UPPSALA UNIVERSITET

Projektrapport från utbildningen i

EKOTOXIKOLOGI

Ekotoxikologiska avdelningen

Nr 123

Neonatal Exposure to Nicotine Alters Adult Susceptibility to Donepezil in Mice

Tommy Strömberg

TABLE OF CONTENTS

PREFACE

This Master's thesis was done as the final part of the Ecotoxicology graduate studies. The Department of Environmental Toxicology, Uppsala University, Uppsala, Sweden has been the home of this work, with some appearances at the Department of Neuroscience, Psychiatry Ulleråker, Uppsala University. I wish to express my gratitude to the persons who have contributed to my thesis work:

Per Eriksson, my superb supervisor. Thanks for all your support and positive thoughts. Thank you for offering me this research opportunity and spreading your never ending enthusiasm. Thanks for the best term anyone could ever have.

Anders Fredriksson, my "behavioural guy". Thanks for helping me with the behavioural tests and all the good advices.

Niklas Johansson and Henrik Viberg, my "vice advisors" and new found friends. Thank you for all of your good advices, your support and creating a nice atmosphere to work in.

Jesper Säfholm and Hanna Markström, my since long time friends and now "co researchers". Thank you for all your support both by helping in the lab and mental support during this work.

The work received financial support from the Swedish Research Council for Environmental, Agricultural Sciences and Spatial Planning, Foundation for Strategic Environment Research

ABSTRACT

Today it is well known that there can be periods during the mammalian brain development where exposure to xenobiotics causes greater damage and persistent damage which can not be repaired by the body itself. The developmental period and possible critical window occur at different times depending on mammalian species. One of the xenobiotics that have been shown to cause these damages is nicotine which disturbs the development of low-affinity nicotinic receptors in cerebral cortex of the neonatal mouse. It has been shown that exposure to nicotine during this critical window of brain development increases the adult susceptibility to an organophosphate toxicant.

The aim of this work was to study if neonatal exposure to nicotine could alter the susceptibility of donepezil (an agent used in the therapy of Alzheimer's disease) on adult mice. And also to study whether donepezil can cause permanent effects on adult mice neonatally exposed to nicotine.

Neonatal mice was exposed to either 33µg nicotine/kg bw s.c. or 10 ml 0.9% NaCl/kg bw s.c., twice daily between postnatal days 10-14. When the mice reached an age of 3 months they were observed for spontaneous behaviour. There were no differences in spontaneous behaviour between the saline and the nicotine treated groups. Directly after the spontaneous behaviour test the mice were injected with either 0.4 mg donepezil/kg bw or 0.8 mg donepezil/kg bw s.c. After the injection they were put back in the test chamber and observed for another 60 min period. Mice exposed neonatally to nicotine responded to donepezil with a dose-response related decrease in activity, whereas neonatally vehicle exposed mice showed no significant change. This indicates that mice exposed neonatally to nicotine have an altered susceptible to donepezil.

To observe long term effects of adult exposure to donepezil in mice neonatally exposed to nicotine, adult mice received either 0.4 mg donepezil/kg bw or 0.8 mg donepezil/kg bw s.c. once every second day for 7 days. When the mice reached an age of four and a half month they were again observed for spontaneous behaviour. The spontaneous behaviour revealed no significant differences between the different treatment groups.

This study indicates that neonatal exposure to low doses of nicotine alters the adult susceptibility to donepezil, but this altered reaction might not lead to persistent behavioural deficits.

1. INTRODUCTION

1.1. Exposure to toxic agents during life

Individuals are exposed to environmental toxic agents during their whole life, from fertilization up to adult age. The embryo and fetus are exposed via the placenta, and the amount of a toxic compound they are exposed to depend on the mothers intake of toxic agents and the agents ability to pass through the placenta. Newborns can be exposed to toxic agents by direct exposure via inhalation and/or dermal uptake and also through suckling the mother's milk (Court, Cuomo *et al.* 1996).

There are clear evidence by adverse effects of methylmercury poisoning, fetal alcohol syndrome and drug abuse during pregnancy. Studies have shown that there can be windows of vulnerability to toxic agents (Court, Cuomo *et al.* 1996). One of these periods occur during the brain growth spurt (BGS) which take place at different times of development in different mammalian species (Davison and Dobbing 1968).

1.2. Brain development

The development of the brain varies among species. Different parts of the brain are formed in different stages of development in different species. This means that all brain parts have their own critical period, where it is vulnerable to toxic agents. It has been shown that the brain weight and amount of total brain protein increases from the day of birth to 30 days after birth. The increase in whole brain membrane protein on the other hand, is taking place in the first 20 days of life. During this time the weight is nearly 13-fold (Davison and Dobbing 1968)

Although two certain stages can be seen during mammalian brain development. The first one includes development of the adult shape of the brain. This includes organogenesis and multiplication of neuroblasts. Exposure to xenobiotics during this first stage can result in malformation of the brain. The second stage includes maturation of axonal and dendritic growth, glial multiplication, myelinisation and growth in size. This period is also known as "the brain growth spurt". In humans, this period begins prenatally during the third trimester and continues for the duration of the first 1-2 years of life. The same period in rodents occurs neonatally during the first 3 weeks of life (Fig. 1) (Davison and Dobbing 1968).

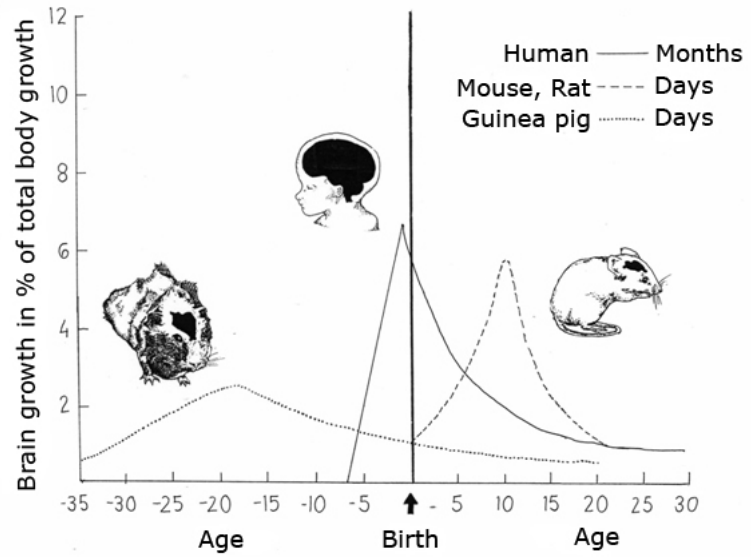

Figure 1. Rate curves of brain growth in relation to body growth and birth in different species. Values are calculated at different time intervals for each species. From Davidson and Dobbing, 1968, and Eriksson (unpublished), with permission. Illustration by Ylva Stenlund.

After the two first weeks of the BGS the development, the proliferation and migration of cells, receptor development and the synaptogenesis is almost over. Now the trimming process starts. Trimming is the process where weak or unimportant synapses are eliminated by selective cell death. This process goes on during year 2 and 3 of human life and completes the brain development (Court, Cuomo *et al.* 1996).

There has already been shown in earlier studies that exposure to low-doses of environmental agents (such as nicotine) during the brain growth spurt in mouse can result in irreversible changes in adult brain function. These effects occurs at doses that do not have permanent effects on adult mice (Nordberg, Zhang *et al.* 1991; Eriksson, Ankarberg *et al.* 2000). Studies also show that there is only for the period of a few days during neonatal development of the mouse brain that these persistent effects can be induced, namely around post-natal day (PND) 10 (Eriksson, Ahlbom *et al.* 1992; Ahlbom, Fredriksson *et al.* 1995; Eriksson 1998).

Earlier studies have shown that mice exposed to low doses (66µg/kg bw) of nicotine during this critical period of neonatal development showed a hypoactive response to nicotine in a spontaneous behaviour test, while mice treated with saline showed a hyperactive response to nicotine in the same test. In addition it was found that mice exposed neonatally to

nicotine lacked nicotinic receptors of low-affinity binding sites (Eriksson, Ankarberg *et al.* 2000).

When being exposed to toxic agents during neonatal life, the susceptibility to xenobiotics in later life stages can increase. This indicates that neonatal exposure to toxic agents can alter susceptibility of adult exposure to xenobiotics (Johansson, Fredriksson *et al.* 1995; Johansson, Fredriksson *et al.* 1996).

1.3. The cholinergic system

In a study from 1905, Langley observed the potency and specificity of drugs. He noticed that some drugs mimicked a biological response. From this study he presented the theory about hormones and neurotransmitters interacting with receptors in the cells and thereby produces their biological effects. Today this receptor concept has been accepted and improved by the actual isolation of macromolecular substances that fit all the criteria of being receptors (Cooper 1996).

The cholinergic system is one of the major transmitter systems in the brain. It fills an important function in pre- and postsynaptic signaling and involved in many physiological processes including memory and learning. (Karczmar 1975).

1.3.1 Acetylcholine (ACh)

Acetylcholine is the main neurotransmitter in the cholinergic system. It was the first substance identified as a neurotransmitter. Ach is manly localized at synapses in the central nervous system, but also in the ganglia of the visceral motor system. The substance is synthesized in nerve terminals. Its precursors are acetyl coenzyme A (acetyl CoA) and choline. Acetyl CoA is derived from the glycolysis via pyruvate. The reaction is catalyzed by choline acetyltransferase (ChAT). The transmitter is loaded into vesicles of the presynaptic neuron (Purves, Augustine *et al.* 2004). When a signal reaches the presynaptic nervending, the vesicles fuses with the membrane and the acetylcholine is released into the synaptic cleft. In the membrane of the postsynaptic cell there are acetylcholine receptors which gets activated when acetylcholine binds to it. When activated these receptors starts the signal in the postsynaptic cell (Cooper 1996; Purves, Augustine *et al.* 2004). The action is terminated by the enzyme, acetylcholinesterase (AChE). AChE is localized in the synaptic cleft and hydrolyses ACh into acetate and choline. 35%-50% of the choline is then reused to produce

new acetylcholine by uptake into the presynaptic nervending by Na^+/choline transporters. (Cooper 1996; Purves, Augustine *et al.* 2004).

The cholinergic system is usually divided into two major receptor families, nicotinic and muscarinic. They are named after the substances binding to them. Both are activated by acetylcholine although they belong to different gene families (Cooper 1996).

During the brain development in rodents the activity of AChE and ChAT reaches adult values at 20 days of age (Fiedler, Marks *et al.* 1987). The specific activity for ChAT increase from 1 to 8% of adult activity between day 15 of gestation and day 7 postnatally. Then the activity increases linearly up to adult values. The concentration of ACh is 22% of adult concentration at 15 days of gestation. At birth the concentration have reached 29% and then rises to adult levels at the age of 4 weeks (Coyle and Yamamura 1976).

1.3.2. Nicotinic receptors

The nicotinic receptors were an unsolved riddle until relatively recently. By using two snake toxins, α-bungarotoxin and *Naja naja siamensis*, the receptor could be isolated and purified. These two toxins specifically bind to the nicotinic receptor and made the labeling easier (Cooper 1996).

α-Bungarotoxin is used as a marker for one type of nicotinic receptors namely α7. It has been shown that α-Bungarotoxin has a very strong affinity for the receptor and also that the binding has a peak at day 10 postnatally. This peak value is almost three times the adult value (Fiedler, Marks *et al.* 1987)

Nicotinic acetylcholine receptors (nAChRs) in the CNS are important for functional processes, such as cognitive and memory functions (Nordberg 2001). The receptors are nonselective cat ion channels that create excitatory responses in the postsynaptic nerve cell. The receptor consists of five subunits (α, β, γ, δ, ε) that is built up in a pentamere around a membrane-spanning pore (Purves, Augustine *et al.* 2004). There are 9 α (α2-α10) subunits and 3 β (β2-β4) subunits that have been cloned (Sargent 1993; Gotti and Clementi 2004). There are two different types of formations, neuromuscular and neuronal. Receptors existing in muscles or electrical organs (*Torpedo* and *Electrophorus*) consists of two α subunits and then various combinations of the other four, while the neuronal type and consists of 3α and 2β subunits. This formation makes the neuronal receptor lack sensitivity to α-bungarotoxin (Purves, Augustine *et al.* 2004).

In brain tissue after autopsy of Alzheimer's disease patients there is a consistent loss of nAChRs. Most of all the findings showed reduced levels of α3, α4 and α7 nAChR subtypes (Nordberg 2001).

1.4. Nicotine

Nicotine is an alkaloid substance that is extracted commercially from the leaves of *Nicotiana tabacum*. Products such as cigarettes, chewing tobacco, snuff and nicotine chewing gum contain nicotine, in the past it was also used as an insecticide. (Benowitz 1986). In pharmacology it is described as a ganglia stimulating substance. Nicotine is today not used therapeutically. The toxicology and pharmacology of nicotine is although of great interest because of the big tobacco consumption (Rosell and Danielsson 1975). Nicotine centrally activates reward systems and that is presumed to be the reason why tobacco is one of the most commonly used dependence-producing products (James and Nordberg 1995). The absorption of nicotine over biological membranes is pH dependent. As nicotine is a weak base (pKa = 7.9) its ionized in acidic environment. Ionized nicotine does not rapidly cross membranes. The pH in cigarette smoke varies from 5.5 – 8.5 depending on the tobacco. When the smoke reaches the alveoli of the lungs it is rapidly absorbed independent of pH. Smokers on average absorb 1 mg of nicotine per cigarette. In snuff and chewing tobacco and chewing gum the pH is buffered to an alkaline level (Benowitz 1986). Once inside the body the nicotine is metabolized by CYP2A6 to cotinin. Cotinin is then metabolized by CYP2A6 to *trans-3'-hydroxycotinine* which is excreted in the urine (Lewis, Miller *et al.* 2007).

Nicotine can cause both acute poisoning symptoms as well give rise to chronic effects. Nicotine exerts its effects by binding to the nicotinic receptor, a subclass of cholinergic receptors. Small doses of nicotine can cause increase in heart rate, elevated blood pressure and tightening blood vessels in the skin. Acute overdose of nicotine have so far only occurred from three different situations. Either it is children that accidentally ingested tobacco products, tobacco workers exposed to wet tobacco leaves or people working with nicotine-containing pesticides. The cause of the acute effects of nicotine is a rapid rise in nicotine levels results in extreme stimuli of nicotinic receptors. This leads to ganglionic paralysis, nausea and increased heart rate. This reaction is often followed by slowing heart rate, a fall in blood pressure. This results in coma and in some cases death as a result of respiratory muscle paralysis (Anthony, Montine *et al.* 2001). The acute oral LD_{50} (lethal dose for 50% of the individuals) for nicotine in rats has been shown to be 50 – 60 mg/kg bw (Benowitz 1986). In

humans symptoms can be seen at 4 mg/kg b.w., and the calculated LD_{50} is around 60 mg/kg b.w. (Rosell and Danielsson 1975)

1.5. Donepezil

Donepezil is a reversible inhibitor of acetylcholinesterase and thereby have an action similar to organophosphate compounds. It prevents the hydrolysis of acetylcholine through the inhibition of AChE (Misson and Kendall 1997). It has been proved that 10 mg donepezil given once daily is an effective symptomatic treatment for Alzheimer's disease (AD) in the long term perspective. The effect ceases with time but still after 3 years the effect can be detected in Alzheimer 's disease Assessment Scale-cognitive subscale (ADAS-cog) and Clinical Dementia Rating-Sum of the Boxes scale (CDR-SB). Donepezil is also used as the active substance in the widely used AD medicine Aricept® (Rogers, Doody *et al.* 2000). The function of an organophosphate is described in Ecobichon (2001). Acetylcholinesterase has an active site with a serine hydroxyl group. This hydroxyl group reacts with organophosphate and replaces one of the side chains (Fig. 2). Without intervention, this inhibition persists until enough quantities of new AChE have been synthesized. The groups named "X", "Y" and "Z" in the picture can be very different. This plays an important role in the action and binding to the active site.

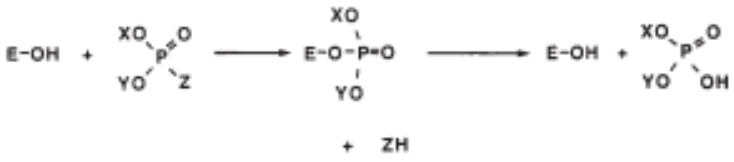

Figure 2. The interaction between an organophosphorus ester and the serine hydroxyl groups in the active site of the enzyme acetylcholine esterase (E-OH). From Ecobichon, 2001.

Studies *in vitro* have shown that the P450 isoenzymes 3A4 and 2D6 are responsible for the metabolism of donepezil. The effect of the AChE inhibitor can be affected by intake of other substances. Ketokonazol and kinidin are inhibitors of P450 enzymes and therefore the metabolism of donepezil slows down. Donepezil concentrations up to 30% higher than before have been measured when ketokonazol was administered. Substances such as rifampicin, fenytoin, karnamazepin and alcohol have enzyme inducing effects and therefore they may reduce the levels of donepezil (FASS® 2007).

Donepezil can be secreted via urine (FASS® 2007). Studies have shown that 57% of the administered ^{14}C-labeled donepezil was secreted via urine (17% as unmetabolized donepezil) and 14.5% via feces. This suggests that secretion through urine and biotransformation are the two major ways of elimination.

1.5.1. Aricept®

Aricept with the active substance donepezil is a medication used to treat Alzheimer ’s disease. Aricept has been on the market since 1996 for the treatment of patients with mild to moderate Alzheimer's. In 2006 it and was approved for the treatment of severe Alzheimer's (Pfizer Inc. and Eisai Inc. 2006).

Aricept are given once daily to Alzheimer’s patients in two different doses of 5 and 10 mg. The plasma concentration and area under curve rise in proportion to the dose, maximum plasma concentration can be measured 3 to 4 hours after taken orally. The half-life for elimination is 70 hours and daily dosage leads to a steady state within about 3 weeks. At steady state the 5 mg and 10 mg pills gives an AChE inhibition of 63.6 and 77.3% respectively (FASS® 2007).

2. AIMS

1. To study the susceptibility of donepezil on adult mice neonatally exposed to nicotine.
2. To study whether donepezil can cause permanent effects on adult mice neonatally exposed to nicotine.

3. MATERIALS AND METHODS

3.1. Chemicals

α-Bungarotoxin, N-[propionyl-^{3}H]-propionylated (55.0 Ci/mmol) where obtained from Amersham, Bucks, England while (-)nicotine-bi-(+)-tartate where obtained from Sigma, St. Louis, U.S.A and donepezil hydrochloride where obtained from TRC, Toronto, Canada.

3.2. Animals

Pregnant NMRI mice were obtained from B&K, Sollentuna, Sweden. After giving birth, each litter (adjusted to 8-14 mice within 48 hours) was kept together with its respective dam in a plastic cage in a room with an consistent temperature at 22°C and a 12 h light/12 h dark cycle.

At 10 days of age the NMRI mice received 33µg/kg body weight (-)nicotine-base (Sigma, St. Louis, U.S.A), s.c. twice daily for 5 days. Based on calculations from SCB (Swedish bureau of statistics) and Russel (1990) this amount of nicotine is absorbed when the average weight person (irrespective of sex) smokes 4-5 cigarettes a day. Before injection, the pH of the (-)nicotine solution was adjusted to about 6.5. This was done to prevent necrosis at injection area in mice.

At the age of 4 weeks the mice were weaned and the males were placed and raised in groups of 4-7 siblings in each litter. The litters were placed in a room for male mice only. The animals were supplied with standardized pellet food and tap water *ad libitum*.

At the age of 3 months the male mice received a single, s.c. dose of 0.4 mg (low dose) or 0.8 mg (high dose) donepezil hydroklorid (TRC, Toronto, Canada)per kg b.w.

3.3. Acetylcholine esterase activity

A dose finding for the inhibition of AChE was done. There were 5-6 male mice for each treatment and a total number of 34 mice. The mice were given a single injection of 0.4, 0.8mg donepezil/kg bw s.c. or 10 ml 0.9 % NaCl/kg bw s.c. After 30 min the mice were euthanized by cervical dislocation. The brain was then dissected out, medulla oblongata was removed

and the brain was then putted in ice cold pH 8 K^+ - buffer. The AChE activity was analyzed as described by Ellman, Courtney *et al*. (1961)

3.4. Behavioural tests

3.4.1. Spontaneous behaviour

The spontaneous behaviour was observed in male mice at an age of 3 months, as earlier described by Eriksson *et al.* (1990). The animals were tested once only and the test was performed between 08.00 and 12.00 under the same light and temperature conditions.

Locomotion, rearing and total activity was measured for 3 x 20 min in an automated device consisting of cages (40 x 25 x 15 cm) placed within two series of infrared beams (low level and high level) (Rat-O-Matic, ADEA Elektronik AB, Uppsala, Sweden) (Archer, Fredriksson *et al.* 1987).

Locomotion was registered when the mouse moved horizontally through the low-level grid of infrared beams.

Rearing was the vertical movement that was registered at a rate of four counts per second, whenever and as long as a single high-level grid was interrupted, i.e. the number of counts obtained was proportional to time spent rearing up.

The total activity registered all types of vibrations within the test cage, i.e. those caused by mouse movements, shaking (tremors) and grooming. This was done by a pick-up (mounted on a lever with a counter weight) which the test cage was in contact with.

3.4.2. Behavioural response to donepezil

The behavioural response to donepezil was first observed in the male mice at the age of 3 months. This test was conducted directly after the spontaneous behaviour test, as previously described (Nordberg, Zhang *et al.* 1991). The mice received either saline or donepezil injections s.c. (0.4 or 0.8 mg/kg b.w.). Subsequently the mice were observed for another 60 min period to record the response of exposure to donepezil.

In order to study persistent effects of donepezil in mice neonatally exposed to nicotine, the second observation on spontaneous behaviour was made 5 weeks after the donepezil exposure. The mice where once again observed for a 60 min period.

3.4.3. Swim maze

This test was performed on mice at the age of 4 months. A total of 45 mice, divided into 3 groups of 15 based on treatment. The animals for each group were chosen from 4 different litters.

The swim maze used was the Morris swim maze (Morris 1981). It was a grey tube, 103 cm in diameter, 35 cm deep, filled with water to 15 cm from the brim giving a water depth of 20 cm. The water temperature was 22°C. A platform was submerged 1 cm beneath the surface in the center of the northeast quadrant of the pool. The platform was a circular metal plate with a diameter of 12 cm. The quadrant opposite the one with the platform was set to be the start quadrant. The mice´s ability to find the submerged platform was observed for 5 days. The mice were given 5trials each day between 8 a.m. and 12 a.m. Before the first trial each day the mouse was placed on the platform for 30 seconds. It was then placed in the southwest quadrant facing the wall of the tube. It then had 30 seconds to locate the platform. If the mouse failed to locate the platform it was placed upon it for 30 seconds before the next trial.

The mice received five trials a day on 4 days consecutive under the same conditions. On the fifth day the platform was moved to the center of one of the quadrants next to the start quadrant. Once and again the mice had five trials with the same procedure as during day 1-4. Trials 1-20 during day 1-4 measured the mice´s spatial learning ability. Trials 21-25 during day 5 measured the mice´s relearning ability.

The observations were recorded with TSE VideoMot 2. A Video system constructed to record and measure the time, movement pattern and swim speed in finding the submerged platform.

3.5. Statistical analysis

Spontaneous and behavioural response to donepezil : The data was subjected to a split-plot ANOVA (analysis of variance) and pairwise testing between treated groups and their corresponding control groups was performed with Turkey HSD (honestly significant difference) test (Kirk 1968)

4. RESULTS

4.1.1. Effects of neonatal exposure to nicotine, spontaneous behaviour and behavioural response to donepezil in adult mice.

In Figs. 3 and 4 the results from locomotion and rearing variables in 3-month-old mice after exposure to 33 µg nicotine/kg bw s.c. twice daily , or to 10 ml 0.9% NaCl/kg bw s.c. twice daily as control between day 10 and 14 postnatally, are shown. The spontaneous motor activity was observed during the first 60 min (Fig. 3).

In both saline- and nicotine treated mice a decrease in activity over time was observed in response to the diminishing novelty to explore the test chambers. No significant group x period interactions were observed [$F_{2,156}$ = 0,22, $F_{2,156}$ = 1,16], for the locomotion and rearing respectively.

In Fig. 4 the response to donepezil in mice treated neonatally with saline or nicotine are shown. The mice were given a single injection of 0,4 or 0,8 mg donepezil/kg bw s.c., or 10 ml 0.9% NaCl/kg bw s.c., and observed for another 60 min period. Significant group x period interactions [$F_{10,108}$ = 57,61, $F_{10,108}$ = 34,94] were observed for locomotion and rearing respectively. In mice neonatally exposed to nicotine there was no difference vs. controls when challenged with saline. However, in mice neonatally exposed to nicotine and challenged with 0.8 mg donepezil/kg bw, a significant decrease in both locomotion and rearing was seen. Also in the mice neonatally treated with nicotine and challenged with 0.4 mg donepezil/kg bw showed decreased activity in locomotion variable during the first 20 min period. Pair wise testing between mice given nicotine neonatally and either 0.4 or 0.8 mg donepezil at the age of 3 months showed a significant dose-response related effect in locomotion variable during the first 20 min period.

In the second period (minute 80-100) the mice were hyperactive when given nicotine neonatally and challenged with 0.8 mg donepezil/kg bw when looking at both locomotion and rearing variables compared to the controls. During the last 20 min period

(minute 100-120) The mice given nicotine neonatally and challenged with 0,8mg donepezil/kg bw showed increased activity on locomotion variables compared to the controls.

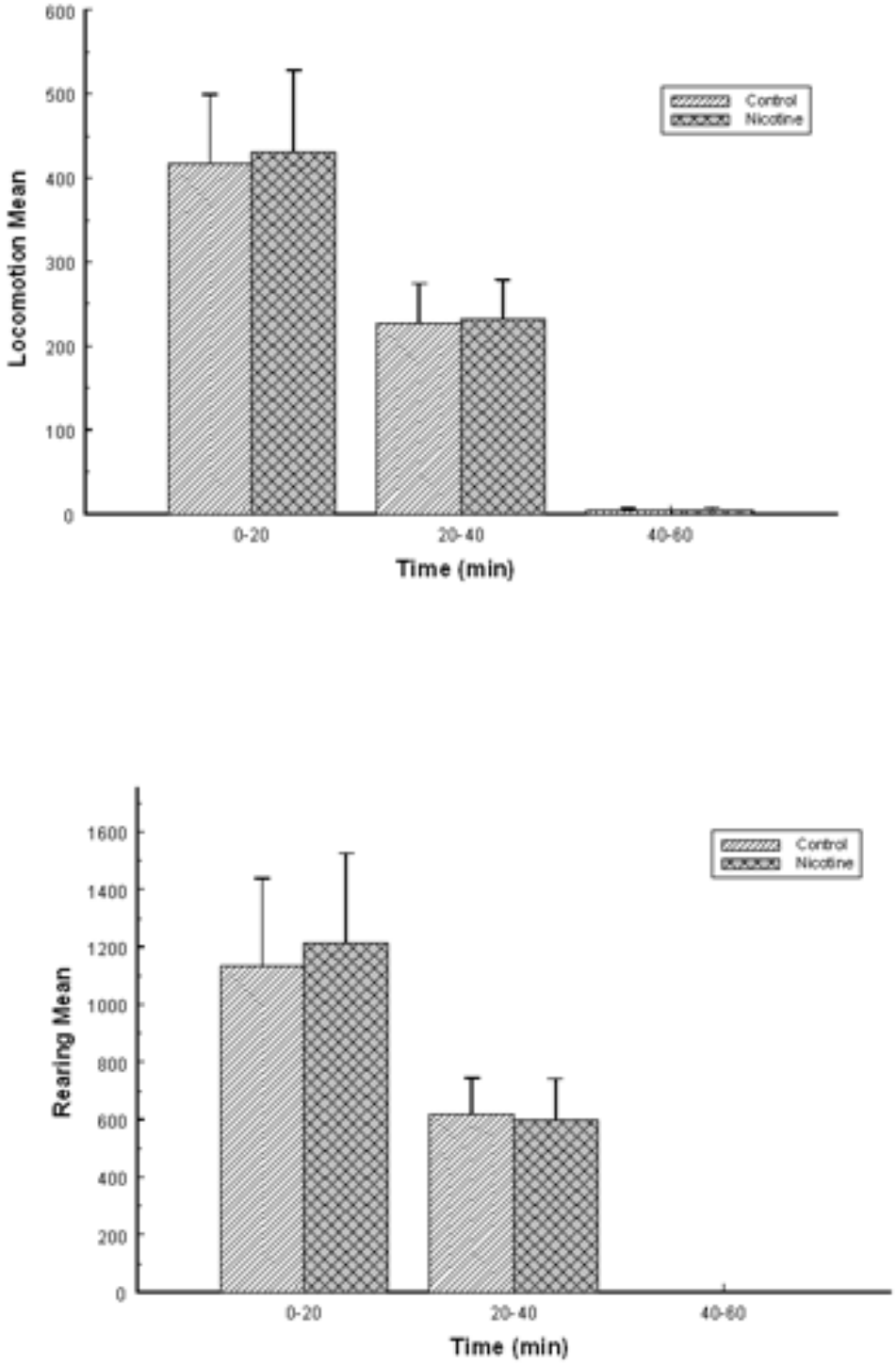

Figure 3: Spontaneous behaviour. Locomotion and rearing variables in 3-month-old male mice after neonatal exposure to either 33µg nicotine/kg bw s.c. (Nicotine) or 10 ml 0.9% NaCl/kg bw s.c. (Control), twice daily between the 10th and 14th postnatal days. For statistic evaluation, an ANOVA with split-plot design was used. The height of the bars represents the mean value + standard deviation.

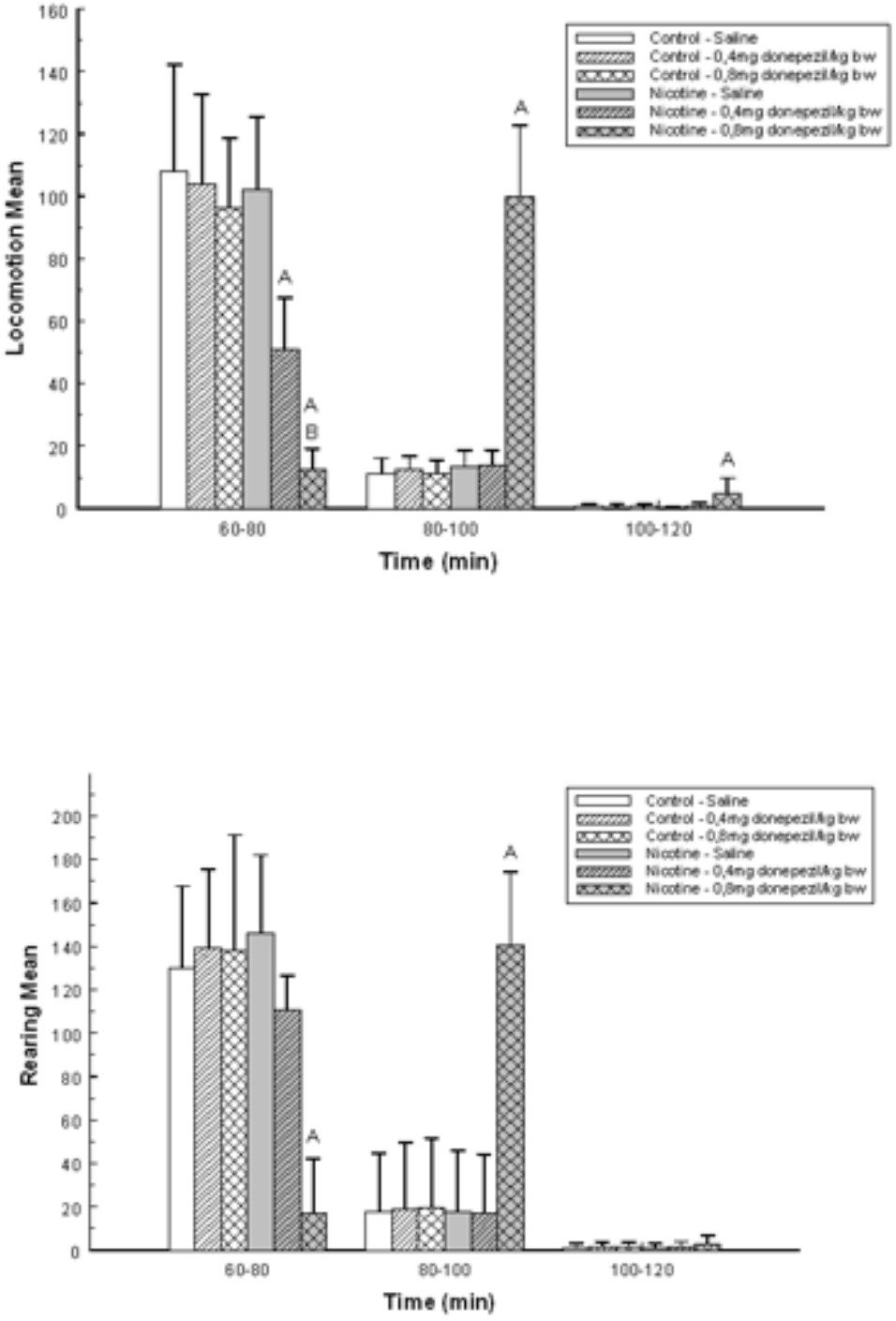

Figure 4: Donepezil-induced behaviour. Locomotion and rearing variables in 3-month-old male mice after neonatal exposure to either 33µg nicotine/kg bw s.c. (Nicotine) or 10 ml 0.9% NaCl/kg bw s.c. (Control), twice daily between the 10th and 14th postnatal days, and challenged with saline, 0.4 or 0.8 mg donepezil/kg bw s.c. For statistic evaluation an ANOVA with split-plot design was used. The height of the bars represents the mean value + standard deviation. Statistical differences are indicated by capital letters for $p < 0.01$ and lower case letters for $p < 0.05$. A means that there is a significant difference in behavior compared to control animals, B that there is a significant difference compared to control animals and mice treated with 0.4 mg donepezil/kg bw.

4.1.2. Long term effects of adult exposure to donepezil in mice neonatally exposed to nicotine

The long term effect to donepezil in adult mice treated neonatally with saline or nicotine are illustrated in Fig. 5. The mice were given 33µg nicotine/kg bw s.c. twice daily , or to 10ml 0.9% NaCl/kg bw s.c. as control between day 10 and 14 postnatally and at the age of 3 months they received an injection of 0.4 or 0.8 mg donepezil/kg bw s.c., or 10 ml 0.9% NaCl/kg bw s.c. every second day for 7 days. At the age of four and a half months they were observed for spontaneous behaviour. No significant group x period interactions were observed [$F_{10,108} = 0,94$, $F_{10,108} = 0,95$], for the locomotion and rearing, respectively.

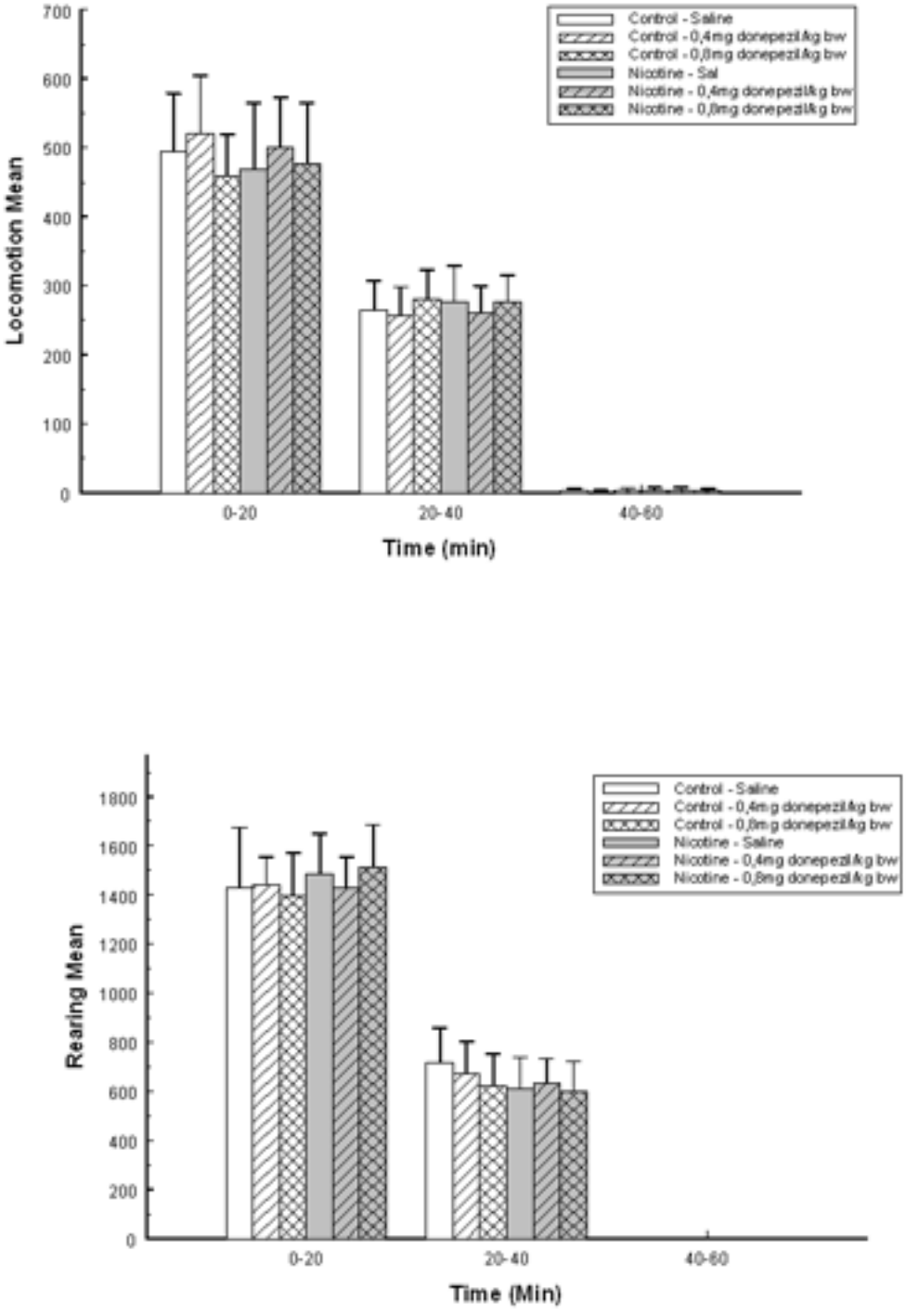

Fig 5: Long term effect, spontaneous behaviour. Locomotion and rearing variables in 4,5-month-old male mice after neonatal exposure to either 33µg nicotine/kg bw s.c. or 10 ml 0.9% NaCl/kg bw s.c. (Control), twice daily between the 10th and 14th postnatal days, and challenged with saline, 0.4 or 0.8mg donepezil/kg bw s.c. every second day for 7 days at the age of 3 months. The height of the bars represents the mean value + standard deviation.

4.1.3. Effects of neonatal exposure to nicotine on performance in Swim maze

Camera and software for the TSE VideoMot 2 measuring system was installed and calibrated for measuring the mice performance in the swim maze. In the test the control mice did not show a learning pattern that is normal in this test. Therefore the results can not be used to make any conclusions on the effects of neonatal exposure to nicotine.

4.1.4. Levels of AChE inhibition by different doses of donepezil

The AChE inhibition in three month old mice neonatally exposed to either 33µg nicotine/kg bw s.c. or 10 ml 0.9% NaCl/kg bw s.c., twice daily between the 10^{th} and 14^{th} postnatal days and then given saline, 0.4 or 0.8 mg donepezil/kg bw s.c. at the age of 3 months, are illustrated in Fig. 6. The low dose of 0.4 mg donepezil/kg bw gave an AChE inhibition of 2,1 and 6,2% compared to the control for saline and nicotine treated mice respectively. The high dose of 0,8mg donepezil/kg bw gave an AChE inhibition of 43.0 and 40.9% compared to the control for saline and nicotine treated mice, respectively.

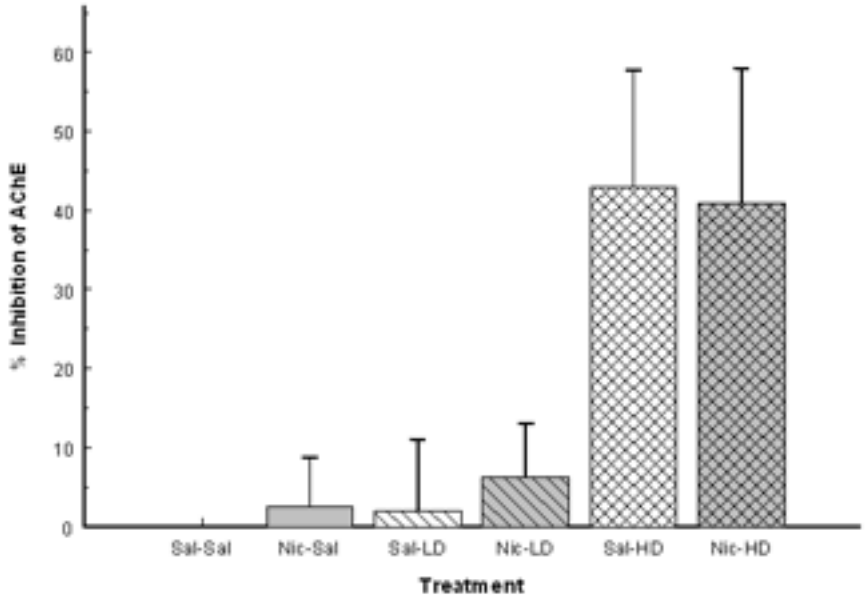

Figure 6: AChE inhibition when given donepezil. AChE inhibition in 3-month-old mice after neonatal exposure to either 33µg nicotine/kg bw s.c. (Nicotine) or 10 ml 0.9% NaCl/kg bw s.c. (Control), twice daily between the 10^{th} and 14^{th} postnatal days, and challenged with saline, 0.4 or 0.8 mg donepezil/kg bw s.c. The height of the bars represents the mean value + standard deviation.

5. DISCUSSION

This study clearly shows that a substance, in this case nicotine, that is known to effect the cholinergic system in doses that obviously do not cause any visual effects by its own, can make the mice more susceptible to another agent known to effect the cholinergic system, such as donepezil.

The activity on the locomotion variable from the behavioural response to donepezil indicates a dose-response effect of donepezil when administered to 3-month-old mice, earlier exposed to nicotine between 10^{th} and 14^{th} postnatal day. The results from the AChE inhibition test shows that a high dose of 0.8 mg donepezil/kg bw gives 6-7 times greater AChE inhibition compared to the dose of 0.4 mg donepezil/kg bw. This indicates a dose-response effect on the inhibition in both control mice and nicotine exposed mice, but a dose-response effect on reaction to donepezil is only seen in mice neonatally exposed to nicotine. The donepezil-induced behaviour study clearly shows of an acute response to the high dose (0.8 mg donepezil/kg bw) on both locomotion and rearing variables. In the first 20

min period the mice displays a hypo active condition. This reaction is also seen in earlier studies with neonatal exposure to nicotine and adult exposure to paraoxon (Ankarberg 2004)

Earlier studies show that exposure to nicotine during early development changes brain nicotinic receptors and increases the susceptibility to nicotine or organophosphates in adult life stages (Nordberg, Zhang *et al*. 1991; Eriksson, Ankarberg *et al.* 2000; Ankarberg 2003; Ankarberg 2004). Earlier reports have shown that neonatal exposure to low doses of nicotine affects the NAChR in the neonatal mouse brain, leading to permanent disorder of brain function of adult mice, revealed as changes in behaviour and in binding properties of nicotinic receptors (Nordberg, Zhang *et al*. 1991). Earlier studies have also shown that mice treated neonatally with nicotine become hypoactive if challenged with nicotine as adult whereas control animals become hyperactive (Ankarberg 2003). When challenging with paraoxon, instead of nicotine, the treated mice become hypoactive but the control mice don't show altered response or any hyperactivity as seen for nicotine (Ankarberg 2004). In the present study, challenging with donepezil, the neonatally nicotine treated mice shows a hypoactive condition and the control mice show no altered response, in similarity to the reaction to paraoxon. However, in the period of 80-100 minutes there is a clear hyperactivity in mice neonatally given nicotine, indicating differences in response to donepezil and paraoxon.

The difference in response to nicotine and the AChE-inhibition in the control animals might be due to different stimulation of NAChR. Nicotine is an agonist and stimulates the release of ACh and thereby increase the levels of ACh. Both paraoxon and donepezil are cholinesterase inhibitors. The effect of both paraoxon and donepezil that is increasing the levels of ACh might be lesser compared to a direct stimulation by nicotine. However this needs further investigations. Taken together all these experiments show that neonatal exposure to nicotine alters the response of the adult cholinergic system. This might be interesting for treatment of neurodegenerative diseases like Alzaimer's Disease.

The acute reaction to the donepezil appears to be reversible, since the mice showed a normal spontaneous behaviour 5 weeks after been exposed to donepezil. None of the treated groups showed any significant difference compared to the control group. In the study by Ankarberg *et. al*.(2004) the hypoactive reaction to paraoxon was permanent. The lack of effect in the present study could be too low doses for ChE to be inhibited. Another fact can be the treatment time with donepezil may have been too short to cause prolonged inhibition of AChE. In humans the steady-state for donepezil levels in the plasma is 3 weeks (FASS® 2007).

Another explanation can be that the effects of donepezil are reversible, which is beneficial regarding to the medical usage. These results call for further studies.

This study indicates that neonatal exposure to low doses of nicotine alters the adult susceptibility to donepezil. The results indicate that differences in adult susceptibility to donepezil, need not to be an inherited condition. Rather it might well be acquired by low dose exposure to nicotine during a critical period of neonatal brain development.

6. REFERENCES

Ahlbom, J., A. Fredriksson, *et al.* (1995). "Exposure to an organophosphate (DFP) during a defined period in neonatal life induces permanent changes in brain muscarinic receptors and behaviour in adult mice." Brain Res **677**(1): 13-9.

Ankarberg, E., Fredriksson, A. and Eriksson, P. (2003). "Neonatal exposure to nicotine increases the susceptibility to nicotine in adult mice." Behav Brain Res.

Ankarberg, E., Fredriksson, A., Eriksson, P., (2004). "Increased susceptibility to adult paraoxon exposure in mice neonatally exposed to nicotine " Toxicological Sciences **82**: 555-561.

Anthony, D. C., T. J. Montine, *et al.* (2001). Toxic Responses of the Nervous System. Casarett & Doull's Toxicology: *The basic science of poisons*. C. D. Klaassen, The McGraw-Hill Companies, Inc. **5:th:** 555-556.

Archer, T., A. Fredriksson, *et al.* (1987). "Marble burying and spontaneous motor activity in mice: interactions over days and the effect of diazepam." Scand J Psychol **28**(3): 242-9.

Benowitz, N. L. (1986). "Clinical pharmacology of nicotine." Annu Rev Med **37**: 21-32.

Berger, F., F. H. Gage, *et al.* (1998). "Nicotinic receptor-induced apoptotic cell death of hippocampal progenitor cells." J Neurosci **18**(17): 6871-81.

Cooper, J. R., Bloom, F. E., and Roth, R. H. (1996). The biochemical basis of neuropharmacology. New York, Oxford University Press.

Court, J., V. Cuomo, *et al.* (1996). IEH report on Perinatal Developmental Neurotoxicity.

Coyle, J. T. and H. I. Yamamura (1976). "Neurochemical aspects of the ontogenesis of cholinergic neurons in the rat brain." Brain Res **118**(3): 429-40.

Davison, A. N. and J. Dobbing (1968). Applied Neurochemistry. Oxford, Blackwell.

Davison, A. N. and J. Dobbing (1968). "The developing brain." Applied Neurochemistry: 178-221, 253-316.

Ellman, G. L., K. D. Courtney, *et al.* (1961). "A new and rapid colorimetric determination of acetylcholineesterase activity." Biochemical Pharmacology **7**: 88-95.

Eriksson, P. (1998). Perinatal developmental neurotoxicity of PCBs. Stockholm, Swedish Environmental Protection Agency**:** 56.

Eriksson, P., J. Ahlbom, *et al.* (1992). "Exposure to DDT during a defined period in neonatal life induces permanent changes in brain muscarinic receptors and behaviour in adult mice." Brain Res **582**(2): 277-81.

Eriksson, P., E. Ankarberg, *et al.* (2000). "Exposure to nicotine during a defined period in neonatal life induces permanent changes in brain nicotinic receptors and in behaviour of adult mice." Brain Research **853**(1): 41.
Eriksson, P., L. Nilsson-Hakansson, *et al.* (1990). "Neonatal exposure to DDT and its fatty acid conjugate: effects on cholinergic and behavioural variables in the adult mouse." Neurotoxicology **11**(2): 345-54.
Falkeborn, Y., C. Larsson, *et al.* (1983). "A comparison of the regional ontogenesis of nicotine- and muscarine-like binding sites in mouse brain." J. Devl. Neuroscience **1**(3): 187-190.
FASS®. (2007). Retrieved 2007-01-31, from http://www.fass.se/LIF/produktfakta/artikel_produkt.jsp?NplID=19970714000031.
Fiedler, E. P., M. J. Marks, *et al.* (1987). "Postnatal development of cholinergic enzymes and receptors in mouse brain." J Neurochem **49**(3): 983-90.
Gotti, C. and F. Clementi (2004). "Neuronal nicotinic receptors: from structure to pathology." Progress in Neurobiology **74**(6): 363-396.
James, J. R. and A. Nordberg (1995). "Genetic and environmental aspects of the role of nicotinic receptors in neurodegenerative disorders: Emphasis on Alzheimer's disease and Parkinson's disease." Behav Gen **25**: 149-159.
Johansson, U., A. Fredriksson, *et al.* (1995). "Bioallethrin causes permanent changes in behavioural and muscarinic acetylcholine receptor variables in adult mice exposed neonatally to DDT." Eur J Pharmacol **293**(2): 159-66.
Johansson, U., A. Fredriksson, *et al.* (1996). "Low-dose effects of paraoxon in adult mice exposed neonatally to DDT: Changes in behavioural and cholinergic receptor variables." Environmental Toxicology and Pharmacology **2**(4): 307-314.
Karczmar, A. G. (1975). Cholinergic influences on behaviour. Cholinergic Mechanisms. P. G. Waser. New York, Raven Press**:** 501-529.
Kirk, R. E. (1968). Procedures for the behavioural science. Belmont, CA, Brooks/Cole.
Lewis, A., J. H. Miller, *et al.* (2007). "Monoamine oxidase and tobacco dependence." NeuroToxicology **28**(1): 182-195.
Misson, J. and M. J. Kendall (1997). "Therapeutic advances: donepezil for the treatment of Alzheimer's disease." Journal of Clinical Pharmacy and Therapeutics **22**: 251-255.
Morris, R. (1981). "Spatial localization does not require the presence of local cues." Learning Motivation **12**: 239-260.
Nordberg, A. (2001). "Nicotinic receptor abnormalities of Alzheimer's disease: therapeutic implications." Biological Psychiatry **49**(3): 200-210.
Nordberg, A., X. A. Zhang, *et al.* (1991). "Neonatal nicotine exposure induces permanent changes in brain nicotinic receptors and behaviour in adult mice." Brain Res Dev Brain Res **63**(1-2): 201-7.
Pfizer Inc. and Eisai Inc. (2006). Retrieved 2007-01-29, from http://www.aricept.com/about/tips.aspx.
Purves, D., G. J. Augustine, *et al.* (2004). Neuro science, Sinauer Associates.
Rogers, S. L., R. S. Doody, *et al.* (2000). "Long-term efficacy and safety of donepezil in the treatment of Alzheimer's disease: final analysis of a US multicentre open-label study." European Neuropsychopharmacology **10**(3): 195-203.
Rosell, S. and B. Danielsson (1975). Farmakologiska Principer. Stockholm, Nya Lagerblads Tryckeri AB.
Russel, M. A. H. (1990). Nicotine intake and its control over smoking. Nicotine Psychopharmacology - Molecular, Cellular, and Behavioural Aspects. S. Wonnacott**:** 374-419.

Sargent, P. B. (1993). "The diversity of neuronal nicotinic acetylcholine receptors." Annu Rev Neurosci **16**: 403-443.
Zhang, X., P. Stjernlof, *et al.* (1987). "Anatoxin-a a potent ligand for nicotinic cholinergic receptors in rat brain." Eur J Pharmacol **135**(3): 457-8.