pharmaceutical etomidate. The levels of these proteins are known to increase during the brain growth spurt (BGS). In this study we will analyze the protein levels in the exposed animals and compare them to protein levels in control animals. In connection to this experiment, behavioral studies determining the effect of etomidate have also been conducted on mice. No harmful behavioral effects could be detected (personal communication, Henrik Viberg). In that experiment, two groups have been exposed to either ketamine or propofol, which are then used as positive (ketamine) and negative (propofol) controls for behavioral disturbances in this experiment.

The desired traits of an anesthetic agent include inducing a reversible sleep, relaxing muscles and suppressing reflexes, but not suppressing cardiovascular or respiratory function. In addition, a fast acting substance, that is inexpensive and easy to administer, with a rapid clearance after ceased exposure is also favourable (Nicholson, 2014). All modern anesthetics have either γ-amino butyric acid (GABA) receptor enhancing or N-Methyl-D-aspartate (NMDA) receptor blocking properties (Jevtovic-Todorovic et al., 2003).

Brain growth spurt

Davidson and Dobbing (1968) described the brain growth spurt (BGS) as a period where brain weight increases rapidly due to glia cell proliferation, the acquirement of neuronal connections, axonal and dendritic outgrowth and myelination, see figure 1. During this process, the brain is more vulnerable and susceptible to xenobiotics present in the environment. An individual who suffers a deficiency of nutrients or is exposed to a toxicant during this period would not recover and regain a fully developed brain regardless of the time for rehabilitation given (Dobbing & Smart, 1974). Dobbing and Smart (1974) concluded that exposures to xenobiotics during the BGS does not delay brain development, they suppress it entirely. Therefore, the individual should desirably not be exposed to xenobiotics during this period.

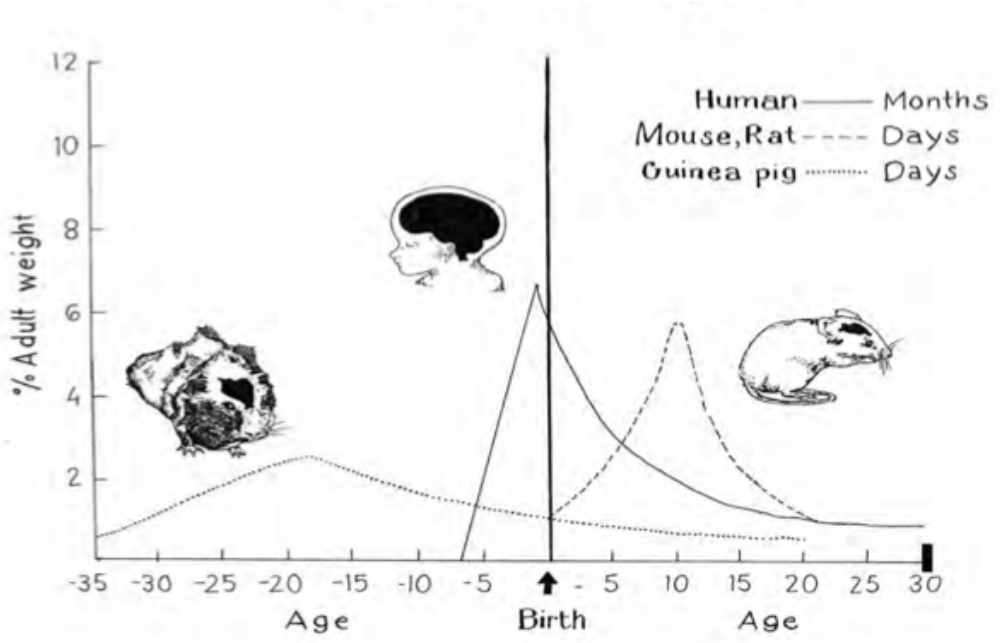


Figure 1. Timespan of the brain growth spurt (BGS) period in man (months), guinea pig (days), mouse and rat (days) in relation to birth (Davidson & Dobbing, 1968; Eriksson unpublished. Illustration by Ylva Stenlund).

Brain development differs between species. Mice have a BGS during the neonatal period, which extends from just after birth to the first few weeks of life, peaking around postnatal day (PND) ten (Dobbing & Sands, 1979). This study focuses on the hippocampus and the cortex regions of the brain; the neuroproteins tested here are involved in the BGS in different ways and have a distinct ontogeny (Viberg, 2009). In rodents, the hippocampus is involved in spatial learning as well as the recognition and working memory. It is also suggested to be involved in more recent memories. Test animals that have lesions in this area perform more poorly in certain spatial learning tasks (Lynch, 2004). The cortex is also involved in spatial learning and working memory. It is used for remote, or long term, memories. Lesions in the cortex area are considered to be more serious than lesion is the hippocampus since they can lead to severe impairments (Lynch, 2004).

Since damage to the hippocampus and cerebral cortex will affect learning and memory it will also affect the behavior of the exposed animal (Viberg et al., 2008), e.g. habituation is connected with these abilities (spatial learning) (Daenen et al., 2001; Giovannini et al., 2001).

The GABA receptor

The human BGS starts in the last trimester and continues during the first few years of life. During this time the GABA and NMDA receptor systems develop (Davidson and Dobbing, 1968; Dobbing & Sands, 1979; Ikonomidou et al., 1989; McDonald et al., 1988). The GABA receptor natural ligand is an amino acid named γ-Aminobutyric acid (GABA). GABA is an inhibitory neurotransmitter accountable for most synaptic inhibition of neurons (1/3 of all synapses) and is located in the central nervous system (CNS) of mammals (Bloom & Iversen, 1971; McCormick, 1989). An excessive activation of this ligand gated ion channel during the BGS can cause an inhibition of the communication, which induces a widespread apoptotic neurodegeneration (Ikonomidou et al., 2001).

The NMDA glutamate receptor

The ionotropic NMDA glutamate receptor is a ligand gated ion channel and therefore highly permeable to calcium ions. In addition, it is involved in many neuronal functions, e.g. spatial memory (Lynch, 2004; Wenthold et al., 2003). As the name suggests, a natural ligand of the NMDA receptor is glutamate, but also glycine (Guerrini et al., 1995). If the NMDA system is blocked, for a period of hours, during synaptogenesis in the developing brain that will cause an extensive apoptotic neurodegeneration in the brain due to the system's sensitivity to excitotoxic degeneration (Ikonomidou et al., 2001). In addition, the acquisition of spatial learning will be inhibited (Lynch, 2004).

Etomidate

The pharmaceutical etomidate (R-1-ethyl-1-(a-methylbenzyl)-imidazole-5-carboxylate) is a heterocyclic, non-barbital, imidazole derivative, which is used as an anesthetic agent. It affects the body by activating the GABA receptor (Uschida et al., 1995). This sedative was synthesized in the 1960's and introduced in Europe a decade later; its introduction to the U.S. was in the early 1980's (Ruth et al. 2001; Jackson, 2005). Today it is generally used on trauma patients, when performing endotracheal intubation (Jackson, 2005). Ever since the use of etomidate began, it was regarded as one of the more prominent anesthetic agents due to its rapid anesthetic properties (10 sec), minimal side effects and fast dissipation of clinical effects after ceased exposure. At first, these properties led to etomidate being used for prolonged sedation during surgical procedures (Fragen et al., 1984; Newberg Milde et al., 1985; Ruth et al., 2001; Streisand et al. 1998). However, it was soon discovered that etomidate increased mortality when used for a long-term sedation. Nowadays, etomidate is only used for short-term sedation (6-8 min) (Desborough, 2000; Jackson, 2005; Plewa et al., 1997).

Etomidate is mainly given intravenously, but can also be delivered through the oral mucosa (trans mucosal), which entails being absorbed through the cheek or under the tongue. This way is preferred to oral (swallowed) because the pharmaceutical reaches the blood stream faster and avoids hepatic metabolism (Ruth et al., 2001; Streisand et al., 1998). The average dose of etomidate in adults is 0.2-0.4 mg/kg body weight or 16-30 µg/kg body weight, depending on single or repetitive induction (de Jong et al., 1984; Fragen et al., 1984; Schenarts et al., 2001; Van Hamme et al., 1978).

Metabolism and excretion

The half-life of etomidate in humans is about 75 minutes. After 4-5 half-lives a substance is considered to be entirely eliminated from the body. The clearance of etomidate and its metabolites, which occurs through ester hydrolysis, should therefore be complete within 24 hours (Bahn et al., 2012). However, Fellows (et al., 1983) noted that adrenocortical suppression after induction of etomidate lasted for 4 days after the use was discontinued. It is not uncommon for the toxic effects of an exposure to outlast the presence of the substance in the body. These effects are also important for the desired traits of an anesthetic. Etomidate is ultimately metabolized in the liver and excreted by the kidneys (Banh et al., 2012). Only a small percentage of etomidate is excreted unchanged (2%) in the urine (Van Hamme et al., 1978).

Adverse effects

Despite the fact that etomidate is one of the most commonly used anesthetics for critically injured people it has some severe side effects. These entail adrenocortical suppression, myoclonus, vomiting and nausea (Fellows et al., 1983; Plewa et al., 1997; Streisand et al., 1998). The suppression of the adrenal cortex is an unusual side effect for an anesthetic, but a very important one for the continued use of etomidate, since the function of the adrenal glands involve secreting hormones that control body functions such as sexual maturation, metabolic processes, and the balance of salt and water (Plewa et al., 1997).

At first, scientists described a suppression of the adrenal gland that was thought to occur only during long-term induction (Fellows et al., 1983; Streisand et al., 1998; Varga et al., 1993). However, de Jong (et al., 1984) claim that short-term induction of etomidate also affects biosynthesis of adrenal steroids in an acute way. Varga (et al., 1993) indicated that the decrease in cortisol levels could be a result of etomidate blocking mitochondrial 11β-hydroxylation activity. This leads to a decreased conversion of 11-deoxycortisol to cortisol (Plewa et al., 1997; Schenarts et al., 2001).

Ketamine

Human use of the pharmaceutical ketamine (2-(2-chlorophenyl)-2-(methylamino)-cyclohexanone) can be either recreational, as an antidepressant, or it can be routinely used as an anesthetic during, for example, pediatric or obstetric medicine. Ketamine is an NMDA antagonist, which means that it has the ability to block these glutamate receptors. The mode of action of ketamine, beyond affecting the NMDA receptor is not fully understood (Ikonomidou et al., 2001).

The use of ketamine as an anesthetic first started in the mid 1960's (Domino et al., 1965). It was considered a safe and unique anesthetic since it induced dissociative anesthesia, which is a form of anesthesia that does not necessarily include unconsciousness.

Metabolism and excretion

To be able to excrete ketamine it has to be converted into a more hydrophilic compound. This is achieved by demethylation of its metabolites, a phase I reaction (oxidation). Once converted into phase I metabolites, they are excreted as glucoronide conjugates through the urine (Meyer & Maurer, 2011). Some metabolites can be detected in the urine within 24 hours and the parent compound has been identified in the urine two weeks after exposure. The concentration of the parent compound in the urine is relatively low compared to the concentration of the metabolites (Meyer & Maurer, 2011).

Adverse effects

As mentioned in the introduction, ketamine is used as a positive control for behavioral disturbances. Intoxication by ketamine can demonstrate cognitive difficulties following hippocampal dysfunction depending on the developmental stage of the exposed individual. The reversibility of detrimental effects caused by ketamine exposure, or use, also depends on age. Studies have indicated that apoptosis is common when the developing central nervous system (CNS), i.e. younger individuals, are exposed to ketamine, while other types of cell death affect more mature individuals (Hayashi et al., 2002; Ikonomidou et al., 1999; Majewski-Tiedeken et al., 2008).

Propofol

The general anesthetic propofol (2,6-diisopropylphenol) is a non-barbiturate used in clinical practice. Propofol is a GABA receptor agonist and NMDA glutamate receptor antagonist. The benefits of propofol are, like etomidate, its rapid onset and short duration. Its effect is exerted on the hippocampus.

Metabolism and excretion

Propofol has the shortest half-life of the pharmaceuticals tested in this experiment. It is metabolized either by direct glucoronidation or by hydroxylation followed by glucoronidation and about 50% is excreted as the parent compound (Meyer & Maurer, 2011).

Adverse effects

Propofol's effect on the NMDA and GABA receptors can cause irreversible neurologic dysfunctions (Cattano et al., 2008; Ikonomidou et al., 2001). Just as in ketamine exposure, the detrimental effects to the brain (by propofol) depend on the developmental stage of the exposed individual (Ikonomidou et al., 2001; Kozinn et al., 2006). This pharmaceutical might be a possible neurotoxicant in the developing human brain, and is therefore not recommended for children under the age of three. Even though, it is still used within pediatric or obstetric procedures (Wilder et al., 2009).

Neuro proteins

Three neuroproteins were analyzed and compared to protein levels in control animals. O'Callaghan (1988) determined that neuroproteins could be useful to help determine damage to the CNS during brain development. Therefore, these following proteins, which are all involved in (and important for) the BGS, are used in this experiment.

PSD-95

The Postsynaptic Density protein 95 (PSD-95) is the most abundant of the four known proteins within the PSD family. As the name suggests, it is present at the postsynaptic membrane in the glutamatergic synapses in the brain (Béïque et al., 2006; Kim & Sheng, 2004; Wenthold et al., 2003). The protein organizes the postsynaptic density (PSD) by binding glutamate receptors (NMDA), and other proteins present at the postsynaptic membrane, to the synapse. The protein also determines size and strength of the synapse (Kim & Sheng, 2004; Sheng & Sala, 2001; Wenthold et al., 2003). In addition, PSD-95 adds AMPA receptors to synapses, which increases synaptic transmission (Ehrlich & Malinow, 2004). Besides regulating synaptic transmission and plasticity it is also involved in synapse maturation and protein trafficking (Béïque & Andrade, 2003; El Husseini et al., 2000). Due to its clustering, PSD-95 can be used as a marker of excitatory synapses (Wenthold et al., 2003).

In vivo studies have presented PSD-95 as a protein that is involved in behavioral responses, learning and memory (Kim & Sheng, 2004). Therefore, a loss of this protein has shown to reduce synapses and affect plasticity and learning (Béïque et al., 2006). If PSD-95 is overexpressed it will promote dendritic spine growth in neurons and excitatory currents mediated by AMPA receptors (Kim & Sheng, 2004). Even though PSD-95 is involved in organizing NMDA receptors, their excitatory currents are not affected by up- or down regulation of the protein. The ontogeny of PSD-95 includes an increase at synapses during developmental AMPAfication (Sans et al., 2000). Thereafter, there is a deletion of PSD-95 (Béïque et al., 2006).

GluR1

Glutamate receptors are abundant in the vertebrate central nervous system (CNS) and glutamate, which is involved in synaptic plasticity, is a major neurotransmitter in the brain. The entry of calcium through glutamate receptor channels is considered important during development since it has been suggested that this receptor is connected to learning and memory (Hollmann & Heinemann, 1994; Dingledine et al., 1999).
The glutamate receptor subtype 1 (GluR1) is a subunit of an ionotropic glutamate receptor named α-amino-3-hydrozsay-5-methylisoxazole-4-propionic acid (AMPA). This receptor is a tetrameric ion channel located at postsynaptic membranes and mediates synaptic strength and fast excitatory synaptic transmission. The activation of AMPA results in depolarization of the synaptic terminal and imbalances in AMPA expression can be involved in mood disorders and psychosis. (Kerner, 2009; Lee et al., 2003).

If the AMPA receptor becomes increasingly permeable to Ca^{2+} early death of the organism can occur, which reveals detrimental effects (Rozov et al., 1998; Meyers et al., 1999). A reduction in GluR1 levels can block sensitization, for example to morphine by contributing to behavioral adaptations induced by drugs (Dingledine et al., 1999).

SYP

One of the first synaptic vesicle (SV) proteins to be identified and cloned was synaptophysin, a component in the membrane of SV at pre-synaptic nerve endings (Weidenmann & Franke, 1985; De Camilli et al., 1988). It is involved in formation and cycling of SV, which in turn is needed for communication between neurons, although its exact function in the SV life cycle is not yet fully understood (Evans & Cousin, 2005; Sarnat & Born, 1999). It is known that the function of this integral membrane protein includes neurotransmitter release, by exocytosis, and it can be used to determine neuronal density (Valtorta et al., 2004; Sarnat & Born, 1999). Kwon and Chapman (2011) demonstrated that the abundant membrane protein (8% of total SV protein) SYP was needed for endocytosis in hippocampal neurons. Recent studies have also shown that loss of SYP can lead to learning deficits and retardation due to effects on long-term potentiation (LTP) (Lynch, 2004; Schmitt et al., 2009; Tarpey et al., 2009). A decrease in SYP will also reduce long term synaptic plasticity, while an overexpression will increase the regularity of synaptic currents; however, it will not increase the amount (Janz et al., 1999; Valtorta et al., 2004).

The ontogeny of synaptophysin in mice involves a great, almost linear, increase of protein directly after birth and continuing for the first four weeks of life. The speed (of protein increase) in the cerebral cortex peaks around PND ten, in line with the BGS, and somewhat earlier in the hippocampus (Viberg, 2009).

Aim

The aim of this study is to determine how etomidate affects the developing brain. Neuroproteins, that are important for brain development, are used as markers to detect indications of neurotoxicity in an early state of life. The human brain develops in the pre- and postnatal state. Since late term fetuses, infants and toddlers are being increasingly exposed to toxic substances, it is important to map the vulnerability of the developing human brain.

Materials and methods

The method of this experiment was carried out according to Viberg (et al.) 2008 and 2009. Altogether, there were six groups with individuals from twenty different litters. This experiment and another experiment for behavioral testing had a total of 120 individuals combined.

In the experiment that resulted in this report five groups were exposed to one of the pharmaceuticals etomidate, ketamine or propofol and the sixth group was used as a control. The test animals were exposed subcutaneously to a control (0.9% saline solvent), a low dose (0.3 mg/kg BW), a medium dose (3 mg/kg BW) or a high dose (10 mg/kg BW) of etomidate. The last two groups were exposed to propofol (60 mg/kg BW) or ketamine (50 mg/kg BW).

In this study, half of all animals were used (60). In connection to this study, the other half was allowed to reach adulthood. After four months they were tested for behavioral disturbances (Viberg, personal communication). The groups exposed to ketamine and propofol were used as positive (ketamine) and negative (propofol) controls for behavioral disturbances.

Exposure

Etomidate (Etomidate-Lipuro 2 mg/ml Emulsion for injection B. Braun Melsungen AG) and Propofol (Diprivan 10 mg/ml Astra. Södertälje, Sweden) were purchased from Apoteksbolaget. Ketamine (Ketalar® 50 mg/ml Pfizer Inc. New York, USA) was purchased from Pfizer Inc. Pregnant Naval Medical Research Institute (NMRI) mice were purchased from Scanbur, Sollentuna, Sweden and housed individually in plastic cages in a room with an ambient temperature of 22 °C. They had 12/12 h cycle of light and dark and free access to standardized food pellets (Lactamin, Stockholm, Sweden) and tap water *ad libitum*.

The day of birth was assigned PND 0; the litters were culled to 10–14 pups within 48 h after birth. The experiment was conducted in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC), after approval from the local ethical committee (Uppsala University and Agricultural Research Council) and by the Swedish Committee for Ethical Experiments on Laboratory Animals, approval number C185/9.

On PND 10, the mice were given 0.3, 3 or 10 mg etomidate/kg BW; control mice received 10 mL/kg BW of a 0.9% saline solution. The mice exposed to propofol received a dose of 60 mg/kg BW and the mice exposed to ketamine received 50 mg/kg BW. All animals received the pharmaceuticals as a single subcutaneous injection to the neck. The animals were euthanized 24 hours after the exposure to etomidate, ketamine or propofol. The cerebral cortex and hippocampus brain regions were collected, frozen in liquid nitrogen and stored in –80 °C until protein analysis.

Homogenization

Cerebral cortex and hippocampus were homogenized in RIPA cell lysis buffer (50 mM Tris–HCl; pH 7.4; 150 mM NaCl; 1 mM EDTA; 1 mM EGTA; 1% Triton X-100; 20 mM sodium pyrophosphate; 2 mM sodium orthovanadate; 1% sodium deoxycholate and 0.1% SDS) with the addition of 5 μL protease inhibitor cocktail (Protease Inhibitor Cocktail Set III, Calbiochem) per mL of RIPA cell lysis buffer. The homogenate was then centrifuged at 14,000 × *g* for 10 min at 4 °C and the supernatant was collected and stored in –80 °C until use.

BCA

The total protein concentration (μg/μl) in the supernatant from the test animal's hippocampi and cortices was determined using the BCA assay method (Pierce).

Slot Blot

The total protein amount used for PSD-95 was 5 μg, for GluR1 and SYP 3 μg was used. The protein supernatants were diluted in sample buffer to a final volume of 200 μL containing the desired protein amount. The diluted supernatant was then applied in duplicates to a nitrocellulose membrane (0.45 mm, Bio-Rad) soaked in TBS buffer [NaCl (0.9%), Tris–HCl (42.1 mM) and Tris–Base (7.5 mM)], using a Bio-Dot SF microfiltration apparatus (Bio-Rad). The membranes were dried at 60 °C for 5 min, fixed in a 25% isopropanol and 10% acetic acid solution, washed in 1% TBS, and blocked for 1 h at room temperature in 5% non-fat dry milk containing 0.03% Tween-20. The membranes were then incubated overnight at 4 °C with either a mouse monoclonal PSD-95 (Millipore, MABN68) antibody (0.1 μL/mL), a rabbit polyclonal GluR1 (Millipore, AB-1504) antibody (1:1000), or a mouse monoclonal synaptophysin (Calbiochem, 573822) antibody (1:5000). Immunoreactivity was detected using a horseradish peroxidase-conjugated secondary antibody against mouse (KPL 074-1806, 1:20,000) or rabbit (KPL 074-1506, 1:20,000). Immunoreactive bands were detected using an enhanced chemiluminescent substrate (Pierce, Super Signal West Dura) with imaging on a LAS-1000 (Fuji Film, Tokyo, Japan). The intensity of bands was quantified using IR-LAS 1000 Pro (Fuji Film). The protein levels were expressed as a percentage of controls.

The specificity of the primary antibodies; PSD-95, GluR1 and synaptophysin was previously evaluated by Western Blot analysis (Viberg, 2009; Åberg, 2011). The antibodies were concluded to be specific for the respective proteins, as the analysis showed only the presence of one band at the appropriate molecular weight. Therefore, the antibodies were considered suitable for use in Slot Blot analysis. In the Slot Blot analysis the chemiluminescence increased with increased protein load. The antibodies recognize both phosphorylated and nonphosphorylated forms of the proteins.

Statistical analysis

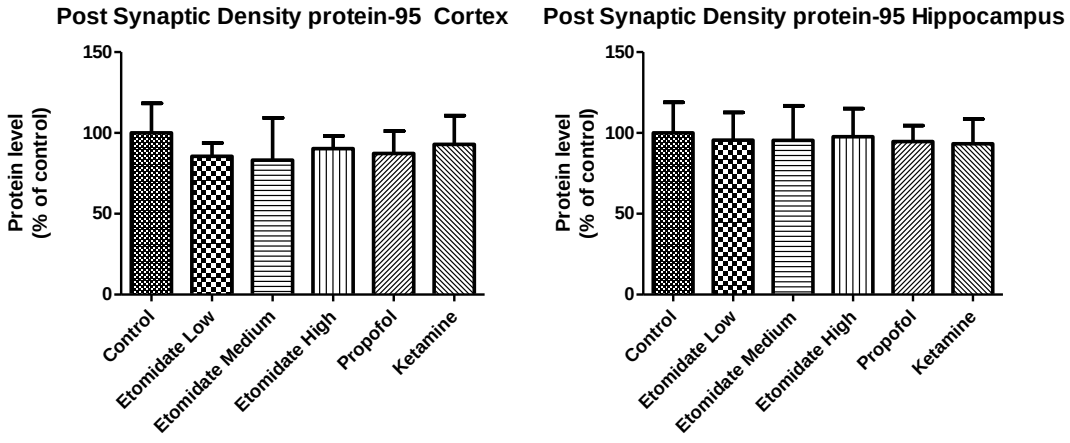
The statistical analysis compared animals from the different treatment groups, taken from multiple litters. The mean values of the chemiluminescent data from the animals were analyzed for all treatments by one-way ANOVA and Newman-Keuls Multiple Comparison Test (GraphPad Prism 5.01, San Diego, CA-USA). An ANOVA (a parametric statistical test) was chosen because our data was normally distributed. The ANOVA was used because it can compare all treatment groups against each other in addition to comparing each treatment group to the control group. A one way-ANOVA was chosen since only one variable (exposure) was of importance in this experiment.

Results

There were no visual signs of toxicity or significant deviations in body weight in the exposed animals compared to the controls throughout this experiment (data not shown).

Protein levels of PSD-95 in neonatally exposed mice

Below is a presentation of the expression of post synaptic density protein-95 (PSD-95) accompanied by the results of the statistical analysis (one-way ANOVA) that show neuroprotein expression in the cerebral cortex and hippocampus, see Figure 2.



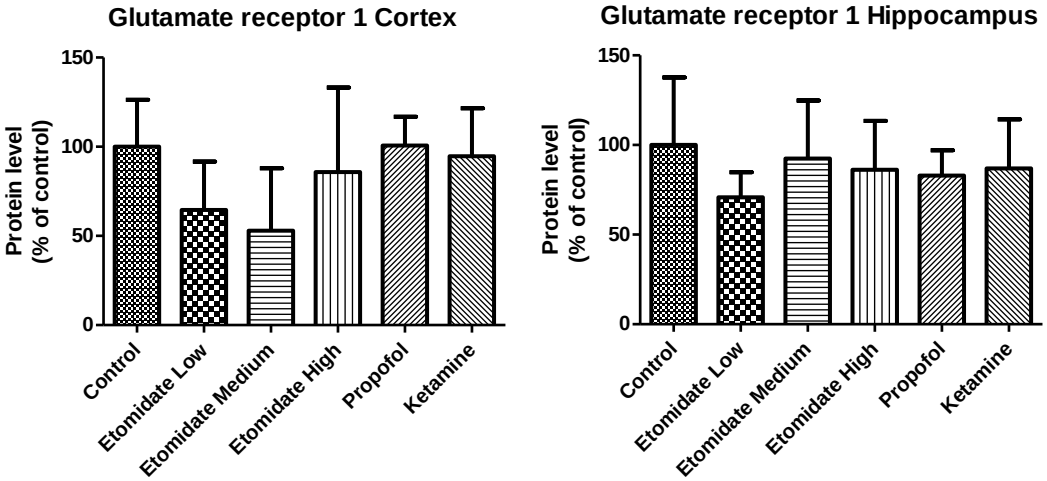
(A) **(B)**

Figure 2. PSD-95 level (mean + standard deviation) expressed in cortex (A) and hippocampus (B) as a percentage of controls in NMRI male mice exposed to control (saline solution 0.9%, n= 8), etomidate 0.3 (n=6), 3 (n=6) or 10 (n=6) mg/kg BW, ketamine (50 mg/kg BW, n=6) or propofol (60 mg/kg BW, n=6) on postnatal day ten and sacrificed 24 hours later.

After exposure to a single subcutaneous dose of etomidate (0.3, 3 or 10 mg/kg BW), propofol (60 mg/kg BW) or ketamine (50 mg/kg BW) on postnatal day ten, the mice were euthanized 24 hours later. The statistical analysis showed no significant differences in protein levels of PSD-95 in the test animals compared to control animals, neither in the cortex ($p = 0.5690$, $F=0.7844$) nor in the hippocampus ($p = 0.9829$, $F = 0.1356$). Furthermore, there were no significant differences between the different treatment groups.

Protein levels of GluR1 in neonatally exposed mice

The levels of GluR1 in cortex ($p = 0.0579$ $F = 2.431$) and hippocampus ($p = 0.5407$ $F = 0.8251$) did not differ significantly between the exposed mice and the controls. In addition, the different treatment groups were not significantly different from one another, see Figure 3.

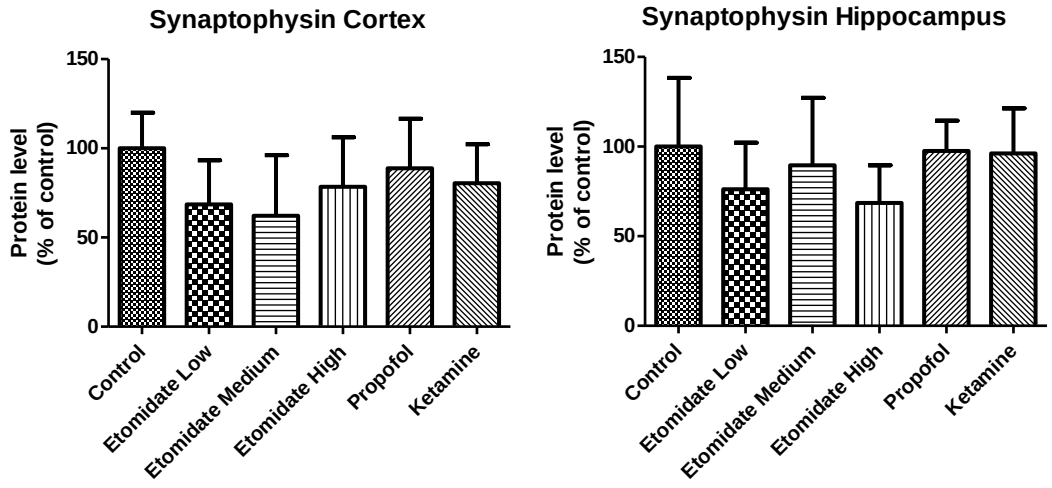


(A) **(B)**

Figure 3. GluR1 (mean + standard deviation) expressed in cortex (A) and hippocampus (B) as a percentage of controls in NMRI male mice exposed to control (saline solution 0.9%, n= 8), etomidate 0.3 (n=6), 3 (n=6) or 10 (n=6) mg/kg BW, ketamine (50 mg/kg BW, n=6) or propofol (60 mg/kg BW, n=6) on postnatal day ten and sacrificed 24 hours later.

Protein levels of SYP in neonatally exposed mice

Synaptophysin levels in the exposed animals were not significantly different from the controls in cortex ($p = 0.1973$ $F = 1.579$) or hippocampus ($p = 0.2718$ $F = 1.338$), nor were the results significantly different between the treatment groups, see Figure 4.



(A) **(B)**

Figure 4. SYP (mean + standard deviation) expressed in cortex (A) and hippocampus (B) as a percentage of controls in NMRI male mice exposed to control (saline solution 0.9%, n= 8), etomidate 0.3 (n=6), 3 (n=6) or 10 (n=6) mg/kg BW, ketamine (50 mg/kg BW, n=6) or propofol (60 mg/kg BW, n=6) on postnatal day ten and sacrificed 24 hours later.

Discussion

In this study, neonatal mice were exposed to a single subcutaneous dose of etomidate (0.3, 3 or 10 mg/kg BW), ketamine (50 mg/kg BW) or propofol (60 mg/kg BW) on PND ten. The animals were euthanized 24 hours later. The results did not show any significant differences in neuroprotein levels between the exposed animals and the controls, or between the different treatment groups.

The results of this study generate two main topics of discussion, besides discussing the three neuroproteins, which are cocktail the effects of mixed pharmaceuticals and differences in sensitivity/ susceptibility of humans compared to the animals used in exposure experiments.

Etomidate effect on PSD-95

The lack of significance in the results of PSD-95 protein levels can be due to an elevated variation in the individuals in this group. The standard deviation (SD) of the protein levels in the cortex region of the individuals who received the medium dose of etomidate (3 mg/ kg BW) can be considered to be somewhat higher than the rest of the treatment groups. This can most likely be due to inconsistencies in lab performance, e.g. when dissecting or homogenizing the brains. Another reason can be that the sample size is too low; if the sample size is increased, the SD will most likely decrease. If the variation of the individuals in the group was lower, i.e. if the individual samples were more similar, it might have resulted in a different effect since the slot blot results did indicate a decreasing trend in protein levels. However, one important thing to keep in mind is that populations will always vary because individuals will always be distinct

from one another (especially in these outbred mice), which in turn will govern the SD.

Etomidate effect on neuroproteins GluR1 and SYP

After each Slot Blot, a student's t-test was performed to get a quick estimate of differences between each treatment group and the control group. There was an evident trend that could be seen in the low (0.3 mg/kg BW) and medium (3 mg/kg BW) dose exposure of etomidate. The t-tests showed a significant decrease in neuroprotein level both in SYP and GluR1, see Table 1. These results were only seen in one of the brain regions analyzed, namely the cerebral cortex. An ANOVA performed only on etomidate exposure, compared against the control and each other, still generated significant results. However, when combined with the rest of the treatment groups (ketamine and propofol) in a one-way ANOVA, the mathematical algorithm of that analysis raised the bar for significance. This cancelled out the significant results from the t-tests (control vs. low and control vs. medium), and also the results from a previous one way-ANOVA where only the etomidate doses were compared.

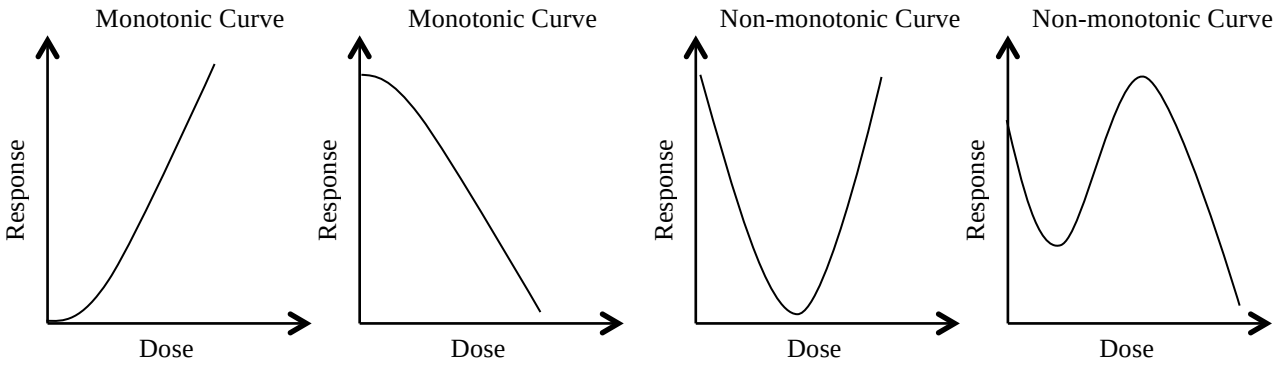
Table 1. p-values for Slot Blot results of GluR1 and SYP in the cortex and hippocampus of NMRI mice neonatally (on PND 10) exposed to 0.3 (n=6) or 3 mg etomidate/ kg BW (n=6) by a single subcutaneous injection to the neck. The animals were sacrificed 24 hours later.

Treatment group	Neuro protein	p-value	Brain region
Low (0.3 mg/ kg BW) and Medium (3 mg/ kg BW)	GluR1	> 0.1 < 0.05	Hippocampus Cortex
Low (0.3 mg/ kg BW) and Medium (3 mg/ kg BW)	SYP	> 0.1 < 0.05	Hippocampus Cortex

One puzzling thing about the results from an ANOVA comparing all the treatment groups is the disappearance of the significant value ($p = 0.0234$) of etomidate's low and medium dose compared to the control. When etomidate is used in a medical procedure, the patient, or child, is most likely to be exposed to the low dose (0.3 mg/ kg BW) of etomidate tested in this experiment, because this is the normal dose for humans (de Jong et al., 1984). Since the one-way ANOVA showed that there was a significant decrease of GluR1 and SYP protein levels in the cerebral cortex of the exposed animals, this should be of importance for future use of etomidate in the ER.

If we take a closer look at the one-way ANOVA, omitting the treatment groups with ketamine and propofol, etomidate exposure has a decreasing effect on protein levels in the two lower treatment groups (0.3 and 3 mg/kg BW) where the decrease is more pronounced the stronger the dose is. However, the decrease in the highest treatment group (10 mg/kg BW) is less than the decrease in the lower doses. This can be seen for both GluR1

and SYP protein levels in the exposed individual's cortex area, and it raises the question whether or not etomidate potentially has a non-monotonic dose-response curve, see Figure 5. Commonly, xenobiotics have a dose-response curve, which entails that the increase of a dose results in an increase or decrease in, for example, protein level. Nowadays, it is known that not all xenobiotics (e.g. endocrine disruptors) and/or endpoints follow a so-called monotonic response curve. However, I have not yet found any studies that have confirmed these suspicions



(A) **(B)**

Figure 5. (A) Standard monotonic dose-response curves follow a correlation between dose and effect. An increase in exposure increases the effect or contrariwise. (B) Non-monotonic dose response curves do not show a correlation in effect and dose, which can be seen in xenobiotics that affect the endocrine system.

Propofol and Ketamine effect on neuroproteins

In the introduction it is mentioned that the clearance of these pharmaceuticals is quite fast in humans. Despite this, effects (neuroapoptosis) of the exposure can be seen for an extended time in the individual (Ikonomidou et al., 2001). This is why studies like this are of importance.

As shown in the previous sections, propofol and ketamine did not produce significant changes in protein levels in this study. This is in agreement with Pontén (et al., 2011) where SYP was not affected by neonatal exposure to propofol. However, in the case of propofol, the lack of significant changes in protein levels might be explained by the fact that the dose used in this experiment was on the low range concerning effects, as seen in earlier studies (Cattano et al., 2008; Ikonomidou et al., 2001) The dose required for reaching a surgical plane of anesthesia in a young mouse is 200 mg/kg BW and the lowest dose that has yielded an effect (neuroapoptosis) is 50 mg/kg BW. Therefore, 60 mg/kg BW might be considered to be on the lower range of a relevant dose regarding rodent sedation by propofol (Cattano et al., 2008; Ikonomidou et al., 2001; Pontén et al., 2011). However, it might be relevant for neuroapoptosis even though our study did not see any visual signs of this. Moreover, the dose of propofol used in this study reflects the actual dose given to patients at hospitals (personal communication, Viberg).

An alarming discovery following Cattano's (et al., 2008) study on propofol showed neuroapoptotic effects in mice at 50 mg/kg BW. One major concern is that humans are given a slightly higher dose, while more sensitive. A translation of doses between species is needed so that suitable doses for humans can be administered, in order to ensure the safety of patients sedated with propofol. Nowadays, there is a difficulty in translating what a secure dose is equivalent to in a different species. Nevertheless, evidence of detrimental effects in rodents is accumulating and they bring grounds for concern (Fredriksson et al., 2007).

As mentioned in the results, there were no visible signs of toxicity in any of the exposed individuals in this study. However, animals exposed to a lower concentration of ketamine (10 and 25 mg/kg BW) at the same stage in life (PND 10) in another study showed an altered behavior after approximately two months (55 days). They became more hypoactive in the beginning and hyperactive in the end of behavioral tests compared to the controls (Viberg et al., 2008). Behavioral variables such as locomotion, rearing and total activity were analyzed. Viberg (et al., 2008) presented a clear dose-response curve for ketamine's irreversible effect on spontaneous behavior. The results of behavioral test performed on animals exposed to ketamine in connection to the present study also resulted in behavioral disturbances, which is in line with the results from previous, and other, studies (personal communication, Viberg).

Cocktail effects

Jevtovic-Todorovic (et al., 2003) claim it is common to use the two types of anesthetics (NMDA antagonists and GABA agonists) in combination to induce and keep a surgical plane of anesthesia when performing obstetric or pediatric surgical procedures. The combination of etomidate, propofol and ketamine is not examined in this study. However, improving the knowledge on how these pharmaceuticals (at different doses) affect the human body is important in order to determine their continued use in medical procedures. The effects of a dose that is harmless can be potentiated when combined with another pharmaceutical, depending on the mechanisms. Therefore, the ideal dose would be one that is strong enough to induce and keep a surgical plane of anesthesia, while not being high enough to produce detrimental effects when combined with a pharmaceutical that induces potentiation.

Since combinations of NMDA antagonists and GABA agonists are commonly used nowadays it is important to try and connect effects of early exposure with signs of toxicity later in life. There is seldom any morphological evidence. Fetal Alcohol Syndrome (FAS) was one of the first found by researchers, but the signs of toxicity from these pharmaceuticals are not gross enough to be visibly seen, even though they are of great importance. Time is progressing, and so are the methods used for determining damage during brain development. In experiments studying brain damage nowadays, visual effects (morphology) are not considered the most sensitive indicators. Therefore, a more suitable tool for determining detrimental effects during brain development is using a molecular marker, such as neuroprotein concentration (Jones et al., 1973; O'Callaghan, 1988).

Sensitivity of rodents and humans

There are some controversies regarding human and rodent sensitivity to these anesthetics. While Hayashi (et al., 2002) suggested humans are more resistant to a blockage of NMDA receptors than rodents due to our ability to compensate for neuronal damage, it is known that humans still are more sensitive to these anesthetics than rodents.

Ikonomidou (et al., 1999) determined that apoptotic neurodegeneration in the mammalian brain could be seen within 24 hours of NMDA blockage. The animals in this study were subjected to a single dose of ketamine and propofol, which both block the NMDA receptor, and euthanized 24 hours later. Consequently, there is a possibility that neuroapoptosis was induced. One important note that we also have to consider is the fraction of the BGS that 24 hours equals in the mouse, due to their much shorter life span compared to humans. Nevertheless, this does not dismiss grounds for concern on whether ketamine should be used on neonates or not. Blockage of NMDA receptors induces apoptotic effects that are most prominent at PND 5-7 (during the BGS), which coincides with the time the animals in this experiment received the ketamine dose.

Even though evidence from rat and mice are mounting, there is still an interspecies variability that has to be considered. We cannot know for sure that humans will react in the exact same way as the test animals. Nevertheless, the development and improvement of research methods nowadays has allowed researchers to find a test animal that is most alike humans in different areas when testing various substances. Studies revealing how to translate doses between species would form an essential tool for increasing the understanding of other studies that have already been conducted.

Concluding remarks

In conclusion, the effects seen at a single subcutaneous exposure of such low doses of etomidate (0.3 and 3 mg/kg BW) should be considered a contribution to the increasing knowledge on how these anesthetics affect the mammalian brain development. Furthermore, since lower doses of ketamine and propofol, than the ones used in this study, have showed detrimental effects in the developing rodent brain, there can be grounds for clinical significance concerning the future use of etomidate. This is mainly because the BGS of humans is more extended and the fact that we are more sensitive to these pharmaceuticals than rodents.

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