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Cholinergic Receptors in Human Prenatal Brain; presence, distribtion and influence of nicotine and ethanol

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Cover page: Two different primary cell cultures of human prenatal cortex. A mixed cell culture showing cells that are immunoreactive to both the neuronal marker β-tubulin (class III) (green) and the astrocytic marker GFAP (red) (left) and a cell culture with mainly cells immunoreactive to β-tubulin (class III) (right). For details see page 25.

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ABSTRACT

Events early in life, including exposure to various compounds, may cause disturbances in brain development. Both maternal smoking and alcohol drinking during pregnancy can induce pre- and postnatal death, cognitive and neurobehavioral disturbances. Nicotine can, through stimulation of nicotinic receptors (nAChRs), interfere with proliferation, differentiation and synaptogenesis during brain development. Ethanol affects both proliferation and induction of apoptosis and is able to modulate the function of nAChRs.

This thesis focuses on the development of nAChRs, especially the α7 nAChR subtype, in the human prenatal brain and the effects of nicotine exposure. A significant positive correlation between gestational age (5-12 weeks of gestation) and the expression of both α7 nAChR protein and α7 mRNA was found. Both the number and the gene expression of α7 nAChRs were significantly higher in the medulla oblongata, pons, mesencephalon and spinal cord compared to cerebellum, cortical and subcortical forebrain at 9-11 weeks of gestation. Comparison of the presence of nAChRs in the prenatal (9-11 weeks of gestation), middle-aged and aged brain, showed a significantly higher expression of α7 mRNA in prenatal pons, medulla oblongata, cortex and cerebellum than in corresponding regions in adult brain, except for aged cortex. Nonetheless, higher prenatal density of the α7 nAChRs themselves ([^{125}I]-α-bungarotoxin binding) was observed only in brainstem, which is more mature at this stage.

The presence of muscarinic receptors (mAChRs) already at gestational week 5 was observed for the first time. Exposure to maternal smoking significantly changed the age-related number of the α4 nAChRs ([^{3}H]-cytisine binding) but not the m_2 mAChR ([^{3}H]-AF-DX 384 binding) in medulla oblongata, pons and cerebellum. In addition, the α4 and α7 mRNA expression was altered in all regions as was the mRNA levels for some mAChR subtypes.

A significantly dose-dependent decrease in cell survival was observed after co-exposure to nicotine and ethanol, at physiologically relevant concentrations, in primary cell cultures from human cortex (6-10.5 weeks of gestation). Neuronal cell cultures were more susceptible to exposure to low concentrations of ethanol than cultures with both neurons and astrocytes. High concentrations of nicotine and ethanol decreased the proliferation of cells in cultures with both neurons and astrocytes.

Conclusions: Subtypes of nAChRs and mAChRs are present early during development. Nicotine exposure during the first three months of gestation will affect the normal expression of these receptors. Maternal smoking provides excessive nAChR stimulation and discoordinate the numerous of events that are necessary for proper assembly of the human

brain. Pregnant women should be advised to totally refrain from smoking and ethanol intake during gestation.

LIST OF PUBLICATIONS

This thesis is based upon the following publications, which are referred to in the text by their Roman numerals I-IV

I. Falk L., Nordberg A., Seiger Å., Kjældgaard A. and Hellström-Lindahl E. (2002) The α7 nicotinic receptors in human fetal brain and spinal cord. *Journal of Neurochemistry* 80: 457-465

II. Falk L., Nordberg A., Seiger Å., Kjældgaard A. and Hellström-Lindahl E. (2003) Higher expression of α7 nicotinic acetylcholine receptors in human fetal compared to adult brain. *Developmental Brain Research* 142: 151-160

III. Falk L., Nordberg A., Seiger Å., Kjældgaard A. and Hellström-Lindahl E. (2005) Smoking during early pregnancy affects the expression pattern of both nicotinic and muscarinic acetylcholine receptors in human first trimester brainstem and cerebellum. *Neuroscience* in press

IV. Falk L., Nordberg A., Sundström E., Seiger Å., Kjældgaard A. and Hellström-Lindahl E. Increased cytotoxic effects of combined exposure to nicotine and ethanol to human prenatal cortex compared to only one drug. *Manuscript*

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LIST OF ABBREVIATIONS

ACh	acetylcholine
AChE	acetylcholinesterase
ADHD	attention deficits and hyperactivity disorder
CNS	central nervous system
ChAT	cholineacetyltransferase
DA	dopamine
FBS	fetal bovine serum
GABA	γ-amino-butyric acid
GFAP	glial fibrillary acidic protein
5-HT	serotonin
mAChR	muscarinic acetylcholine receptor
mRNA	messenger RNA
NA	noradrenaline
nAChR	nicotinic acetylcholine receptor
RT-PCR	reverse transcription polymerase chain reaction
SIDS	sudden infant death syndrome

INTRODUCTION

The human brain undergoes dynamic changes during the whole life span. Despite the great plasticity of the brain, slight perturbations due to environmental exposure of the young organism can cause aberrations - profound or subtle that may not become evident until relatively late in development or at adult age (Rice and Barone Jr, 2000). Neurotransmitters seem to exert unique trophic roles in brain development. The ontogeny of different parts of the brain is dependent on the temporal and regional emergence of critical developmental processes, regulated by the interplay between neurotransmitters and growth factors (Hohmann and Berger-Sweeney, 1998; Ma et al., 2000). Neurotoxicants that promote or interfere with neurotransmitter function evoke neurodevelopmental abnormalities by disrupting the timing or intensity of the neurotrophic action (Levitt, 1998). Several epidemiological studies have demonstrated harmful effects of exposure to nicotine and ethanol during pregnancy. Experimental studies from animals have revealed that nicotine and alcohol can modulate early environmental signals in the prenatal brain, which results in long-lasting alterations in structure and function of the central nervous system (CNS) (Mick et al., 2002; Olney, 2002).

BRAIN DEVELOPMENT

Between 2 weeks and 9 months of gestation the human CNS develops from a thin layer of ectodermal tissue into a highly complex system that can process sensory input and organize actions. The caudal parts of the brain develop earlier than the more rostral parts and medial mature earlier than more lateral structures (Rice and Barone Jr, 2000). Eight prenatal processes are involved in this progression: neural induction, neurulation, proliferation, migration, differentiation, axonal outgrowth, synaptogenesis and apoptosis (Monk et al., 2001). The embryo consists from the beginning of three layers: the endoderm, the mesoderm, and the ectoderm. The neural plate is formed from the dorsal ectoderm at around 18 days of gestation in humans; this process is called neural induction (Slack, 1991). A few days later neurulation occurs, i.e. the neural plate folds into a tube.

Proliferation of cells in mammalians occurs in two stages: *symmetrical*, one progenitor cell produces two new progenitor cells, and *asymmetrical*, when a progenitor cells give rise to one progenitor cell and one postmitotic neuron, which no longer can divide. The asymmetrical proliferation begins at approximately 7 weeks of gestation in humans and continues until midgestation (Rakic, 1978). Several growth

factors together with the neurotransmitters acetylcholine (ACh), γ-amino-butyric acid (GABA) and α-amino-3-hydroxy-5-methylisoxazolepropionic acid (AMPA) are involved in regulating the proliferation (Ma et al., 2000; Monk et al., 2001). The proliferation of progenitor cells in the ventricular zone leads to bulges in the neural tube that take form of vesicles. At 20 days the human nervous system is comprised of three primary vesicles, which has further developed to a five-vesicle stage at 5 weeks of gestation (Fig. 1) (Monk et al., 2001).

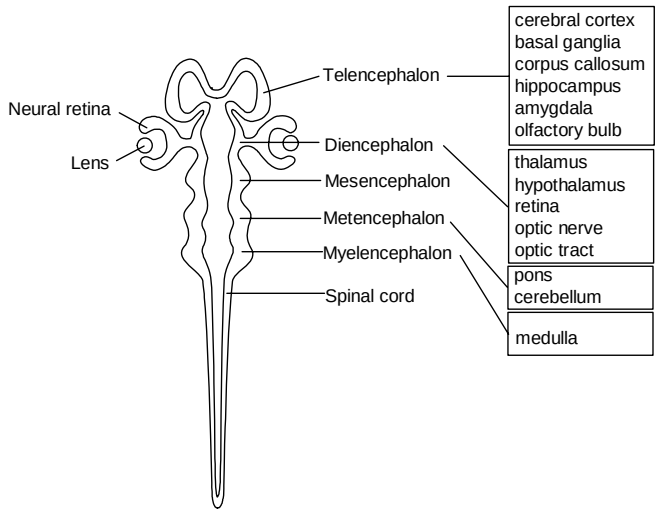

Figure 1. The five-vesicle stage at the 5th week of human gestation. The telencephalon, which gives rise to the cerebral cortex, basal ganglia, corpus callosum, hippocampus, amygdala and olfactory bulb and the diencephalon, which originate in the thalamus, hypothalamus, retina and optic nerve and tracts. In addition, hindbrain is segmented to metencephalon (pons and cerebellum) and myelencephalon (medulla oblongata).

In human brain, neuronal migration starts at around 8 weeks of gestation and ends at approximately 4.5 months of gestation (Rakic, 1972). Neuronal precursor can travel in a radial direction along radial glia cells from the proliferation zones to the outer areas of the CNS or in a tangential direction parallel to the surface of the developing brain to enter different brain regions (Rakic, 1990). Cell adhesion molecules, growth factors, ion channels and neurotransmitter receptors are necessary for a successful migration.

Axonal outgrowth and synaptogenesis are crucial for the brain function. Growth cones transduce cues from the extracellular environment to guide axons to their synaptic destination (Stoeckli and Landmesser, 1998). Several molecules attract or repel the growth cone (Tessier-Lavigne and Goodman, 1996) and many of them provide bifunctional guidance cues: they attract some types of neurons and inhibit others (Mueller, 1999). Formed synaptic connection are strengthened by neurotrophic factors which are necessary for survival (Hendersen, 1996). Refinement of the synaptic

connections and elaboration of dendritic arbors are influenced by both experience and spontaneous neural activity.

During development, roughly half of all brain cells die, through apoptosis (Raff et al., 1993; Costa et al., 2004). Neurotrophic factors are the principal modulators of apoptosis and young neurons obtain these from the target cells they innervate. Apoptosis may perform the house-keeping function of eliminating cells with poor and perhaps unnecessary, synaptic connections (Hendersen, 1996).

There are regional differences in the timing of neuronal maturation, e.g. the late developing region cerebellum remains immature until the late fetal and early postnatal period in humans (Kwong et al., 2000). The fundamental organization of the brain stem differs from the extremely specialized structure of the forebrain and cerebellum. The brain stem represents the cranial continuation of the spinal cord and its organization is similar with some alterations as some groups of neurons migrate away from their site of origin, the neuroepithelium lining the neural canal, to establish nuclei elsewhere (Larsen, 1997).

The main processes in the human brain during the first trimester, the first three months of the pregnancy, are proliferation, differentiation and migration (Rice and Barone Jr, 2000). This thesis will focus on the cholinergic receptors during this period, the influence of smoking on the receptor proteins and gene transcripts and the effects of nicotine and ethanol on cell survival and proliferation.

THE CHOLINERGIC NERVOUS SYSTEM

The endogenous transmitter ACh mediates cholinergic neurotransmission through interaction with two types of receptors, nicotinic (nAChRs) and muscarinic (mAChRs) acetylcholine receptors, which exist in both the central and peripheral nervous system. In the mature CNS two major cholinergic subsystems can be identified. In the first system, cholinergic neurons from the basal forebrain project to the entire cerebral neocortex, hippocampus, amygdala and several thalamic nuclei. The second system arises from cholinergic neurons in the mesopontine tegmentum and provides a widespread innervation mainly to the thalamus and midbrain but also to the reticular formation in pons and medulla oblongata (Paterson and Nordberg, 2000; Gotti and Clementi, 2004). Cholinergic interneurons are also found in the brain stem and forebrain (Semba and Fibiger, 1989; Dani, 2001). One characteristic of the cholinergic system is that relatively few cholinergic neurons make sparse projections that reach broad areas thereby influencing relatively large neuronal structures. The cholinergic neurotransmission is involved in higher brain functions such as cognition, learning, memory and arousal (Paterson and Nordberg, 2000; Changeux and Edelstein, 2001; Levin, 2002).

Subtypes of nicotinic receptors in the human brain

The nAChRs are ligand-gated ion channel receptors widely distributed in the brain and not restricted to the well-defined brain cholinergic pathways. In comparison to mAChRs they are present at relatively low density in the human adult brain (Nordberg et al., 1992; Hellström-Lindahl et al., 1993). The nAChRs are composed of five transmembrane subunits arranged like rods surrounding a central canal. nAChRs belong to the gene super-family of homologous receptors that also includes $GABA_A$, glycine and serotonin receptor-3 (5-HT_3 receptors) (Paterson and Nordberg, 2000). Six α (α$_2$-α$_7$) and three β (β$_2$-β$_4$) subunits have been cloned and sequenced in human brain. Multiple combinations of α and β subunits (Fig. 2A) or the assembly of only α subunits (Fig. 2B) form a variety of functional receptors that differ in terms of their speed of activation, ionic current, rate of desensitization and recovery from desensitization, pharmacology and regulatory control of the agonist response (McGehee and Role 1995; Gotti et al., 1997; Paterson and Nordberg, 2000; Sargent, 2000; Dani and De Biasi, 2001).

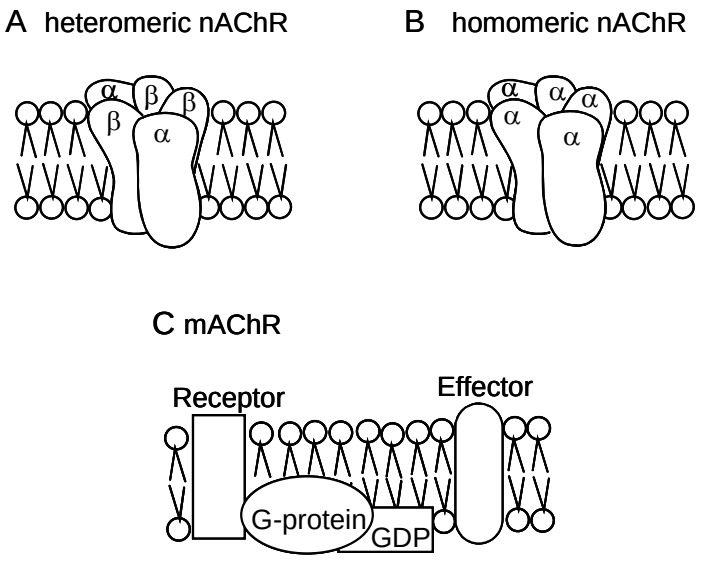

Figure 2. Heteromeric α4β2 (A) and homomeric α7 (B) nAChRs are ion channels, whereas the mAChRs (C) are G-protein coupled receptors.

Most nAChRs in the mammalian brain contain either the α4 (assembled with β2), α7 or α3 subunit (Paterson and Nordberg, 2000; Dani, 2001). Although one subtype of nAChRs often predominates within a region, more than one subtype is often present. In addition, individual neurons can express multiple subtypes of nAChRs (Dani, 2001).

[^{3}H]-nicotine binding sites are found of high density in thalamus, caudate nucleus and substantia nigra, a distribution that more or less coincides with that of the α4β2 subtype. [^{125}I]-α-bungarotoxin binding parallels the distribution of the α7 nAChR and is observed at high densities in hippocampus, substantia nigra and certain cortical layers (Marutle et al., 1998; Court et al., 2000; Paterson and Nordberg 2000; Gotti and Clementi, 2004).

nAChRs are found in various locations in the cell. Presynaptic nAChRs are able to modulate the release of several transmitters (McGehee and Role, 1995; Role and Berg, 1996; Wonnacott, 1997; Vizi and Lendvai, 1999). The α4 containing nAChRs have been implicated in the release of dopamine (DA) (Wonnacott, 1997) whereas the release of glutamate mainly is controlled by the α7 subtype (McGehee and Role, 1995). The β2 subunit is important in modulating GABA release in mice (Lu et al., 1998). The nAChR responsible for modulating the release of noradrenaline (NA) in hippocampus might contain the α3 and β4 subunits (Wonnacott, 1997). nAChRs have also been reported at preterminal and axonal locations where they are thought to depolarize the membrane locally, allowing modulation of transmitter release (Dani, 2001). Postsynaptic nAChRs mediating fast nicotinic transmission has been detected in a few neuronal areas e.g. hippocampal interneurons. Although, direct nicotinic excitation of a neuron rarely predominates, it could influence the excitability of a group of neurons and owing to the broad cholinergic projections into specific areas, the rhythmic activity of these area can be modulated (Dani, 2001). Recently α3, α7 and β4 subunits have been demonstrated on astrocytes in human postmortem brain (Graham et al., 2002; 2003; Yu et al., 2005).

Activation of nAChRs leads to influx of cations, mostly Na^{+} and K^{+} but also Ca^{2+}. Of special interest in the developing brain are the homomeric α7 nAChRs, which are characterized by fast kinetics. They are rapidly activated and inactivated (desensitized) and have the highest permeability to Ca^{2+} among the ligand-gated ion-channels, which may indicate their function. By modulating the intracellular Ca^{2+} levels, the α7 receptor may regulate the function of the target cell via Ca^{2+}-dependent processes, such as activation of second messenger systems and induction of immediate-early gene expression, and thereby influence important processes during brain development (Albuquerque et al., 1995; Role and Berg, 1996; Broide and Leslie, 1999; Dani, 2001).

Subtypes of muscarinic receptors in the brain

The mAChRs are G-protein coupled receptors (Fig. 2C) and stimulation of mAChRs is associated with regulation of intracellular levels of the second messengers adenosine 3´, 5´-cyclic monophosphate (cAMP) and guanosine 3´, 5´-cyclic monophosphate (cGMP), inositol phospholipids, and the opening or closing of K^{+}, Ca^{2+} and Cl^{-} ion channels (Caulfield, 1993). Five different subtypes of mAChRs have

been identified. Subtypes m_1, m_3 and m_5 are preferentially coupled to G-proteins that stimulate phospholipase C, whereas the G-proteins that are coupled to m_2 and m_4 mAChRs are generally associated with the inhibition of adenylyl cyclase (Caulfield, 1993; Kinney et al., 1995b). Different brain regions show varying proportions of mAChRs subtypes. Thus, relatively high densities of the m_1 mAChR subtype are found in the cortex, hippocampus, dentate gyrus and striatum. The m_2 subtype predominates in thalamus and cerebellum and the m_4 mAChR in striatum (Caulfield, 1993; Piggot et al., 2002). Human fetal astrocytes express all five subtypes of mAChRs (Elhusseiny et al., 1999) and activation by carbachol increases the proliferation of rat cortical astrocytes (Guizzetti and Costa, 1996).

CHOLINERGIC RECEPTORS DURING DEVELOPMENT

The neurotransmitters probably play a role distinct from classical synaptic communication during development. In the mature system, receptor stimulation results in a short-term response and continued stimulation elicit compensatory adjustments that offset the stimulation. Receptor stimulation during development – and only during development - communicates with genes that control cell replication, termination of replication and initiation of differentiation, apoptosis, and that determines the ability of the cell to respond to future stimulation (Fig. 3) (Slotkin, 1998; Lauder and Schambra, 1999). The same transmitter may influence multiple genes.

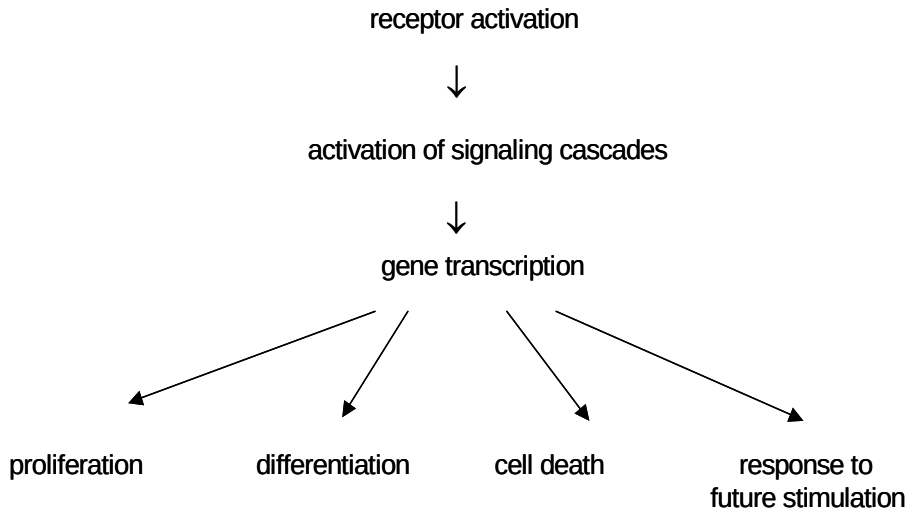

Figure 3. Schematic representation of the linkage of neurotransmitters to cell development. Depending on the context in which the receptor stimulation occurs, the same neurotransmitter, operating through the same receptors, can elicit different responses.

Classical neurotransmitters such as ACh, DA, NA, 5-HT and GABA are present in concentration gradients in tissues and seem to act as developmental signals in various animal species (Lauder and Schambra, 1999; Torrao and Britto, 2002). Their regulatory roles exist throughout ontogenesis, including stages prior to development of the CNS (Lauder and Schambra, 1999). Neuroproliferation, apoptosis and synaptic rearrangements continue in the brain into adolescence (Slotkin, 2004).

In virtually all stages of brain maturation, ACh plays a critical role. In the early chick embryo, prior to brain development, ACh has been reported to be involved in regulating gastrulation, an effect mediated through mAChRs (Laasberg et al., 1987). ACh also promotes cell proliferation through mAChRs (Guizzetti et al., 1996; Lauder and Schambra, 1999; Ma et al., 2000). Through nAChRs, ACh initiates the switch from replication to differentiation (Slotkin, 1998; Slotkin, 2004) and modulates axogenesis and synaptogenesis (Navarro et al., 1989; Chan and Quik, 1993). Depending on the developmental stage, ACh promotes or prevents apoptosis: in poorly differentiated cells it induces, whereas in differentiated cells its effect is anti-apoptotic (Berger et al., 1998; Roy et al., 1998; Atluri et al., 2001). Even in the late phases of brain development the trophic effects of ACh are important. Interfering with the cholinergic signaling at this stage may disrupt the final architectural assembly of brain regions containing cholinergic target zones (Hohmann and Berger-Sweeney, 1998).

The presence of nAChRs has been demonstrated in the human prenatal brain from 4-5 weeks of gestation (Hellström-Lindahl et al. 1998) as well as the enzymes cholineacetyltransferase (ChAT) and acetylcholinesterase (AChE) (Candy et al., 1985; Perry et al., 1986; Court et al., 1995). Functional nAChRs have been reported on stem and progenitor cells in primary cultures of embryonic mouse cortex (Atluri et al., 2001; Schneider et al., 2002). In the human brain, the mRNA for several nAChR subunits is expressed at higher levels during the first three months of gestation than in aged individuals (Hellström-Lindahl et al., 1998) and a decrease in the density of nAChRs is observed during aging (Hellström-Lindahl and Court, 2000). The early expression of nAChRs and the high Ca^{2+} permeability suggest a role for nAChRs in regulation and expression of early genes such as c-fos (Greenberg et al., 1986). Stimulation of neuronal nAChRs may also contribute to pathfinding and target selection during axonal outgrowth and synapse formation (Candy et al., 1985; Lipton and Kater, 1989; Kinney et al., 1993; Zheng et al., 1994; Coronas et al., 1998; Coronas et al., 2000; Torrao and Britto, 2002). The release of ACh increases in contact with a presumptive postsynaptic cell (Xie and Poo, 1986) which may activate nAChRs on the growth cone, altering the level of intracellular Ca^{2+} and thereby halting the movement of the growth cone motility and stabilizing the location (Kwong et al., 2000).

mAChRs have been demonstrated in human prenatal brain from 10 weeks of gestation with a similar distribution as in the adult brain (Gremo et al., 1987). Several mAChR subtypes seem to reach their maximal levels in the hippocampus during infancy or early childhood followed by a slow decrease during aging (Court et al.,

1997). In vitro stimulation of mAChRs by ACh as well as the agonist carbachol promotes DNA synthesis, increases the number of cells and enhances differentiation of neurons (Guizzetti et al., 1996; Ma et al., 2000).

The presence of machinery both for synthesizing ACh and responding to it during early embryogenesis suggests important roles for cholinergic signaling early in the neural development.

EFFECTS OF NICOTINE ON THE DEVELOPING CNS

Prenatal nicotine exposure is associated with an increased risk of complications during pregnancy and early infancy but also later in childhood. The consequences of smoking during pregnancy have been demonstrated in several epidemiological studies. There is an increased risk of miscarriages, perinatal mortality, sudden infant death syndrome (SIDS), behavioral problems and attention deficits and hyperactivity disorder (ADHD) (Slotkin, 1998; Anderson et al., 2005). Aggressiveness and a tendency to seek conflicts, depression and anxiety are more common among children who have been exposed prenatally to nicotine (Orlebeke et al., 1999; Wasserman et al., 1999) as are cognitive disturbances, learning deficits, and dependence of both nicotine and alcohol later in life (Slotkin, 1998; Hellström-Lindahl and Nordberg, 2002). Despite the adverse publicity of smoking and a reduction in the number of pregnant women who use tobacco, still 15-25 % of the pregnant women in the Western world continue to smoke during pregnancy (Law et al., 2003). Smoking is estimated to be the largest single reason for fetal death causing 20 % of all prenatal deaths (Dani and De Biasi, 2001). The plasma nicotine concentration found in smokers is 60-600 nM (Benowitz et al., 1990; Henningfield et al., 1993). Nicotine is efficiently transferred to the fetal compartment and although the metabolism of nicotine and its metabolite cotinine is accelerated during pregnancy (Demsey et al., 2002) the fetus is exposed to higher nicotine concentration than the smoking mother herself (Luck et al., 1985). In addition, the blood brain barrier is not fully developed until after birth (Rodier, 1994). After birth the infant may be exposed to nicotine through environmental tobacco smoke or via the breast milk, where the concentration is up to three times higher than in the mother´s blood (Buka et al., 2003; Slotkin, 2004). A dose-dependent increase in nAChR expression in cortex of suckling rat pups exposed postnatally to nicotine via maternal milk has been reported (Narayanan et al., 2002).

Several mechanisms have been proposed to explain the deleterious effects associated with maternal smoking, including fetal exposure to carbon monoxide and cyanide, restriction of blood flow to the placenta and poor nutritional status of the mother (van de Kamp and Collins, 1994; Slotkin, 1998; Ernst et al., 2001). Apart from these indirect effects of smoking, nicotine also has been reported to directly influence the development of the fetus through interaction with nAChRs, even at very low

concentrations. It has been demonstrated that nicotine is a neuroteratogen in concentrations that do not retard growth (Levin and Slotkin, 1998; Slotkin, 1998; Law et al., 2003). Prenatal nicotine exposure leads to changes in neuronal cell replication and differentiation, followed by reactive gliosis. DNA synthesis is inhibited, and the magnitude of the inhibition is proportional to the density of nAChRs (Slotkin et al., 1987b; McFarland et al., 1991; Slotkin, 1992; Roy et al., 2002). A severe disruption of the specific morphology of both cells and cell organelles of rats exposed to nicotine in utero has been reported (Joschko et al., 1991; Roy and Sabherwal, 1998). Prenatal nicotine treatment has been demonstrated to induce increased cell death in both cortex and cerebellum (Slotkin, 1999) as well as increased gene expression of c-fos indicating increased induction of apoptosis (Slotkin et al., 1997).

Both pre- and postnatal nicotine exposure induced up-regulation in [^{3}H]-nicotine binding sites in several regions in rodent brain (Hagino and Lee, 1985; Tizabi et al., 1997; Miao et al., 1998) and lead to a different behaviour in response to nicotine when the animal has reached adulthood (Nordberg et al., 1991). Prenatal nicotine exposure results in up-regulation of [^{125}I]-α-bungarotoxin binding sites in rat forebrain, while postnatal administration of nicotine down-regulate the number of [^{125}I]-α-bungarotoxin binding sites in several brain regions such as brain stem and cerebellum (Tizabi et al., 2000; Slotkin et al., 2004).

Secondary effects of nicotine exposure on the expression of mAChRs have also been observed (Zhu et al., 1996; 1998; Slotkin et al., 1999). Since presynaptic nAChRs are involved in modulation of neurotransmitter release also in other transmitter systems, it is likely that in utero exposure to nicotine may affect multiple neurotransmitter pathways and the programming of synaptic competence. Indeed, effects on the dopaminergic system (Ribrary and Lichtensteiger, 1989; Muneoka et al., 1999) as well as the serotonergic system (Muneoka et al., 2001; Xu et al., 2001) have been found in animal studies. Disruption of the development of the catecholaminergic systems may explain the increased incidence of ADHD in individuals prenatally exposed to nicotine (Schweitzer et al., 2000).

EFFECTS OF ETHANOL ON THE DEVELOPING CNS

Maternal alcohol drinking during pregnancy, can adversely affect neuronal development of the offspring. The effects are dose-dependent, ranging from mild cognitive impairment to severe perturbations such as fetal alcohol syndrome (FAS), characterized by growth deficiency, CNS disorder and a pattern of distinct facial features. Neurobehavioral disturbances, depression and psychotic illnesses in adulthood are also observed after prenatal ethanol exposure (Olney et al., 2000). The ethanol concentration in blood found after social drinking ranges from 10 to 40 mM (Aistrup et al., 1999). Ethanol can adversely affect the physiological status of the mother through

malnutrition or abuse of other drugs, but can also interfere with the supply of nutrients to the growing fetus. In addition, ethanol has direct effects on the fetus and numerous mechanisms have been suggested to be involved. The two main effects of ethanol on brain development are altered proliferation (Luo and Miller, 1998) and enhanced apoptotic cell death (Ikonomidou et al., 2001; Farber and Olney, 2003), but altered neuronal migration, gene expression and cell adhesion have also been observed, as well as direct effects on the neurotransmitter systems. Ethanol is known to modulate the function of numerous neurotransmitter-gated ion channels including the glutamatergic, GABAergic, cholinergic, and serotonergic systems and it also inhibits voltage-gated calcium channels (Aistrup et al., 1999; Costa and Guizzetti, 1999; Goodlett and Horn, 2001; Jacobs and Miller, 2001). Accumulating evidence from electrophysiological, pharmacological and neurochemical studies supports that ethanol interacts with the nAChRs (Söderpalm et al., 2000; Narahashi et al., 2001; Larsson and Engel, 2004). The increased release of DA in the brain reward system after ethanol intake is blocked by α-conotoxin MII, an α3β2, β3 and α6 antagonist (Larsson et al., 2005). In addition, the nAChR that contain α4 subunit have been suggested to be implied in ethanol and tobacco abuse (Tritto et al., 2001). Severe effects on the development of the forebrain CNS cholinergic system were observed in mice prenatally exposed to ethanol (Lauder and Schambra, 1999). The outcome of the ethanol exposure depends on the dose and duration of the ethanol exposure, the cell type, and the stage of development (Goodlett and Johnson, 1999; Dunty Jr et al., 2001). Neural stem cells are known to be particularly sensitive to alcohol. Proliferation of human fetal astrocytes is reduced after exposure to low concentrations of ethanol (Kötter et al., 2000; Guizzetti et al., 2003). The immature brain is also especially vulnerable to the effects of alcohol during the brain growth spurt period. Regional differences are observed; cerebral cortex, hippocampus and cerebellum are particularly susceptible to prenatal alcohol exposure but basal ganglia and corpus callosum are also affected (Mattson et al., 2001; Riley et al., 2001).

AIMS OF THE THESIS

Signalling through nAChRs seems to be important early in brain development. The high Ca^{2+} permeability of the α7 nAChR subtype, supporting a functional role in regulating early gene expression and activation of second messenger systems, put this nAChR subtype in focus in the studies I and II where the main purpose was to

- explore the regional expression of α7 nAChR subtype in human first trimester CNS both on protein and mRNA level (paper I)

- compare the receptor densities and the gene transcripts of the α7 nAChR subtype in the first trimester with middle-aged and aged brain (paper II).

The adverse effects of smoking during pregnancy have been reported in numerous studies but few have been examined the effects in detail at the molecular level in the human prenatal brain. In the study III the aim was to

- investigate the effect of nicotine exposure in utero during the first trimester on the expression of nAChRs and mAChRs.

Several reports describe effects of prenatal exposure to nicotine and ethanol on proliferation and cell death but the effects of co-exposure to both drugs, especially in the human brain, have received very little attention. In paper IV we wanted to

- study the effect on cell viability and proliferation in primary cell cultures of human first trimester cortex exposed to nicotine or ethanol alone or in combination.

MATERIALS AND METHODS

HUMAN PRENATAL BRAIN

Human brain tissue was obtained following routine abortions by vacuum aspiration in the first trimester of pregnancy (5-12 weeks of gestation). The collection of tissue was approved by the Human Ethics Committee of the Karolinska University Hospital, Huddinge and followed the guidelines of the Swedish Society of Medicine and the US Public Health Service, which include informed consent from the pregnant woman. Dissections of the brain tissue were performed within 1 to 1.5 hours after the abortion. Samples were either weighed and stored at -80º C (paper I, II and III) or prepared for primary cell cultures (paper IV). The gestational age of the aborted fetus was determined by examination of anatomical landmarks and size of the fetus according to the England atlas (England, 1990). Brain regions were separated on the basis of their morphological appearance (Seiger, 1989). Information about the smoking habits of the women was available in very few cases in paper I and II, for all cases in paper III and most of the cases in paper IV.

HUMAN ADULT BRAIN

Adult brain tissue (paper II) from non-smoking subjects was obtained from the Netherlands Brain Bank. The samples of the brain tissue from middle-aged and aged subjects used in this study were collected from the superior and medial frontal gyri. Pons from middle-aged subjects was not available from any of the four brain banks that were contacted.

MEMBRANE PREPARATION (PAPER I, II AND III)

Crude synaptosomal fractions (P2) were prepared by homogenizing the tissue in 0.32 M sucrose solution and then centrifuging at 900 x g for 10 min at 4ºC. The resulting supernatant was re-centrifuged at 12 500 x g for 20 min at 4ºC. The final pellet was re-suspended in either 10 volumes of a phosphate buffer ([^{125}I]-α-bungarotoxin binding assay) or fresh 0.32 M sucrose ([^{3}H]-cytisine and [^{3}H]-AF-DX 384 binding assays). The protein content in all preparations was measured according to the method of Lowry using bovine serum albumin as the standard (Lowry et al., 1951). In order to obtain enough protein for receptor binding assay, specimens from early gestational stages were pooled together.

RECEPTOR BINDING ASSAYS

[^{125}I]-α-bungarotoxin binding (paper I and II)

The number of α7 nAChRs was determined by measuring the binding of the α7 nAChR antagonist α-bungarotoxin. Membrane fractions were incubated with 2.0 nM [^{125}I]-α-bungarotoxin (specific activity 133 Ci/mmol, DuPont/NEN, USA) in 10 mM sodium phosphate buffer containing 50 mM NaCl and 0.1% bovine serum albumin (pH 7.4) for 30 min at 37ºC, using 0.1 mg for the prenatal and 0.2 mg protein for the adult brain. Non-specific binding was determined by pre-incubating the samples with 10^{-6} M α-bungarotoxin for 30 min at 37ºC before adding the [^{125}I]-α-bungarotoxin. Bound and unbound α-bungarotoxin was separated by centrifugation at 11 500 x g for 20 min at 4ºC. The pellet was washed twice with the assay buffer and re-centrifuged at 11 500 x g for 5 min at 4ºC. The bottoms of the tubes with the pellets were cut off and transferred to new tubes and the radioactivity was determined in a γ-counter. The specific binding was calculated from the counted dpm and expressed as fmol/mg protein. The assays were mostly performed in triplicates but sometimes, due to limited amount of tissue, in duplicates for each subject.

[^{3}H]-cytisine and [^{3}H]-AF-DX 384 binding (paper III)

Membrane fractions (0.1 mg protein) were incubated with either 2.0 nM [^{3}H]–cytisine (specific activity 35.2 Ci/mmol, NEN Life Science Products, USA) in 50 mM Tris-HCl buffer (pH 7.4) on ice for 90 minutes or 2.0 nM [^{3}H]-AF-DX 384 (specific activity 120 Ci/mmol, NEN Life Science Products, USA) in 50 mM Na-K phosphate buffer (pH 7.4) at 25°C for 60 minutes. Non-specific binding was determined with 10^{-4} M (-)-nicotine ([^{3}H]–cytisine binding assay) or 10^{-6} M atropine ([^{3}H]-AF-DX 384 binding assay). Incubation was terminated by filtration under vacuum using a cell harvester with Whatman GF/C glass filters. Samples were washed three times with ice-cold binding buffer and transferred to scintillation tubes containing 5 ml scintillation fluid. The radioactivity was measured in a Wallac 1409® scintillation counter. The assays were mostly performed in triplicates but sometimes, due to limited amount of tissue, in duplicates for each subject.

RNA EXTRACTION (PAPER I, II AND III)

Total RNA from different regions was isolated by using either SNAP Total RNA isolation Kit (Invitrogen, Groningen, the Netherlands) or TRI REAGENT™ (Sigma-Aldrich, Sweden) and performed according to the manufacturers´ instructions. In order to remove traces of contaminating genomic DNA, all RNA samples were incubated with RNase-free DNase (Mousavi et al., 2001). Samples from the earliest gestational ages were pooled together to obtain enough RNA for RT-PCR.

RT-PCR (PAPER I, II AND III)

A relative quantification method was used, where the expression of nAChR and mAChR mRNA was compared with the expression of an internal standard. Cyclophilin is constantly expressed in all investigated regions during the first trimester (Falk et al., 2002) and was consequently used as internal standard in all PCR reactions. In order to obtain better separation between the mRNA of interest and the cyclophilin, two different sets of cyclophilin primers were used. nAChR mRNAs were co-amplified with cyclophilin primers giving a PCR product with the size 482 bp and mAChR mRNAs with cyclophilin primers giving a smaller PCR product (235 bp). When investigating the cyclophilin expression in the adult brain (paper II), we found that levels of cyclophilin were decreased compared to prenatal brain. This was also true for the two other housekeeping genes we examined, β-actin and GAPDH. Since the reduction of α7 mRNA expression was more pronounced than the cyclophilin expression we used cyclophilin as an internal standard also for this study. The relative amount of the mRNA of interest was estimated as the ratio between the intensity and the area of the band of receptor and the cyclophilin PCR products.

The RT-PCRs were performed using MuLV reverse transcriptase and Taq DNA polymerase. Experiments to ascertain that there was a linear relationship between the amount of input cDNA and the amplified PCR products were performed for each set of primers. Based on the results of these experiments, different amounts (1.5-4 µl) of RT reaction mixture, annealing temperatures and numbers of cycles of PCR were used in the PCR reactions, depending on the mRNA being amplified.

Since the m_3 mAChR and cyclophilin primers influenced each other's amplification of PCR products, they were not run within the same tube but in parallel in separate tubes.

PRIMARY CELL CULTURES (PAPER IV)

The human cerebral cortex was carefully mechanically dissociated into single cells and dispersed in either DMEM F12 medium (Dulbecco´s modified Eagle´s medium/Hamm´s F12 nutrient mixture (Gibco^{TM}, Invitrogen) supplemented with 10% fetal bovine serum (FBS), 5 µg/ml human insulin (Actrapid, Novo Nordisk, Bagsvaerd, Denmark), 1 µg/ml human Fe^{3+}-saturated transferrin, 0.1 mM putrescine, 30 nM sodium selenite, 0.3 nM hydrocortisone, 20 nM progesterone, 1 pM β-estradiol, 25 µg/ml transforming growth factor-α, 25 µg/ml fibroblast growth factor-basic, 0.5 mM L-glutamine and 20 µg/ml gentamicin or in Neurobasal medium (Gibco^{TM}) containing 2% supplement B-27 (Gibco^{TM}), 0.5 mM L-glutamine and 20 µg/ml gentamicin. Cells for the cytotoxicity and proliferation assays were plated at a density of 125 000 cells/cm^2 while a cell density of 35 000 cells/ cm^2 was used for immunocytochemistry.

Human neuroblastoma (SH-SY 5Y) and astrocytoma (U-118 MG) cells were plated at a density of 100 000 cells/cm^2. The SH-SY 5Y cells were cultured in minimum essential medium and the U-118 MG cells in Dulbecco´s Modified Eagle´s medium with 4 mM L-glutamine, both supplemented with 10% FBS. All cells were incubated at 37ºC under a water saturated atmosphere with 5% CO_2. Medium was changed every 2-4 days.

TREATMENT WITH NICOTINE AND ETHANOL (PAPER IV)

When the human cortical cells had been cultured for 5-6 days, the culture medium was replaced with fresh medium containing nicotine (10^{-7}-10^{-4} M) or ethanol (5-150 mM) or combinations of nicotine (10^{-7}, 10^{-6}or 10^{-5} M) and ethanol (10, 25 or 50 mM). The cells were grown for an additional 3 days until they nearly reached confluency. To reduce the evaporation of ethanol from the culture medium, the cell culture plates were covered with parafilm.

The human neuroblastoma and astrocytoma cells were treated with nicotine (10^{-7} or 10^{-6} M) and ethanol (10, 25 or 50 mM) or combinations of nicotine and ethanol for 3 days.

IMMUNOCYTOCHEMISTRY (PAPER IV)

For characterization of the cell cultures, immunocytochemistry was used. Neurons were detected with an antibody against the cytoskeleton protein β-tubulin class III and astrocytes with antibody against glial fibrillary acidic protein, (GFAP).

After 8-9 days in culture, the cells were fixed with 4 % paraformaldehyde and 4% sucrose in 0.1 M phosphate buffer and permeabilized with 0.2 % Triton prior to immunocytochemistry. To reduce the non-specific binding the cells were incubated with goat serum. The primary antibodies used for co-staining were mouse monoclonal anti β-tubulin class III antibody, dilution 1:1200 (Sigma-Aldrich Sweden AB, Stockholm, Sweden) and rabbit polyclonal anti-GFAP antibody, dilution 1:500 (DAKO A/S, Glostrup, Denmark), respectively. Incubation was performed overnight at 4ºC. For visualization the cells were incubated for 1 hour in RT with appropriate secondary antibodies conjugated with Alexa Fluor® 488, dilution 1:800 (Molecular Probes Europe BV, Leiden, The Netherlands) or Cy 3, dilution 1:400 (Jackson Immuno Research Laboratories Inc., West Grove, PA, USA). Negative controls were processed as above, but with the primary antibodies omitted.

IMAGE ANALYSIS (PAPER IV)

All images were generated by an Axiophot microscope (Carl Zeiss AG, Göttingen, Germany) and a Hamamatsu digital camera (Hamamatsu Photonics K.K., Hamamatsu City, Japan) and connected to a computerized imaging system (Openlab, version 2.1 for Mac OS, Improvision, Coventry, England). Post-processing was performed using Adobe PhotoShop (version 7.0) software for PC.

STATISTICAL ANALYSIS

Simple linear regression analysis and Pearson´s product-moment correlation was used for evaluation of correlations between the binding of [^{125}I]-α-bungarotoxin or expression α7 mRNA and gestational age (**paper I**).

One-factor ANOVA was used for comparing the [^{125}I]-α-bungarotoxin binding or the α7 mRNA expression between different regions in prenatal brain (**paper I**). A significant ANOVA ($p<0.05$) was followed by Fisher´s protected LSD *post-hoc* test (**paper I**).

In **paper II** the non-parametric Kruskal-Wallis test was used. A significant result ($p<0.05$) was followed by Mann-Whitney test.

In **paper III**, Kendall correlation analyses were used to evaluate alterations in receptor binding or mRNA expression of nAChR subunits and mAChR subtypes with gestational age. Simple linear regression graphs were used to visualize a significant result.

Statistical analyses for the influence of nicotine and ethanol on cell viability and proliferation were carried out using Friedmans and Mann-Whitney tests (**paper IV**).

RESULTS AND DISCUSSION

REGIONAL DISTRIBUTION OF THE α7 nAChR SUBTYPE IN HUMAN BRAIN AND SPINAL CORD AT THE FIRST TRIMESTER (PAPER I)

In the first paper the expression of the α7 nAChR subtype was investigated both on protein and mRNA level in medulla oblongata, pons, mesencephalon, cerebellum, subcortical forebrain, cortex and spinal cord from human brain at 4.5 to 12 weeks of gestation. A positive correlation was observed in both the number of α7 nAChRs (Fig. 4A) and α7 mRNA expression (Fig. 4B) with gestational age in most brain regions investigated.

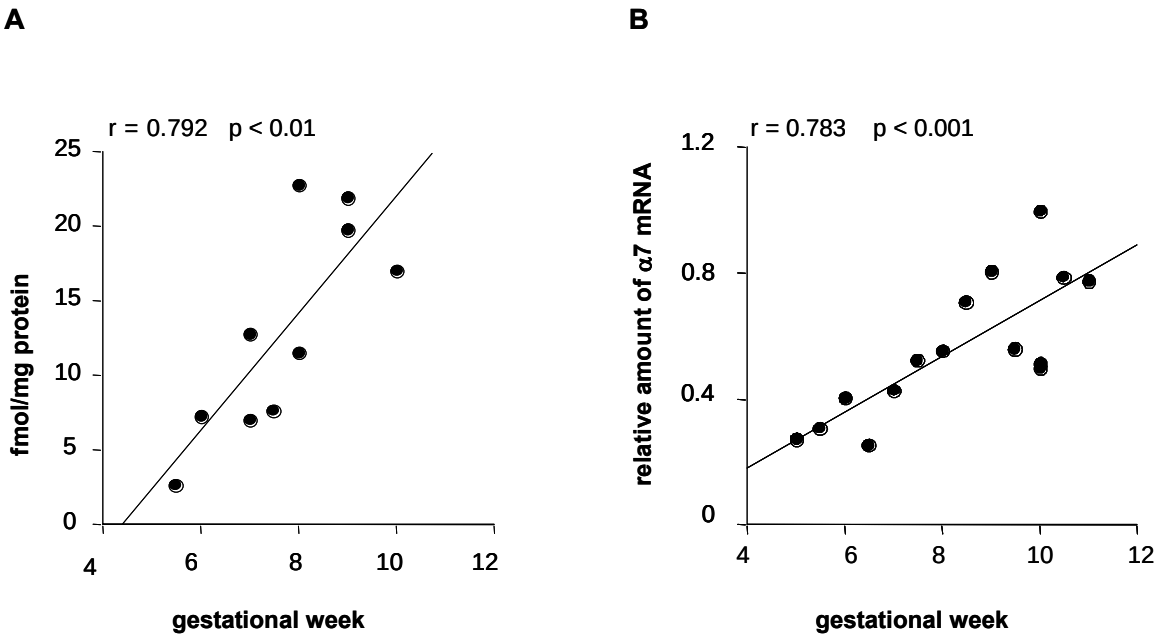

Figure 4. Specific binding of [^{125}I]-α-bungarotoxin (A) and α7 mRNA expression (B) in human pons during the first trimester. The relative amount of the α7 mRNA was estimated as the ratio between the intensity and the area of the band of α7 and the cyclophilin PCR products. r = correlation coefficient

In medulla oblongata, pons, mesencephalon and spinal cord the specific binding of [^{125}I]-α-bungarotoxin (Fig. 5A) and the relative amount of α7 mRNA (Fig. 5B) were approximately 2-3 times higher than in cortex, subcortical forebrain and cerebellum at 9-11 weeks of gestation.

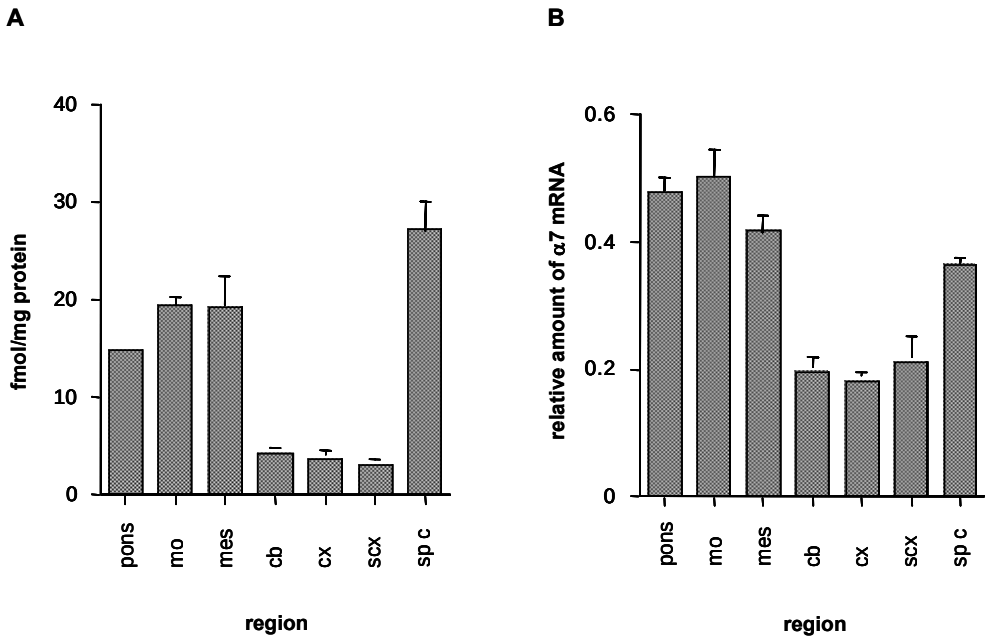

Figure 5. Specific binding of [^{125}I]-α-bungarotoxin (A) and α7 mRNA expression (B) in human CNS regions at 9-11 weeks of gestation. The relative amount of the α7 mRNA was estimated as the ratio between the intensity and the area of the band of α7 and the cyclophilin PCR products. Results are expressed as means ± SEM. mo, medulla oblongata; mes, mesencephalon; cb, cerebellum; cx, cortex; scx, subcortical forebrain; sp c, spinal cord.

The significant correlation between the nAChR binding and the mRNA expression in the prenatal brain indicates that the gene expression strongly influences the number of receptors in the brain during early development. The α7 mRNA expression appears to precede the occurrence of [^{125}I]-α-bungarotoxin binding sites in all brain regions. The delay in appearance of the binding sites suggests that other elements also influence the number of receptors. The regional differences in expression of α7 nAChRs found during the first trimester are probably due not only to regional differences in the induction of the α7 nAChR gene, but also to the fact that neurogenesis occurs later in the more rostral parts of the brain, cortex and subcortical forebrain. The density of the α7 nAChR subtype observed in the cerebellum in this thesis agrees with the reported low density during the second trimester (Whyte et al., 1985; Court et al., 1995) and is in accordance with the main proliferation period of cerebellar neurons occuring postnatally in the rat at a time corresponding to the third trimester in humans (Slotkin et al., 1986).

Similar age-related increases during the first trimester have been reported for the nAChR subtypes α3 and α4 in several human brain regions (Hellström-Lindahl et al., 1998).

DENSITY AND mRNA LEVEL OF α7 nAChR SUBTYPE IN PRENATAL COMPARED TO ADULT BRAIN (PAPER II)

A comparison of the number of α7 nAChRs in the prenatal (9-11 weeks of gestation) medulla oblongata, pons, cerebellum and cortex with the numbers in middle-aged (28-51 years) and aged (68-94 years) brain was performed in paper II. In adults, the relative amount of α7 mRNA was significantly lower in all regions investigated compared to the corresponding regions in prenatal brain except aged cortex (Fig. 6B). A corresponding decrease in the number of α7 nAChRs binding sites was also found in the adult medulla oblongata (Fig. 6A) and pons.

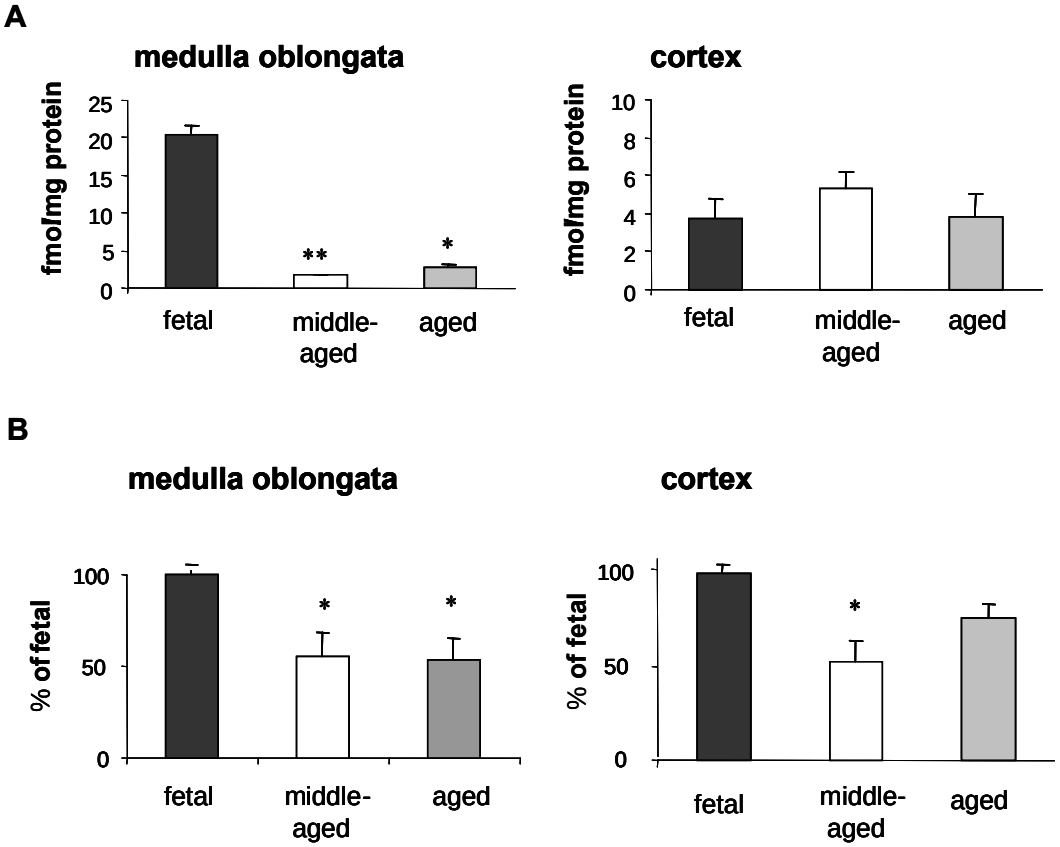

Figure 6. Specific binding of [^{125}I]-α-bungarotoxin (A) and α7 mRNA expression (B) in human fetal, middle-aged and aged medulla oblongata and cortex. The relative amount of the α7 mRNA was estimated as the ratio between the intensity and the area of the band of α7 and the cyclophilin PCR products. * $p<0.05$ and ** $p<0.01$ compared to the fetal region as analyzed with Kruskal-Wallis followed by Mann-Whitney test.

Both the number of [^{125}I]-α-bungarotoxin binding sites and the α7 mRNA expression appeared to be fairly constant from young adulthood throughout life (Fig. 7).

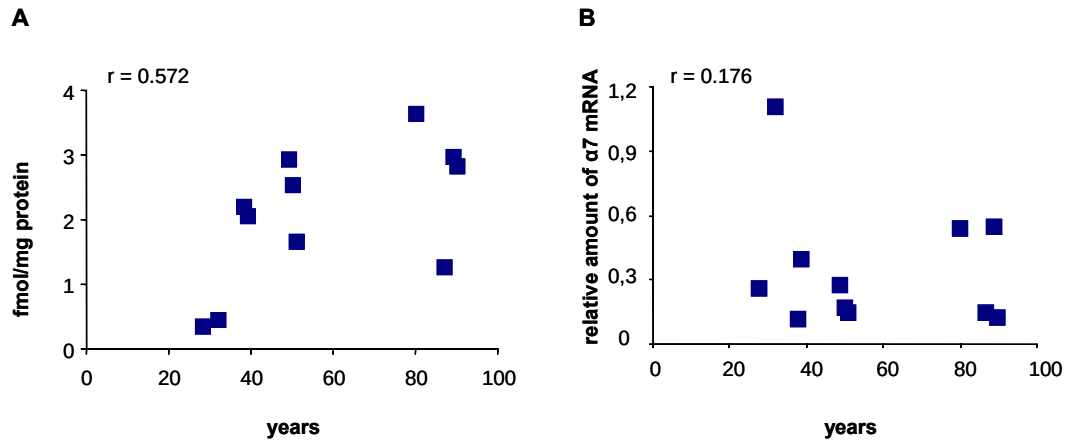

Figure 7. Specific binding of [^{125}I]-α-bungarotoxin (A) and α7 mRNA expression (B) in adult medulla oblongata. The relative amount of the α7 mRNA was estimated as the ratio between the intensity and the area of the band of α7 and the cyclophilin PCR products. No significant correlation was observed between number of [^{125}I]-α-bungarotoxin binding sites or α7 mRNA expression and age using linear regression analysis. r = correlation coefficient.

Later onset of neurogenesis in both cortex and cerebellum could be a reason why the number of α7 nAChRs in these regions was not found to differ significantly in prenatal and adult regions. The density of α7 nAChRs may possibly increase later during gestation, especially in the cortex, where the α7 nAChRs are found at high densities in the adults. In cerebellum other cholinergic markers such as the enzymes ChAT and AChE and the expression of mAChRs, have been reported to rise during late gestation, which appears to coincide with the initiation of cholinergic innervation of this region (Kwong et al.,2000). The highest levels of mAChRs are reached during the first postnatal years, after which they decline and are maintained at a low level throughout the rest of the life (Brooksbank et al., 1978; Kwong et al., 2000).

The high expression of α7 nAChRs during the prenatal period and early childhood suggests an important role during development. The high Ca^{2+} permeability is thought to be essential to the function of this receptor subtype. By influencing the intracellular Ca^{2+} concentration, Ca^{2+}-dependent processes, such as induction of immediate-early gene expression, could be regulated (Ghosh et al., 1994; Role and Berg, 1996; Hohmann and Berger-Sweeney, 1998). Different intracellular Ca^{2+} concentrations may induce different developmental processes. Low concentration of Ca^{2+} has been hypothesized to regulate the motility of neuronal growth cones and

neurite extension (Coronas et al., 2000); higher Ca^{2+} concentrations may be involved in neurite retraction (Pugh and Berg, 1994), growth inhibition, dendritic pruning and synaptogenesis (Bina et al., 1995; Broide et al., 1996; Jones et al., 1999), while the highest Ca^{2+} concentrations triggers apoptosis (Atluri et al., 2001). Induction of apoptosis in hippocampal progenitor cells through activation of α7 nAChRs, but not in more differentiated neurons, may involve the inability of progenitor cells to buffer increases in intracellelular Ca^{2+} levels or the lack of Ca^{2+}-transporting proteins (Berger et al., 1998; Atluri et al., 2001).

INFLUENCE OF SMOKING ON THE NUMBER OF nAChRs AND mAChRs

The influence of smoking on the expression of cholinergic receptors during the first trimester was investigated in paper III. We compared the number of α4 containing nAChRs ([^{3}H]-cytisine binding) and m_2 mAChRs subtypes ([^{3}H]-AF-DX 384 binding) and the gene expression of α4 and α7 nAChRs and m_1, m_2 and m_3 mAChRs in medulla oblongata, pons and cerebellum in aborted fetuses from smoking and non-smoking women. In general, the exposure to nicotine altered the expression pattern and density of nAChRs. An age-related increase in the number of [^{3}H]-cytisine binding sites, α4 and α7 mRNA expressions was found in the cerebellum of fetuses from non-smoking women, but not if the fetus had been exposed to nicotine. In the brain stem, nicotine also disturbed the normal developmental pattern, e.g. in medulla oblongata an age-related increase in [^{3}H]-cytisine binding sites was observed after nicotine exposure, whereas the age-related decrease in α4 mRNA expression in un-exposed tissue was abolished after nicotine exposure (Fig. 8).

Unfortunately, it was not possible to study the α7 nAChRs at protein level due to lack of tissue, but the pattern of α7 mRNA expression was changed in all three regions after exposure to nicotine. In some of the investigated regions the expression also of mAChR subtypes was changed in abortus of smoking women compared to non-smoking women. A positive correlation was observed between both [^{3}H]-AF-DX 384 binding and expression of m_2 mRNA with gestational age in pons exposed to smoking. No difference in the m_2 mAChR binding sites or gene expression with gestational age between aborted fetuses exposed or not to nicotine was found in medulla oblongata. The [^{3}H]-AF-DX 384 binding data from cerebellum were limited, due to the shortage of tissue; this made it difficult to interpret the effects of smoking in this region.

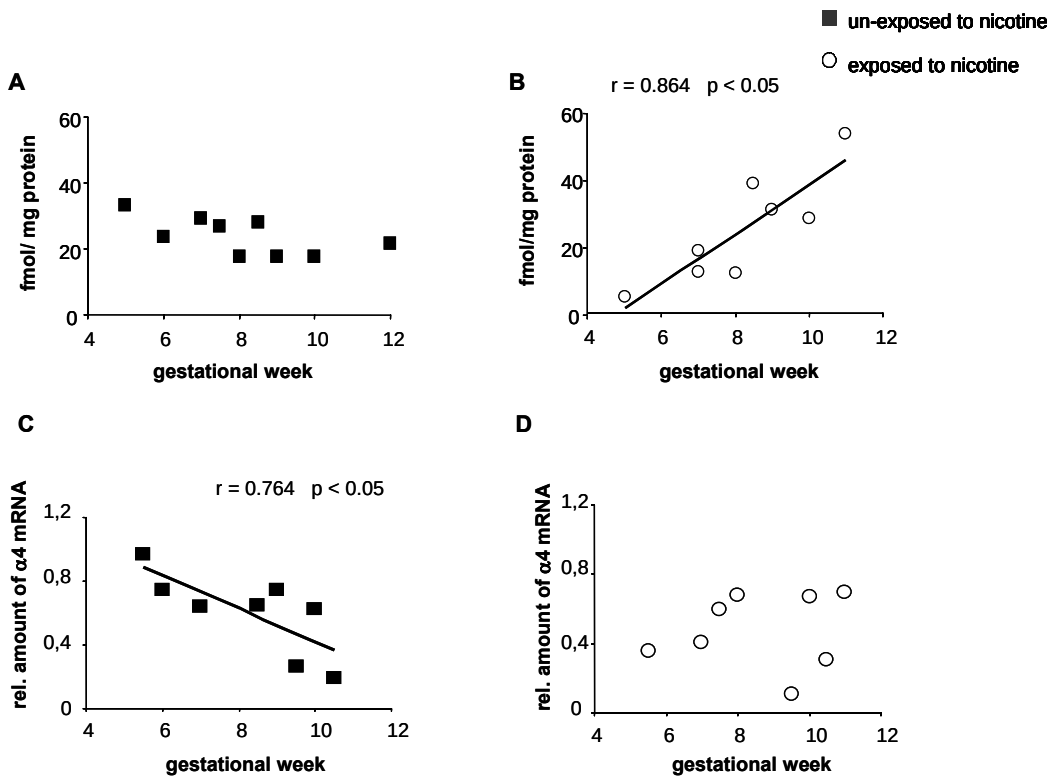

Figure 8. Specific binding of [^{3}H]-cytisine (A) and (B) and expression of α4 mRNA (C) and (D) in human first trimester medulla oblongata isolated from aborted fetuses of smoking and non-smoking women. The relative amount of the α7 mRNA was estimated as the ratio between the intensity and the area of the band of α7 and the cyclophilin PCR products. rel. = relative

The regional variations in the influence of smoking on the α4 receptor subunit may be due to a greater impact of gene expression on the receptor density in cerebellum, where neurogenesis starts later. The effects of nicotine exposure may also be influenced of the proportions of the α4β2 receptor and/or the subunit composition in different regions. The α7 mRNA expression during the first trimester in medulla oblongata, pons and cerebellum was investigated in both study I and III. An age-related increase was observed in study I. In study III we found this increase in medulla oblongata and pons of aborted fetuses exposed to nicotine in utero whereas in cerebellum the increase was found in abortus from non-smoking women. Information about the smoking habits of the women was lacking in most cases in study I. A large proportion of pons and medulla oblongata from abortus exposed to nicotine may have been included in study I.

Nicotine is thought to mimic the roles of ACh as a neurotrophic factor in the developing brain (Navarro et al., 1989; Slotkin, 1998). Proper temporal and spatial distribution of the various growth factors and neurotransmitters is crucial in the

development of the CNS. Since nicotine often is used on daily basis, nAChRs could be stimulated incorrectly and disturb the brain development.

It is well known that smoking up-regulates the number the nAChRs without affecting the gene expression in the human adult brain (Paterson and Nordberg, 2000; Dani and De Biasi, 2001; Mousavi et al., 2003). Both pre- and postnatal nicotine exposure is able to up-regulate the number of nicotine binding sites but only prenatal nicotine exposure influences the nAChR mRNA expression (Hagino and Lee, 1985; Slotkin et al., 1987a; Tizabi et al., 1997; Shacka and Robinson, 1998). Nicotine exposure increased both the number of [^{3}H]-cytisine and [^{3}H]-epibatidine binding sites and α3 and α7 mRNA expression in primary cell cultures of human prenatal cortex but not of subcortical forebrain or mesencephalon (Hellström-Lindahl et al., 2001). Exposure to nicotine during a critical postnatal period is able to cause changes in the nAChR density that persist into adulthood (Miao et al., 1998). The affinity of nicotine to nAChRs in prenatal and adult brain seems to be similar (Hellström-Lindahl et al., 1998). Up-regulation of both α4β2 and α7 nAChR subtypes in brains from smokers has been reported, although the affinity of nicotine to the α4β2 subtype is higher than for the α7 nAChR subtype (Mousavi et al., 2003; Hellström-Lindahl et al., 2004). These studies indicate that the effect of nicotine on the prenatal brain differs from that on the adult brain, where up-regulation is related to changes in receptor turnover, receptor desensitization, secondary structural changes in the receptor, or modification of the receptor by protein kinases or phosphatases rather than gene expression (Peng et al., 1994; Narayanan et al., 2002).

Desensitization may be physiologically important during smoking since a low concentration of nicotine is present for a long time in the brains of smokers (Dani and De Biasi, 2001; Wang and Sun, 2005). 0.5 μM nicotine applied chronically to a DA neuron reduced the ACh-induced inward current by approximately 65 %, indicating that a majority of the nAChRs were desensitized (Pidoplichko et al., 1997). Since nicotine is a drug of abuse that induces strong dependence, people often turn to nicotine replacement products or snuff when trying to quit smoking. Transdermal patches deliver a small and constant dose of nicotine throughout the day that will give a steady-state concentration. This imitates the situation in most smokers and snuff users, who are trying to maintain the steady-state plasma level of nicotine, possibly desensitizing a large proportion of nAChRs. The advantage of nicotine replacement products, though, is avoidance of exposure to the around 3500 other substances present in cigarette smoke. In general, receptors in desensitized states have higher agonist affinities, and over time a majority of receptors will end up desensitized. Perturbations in the development of the brain are likely if a large proportion of nAChRs are desensitized when they are supposed to be activated by pulses of ACh released in the normal ontogenesis of different brain regions.

The presence of mAChRs in all three investigated regions was detected as early as at 5 to 7 weeks of gestation, which is earlier than previous studies have reported (Gremo et al., 1987; Schlumpf and Lichtensteiger; 1987, Kinney et al., 1995b). That

smoking has effects also on the expression of mAChRs strengthens the hypothesis that prenatal nicotine may alter not only the expression of nAChRs but also of muscarinic, dopaminergic and serotonergic receptors. This is in accordance with several animal studies (Ribrary and Lichtensteiger, 1989; Zahalka et al., 1993; Zhu et al., 1996; Muneoka et al., 1999; Muneoka et al., 2001; Xu et al., 2001). Pre- and postnatal maternal smoking has been demonstrated to increase the risk of SIDS due to interference with the central and peripheral autonomic mechanisms that mediate the cardiorespiratory response to hypoxia (Slotkin, 2004; Anderson et al., 2005). Blunting of the cardiorespiratory and arousal response to acute hypoxia after prenatal nicotine exposure has been observed in newborn lambs (Hafström et al., 2002). In mice lacking the β2 nAChR subunit a similar impaired response to hypoxia has been demonstrated (Cohen et al., 2002). An increase in inhibitory m_2 mAChRs and a decrease in stimulatory α-adrenergic receptors in rat hearts have been reported after prenatal nicotine exposure, as well as a reduced density of m_2 mAChRs in the brain stem. The net effect of these alterations is likely to contribute to the excessive bradycardia and cardiac conduction abnormalities identified in SIDS victims. In children who died from SIDS a similar decrease in mAChRs was observed in arcuate nucleus in the medulla oblongata (Kinney et al., 1995a). Nicotine itself has also been reported to induce adenylyl cyclase activity, in contrast to the inhibitory effect on this enzyme that is observed after stimulation of m_2 and m_4 mAChRs with muscarinic agonists (Slotkin et al., 1999; Slotkin, 2004).

EFFECTS OF NICOTINE AND ALCOHOL EXPOSURE ON CELL VIABILITY AND PROLIFERATION IN PRIMARY CELL CULTURES OF HUMAN PRENATAL CORTEX

Effects of 3 days exposure to nicotine and ethanol, both separately and in combination, were investigated in primary cell cultures of human first trimester cortex. Two different media were used, one medium (DMEM F12 supplemented with 10% FBS) directing differentiation to a mixture of neurons and astrocytes and in the other (Neurobasal medium supplemented with 2% B 27) mainly supporting neuronal differentiation (Fig. 9 and the cover).

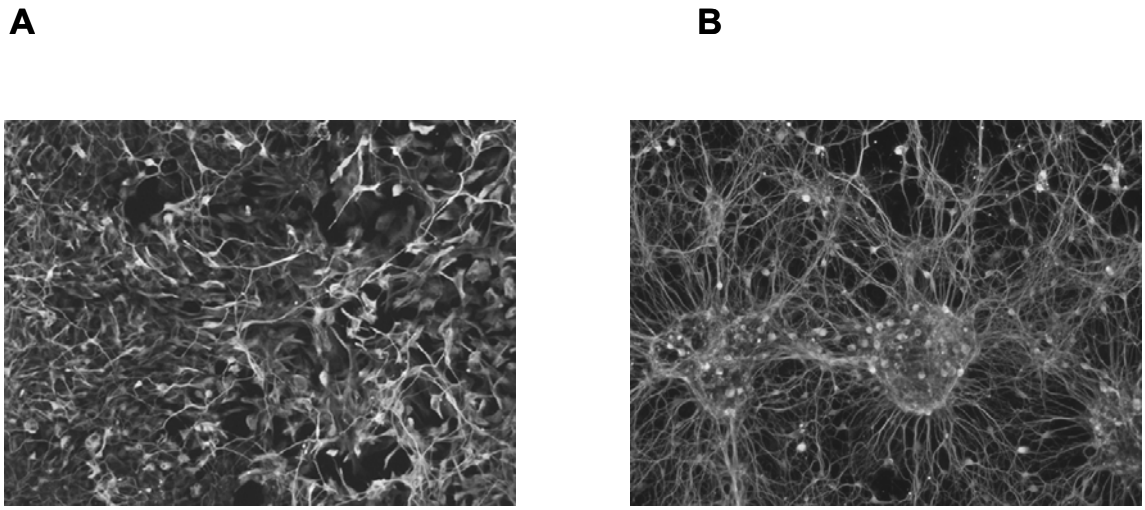

Figure 9. Primary cell cultures of human cortex (9 weeks of gestation) cultured in DMEM F12 supplemented with 10% FBS (A) and Neurobasal medium supplemented with 2% B27 (B) for 8 days. Cultures with neurons and astrocytes, cells immunoreactive to β-tubulin (class III) (green) or GFAP (red), respectively, were found after culturing the cells in DMEM F12 medium. In Neurobasal medium mainly neurons were observed. For colour picture see the cover.

The combined treatment increased the cytotoxic effects in both types of cell cultures (Fig. 10). In general there was a tendency of a dose-related decrease in cell viability at all three nicotine concentrations when ethanol concentration was increased. The mixed cell cultures were more susceptible to the combination of physiologically relevant concentrations of nicotine and ethanol, 10^{-7} M and 10 mM respectively, than pure neuronal cultures. A tendency towards neuroprotective effects was found at increasing concentration of nicotine together with 10 mM ethanol. The neuronal cultures were more sensitive than mixed cell cultures to low concentrations of ethanol alone and high concentration of nicotine.

The proliferation in the mixed cell cultures was decreased after treatment with the combination of 10^{-5} M nicotine and 10, 25 and 50 mM ethanol as well as the combination of nicotine 10^{-6} M and ethanol 50 mM (Fig. 11).

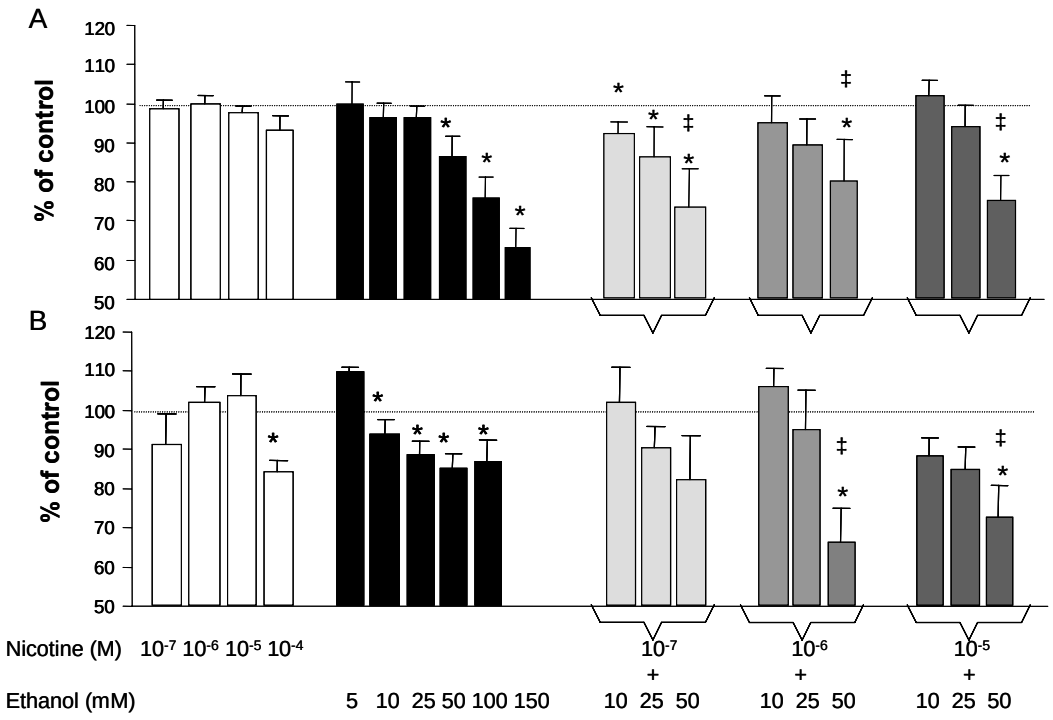

Figure 10. Cell viability in primary cell cultures of human cortex (6-10.5 weeks of gestation) cultured in DMEM F12 medium supplemented with 10% FBS (A) and Neurobasal medium supplemented with 2% B27 (B) after treatment for 3 days with nicotine, ethanol and combination of both drugs. * $p<0.05$ significantly different from control cells, ‡ $p<0.05$ significantly different from corresponding nicotine concentration.

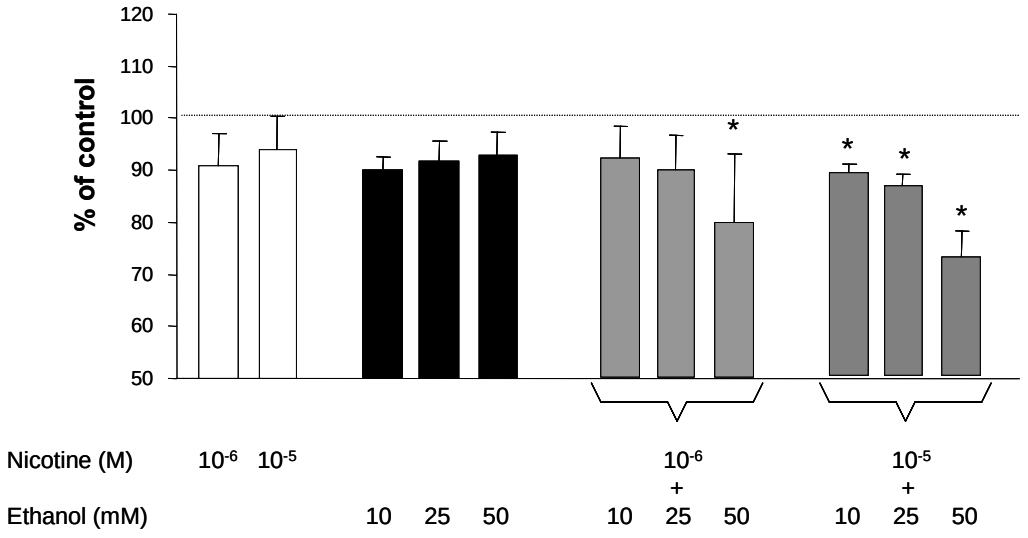

Figure 11. Proliferation, measured as BrdU incorporation, in primary cell cultures of human cortex (6-10.5 weeks of gestation) cultured in DMEM F12 medium supplemented with 10% FBS after treatment for 3 days with nicotine, ethanol and a combination of both drugs.

* $p<0.05$ significantly different from control cells.

No cytotoxic effects were observed in the human neuroblastoma cell line SH-SY 5Y and astrocytoma cell line U118 MG investigated after 3 days exposure to nicotine or ethanol or the two in combination, indicating that primary cells from human fetal brain are much more sensitive than immortalized cells to nicotine and ethanol.

Remarkably, the combination of nicotine and ethanol at concentrations normally found in smokers and during social drinking, elicited a significant reduction in the number of living cells in cultures with both neurons and astrocytes. Many people smoke in connection with alcohol intake, which from our results seems to be devastating to the number of developing cells. Numerous studies have reported an increased cell death in the developing rat brain after exposure to nicotine (Joschko et al., 1991; Roy et al., 1998; Slotkin, 1998) or ethanol (Maier et al., 1999; Mooney and Miller, 2003). Stem and progenitor cells seem to be more susceptible to the effects of both nicotine (Berger et al., 1998) and ethanol (Dunty Jr et al., 2001; Hao et al., 2003) than more mature and differentiated cells. The nicotine-induced apoptosis observed in hippocampal progenitor cells is mediated through the α7 nAChR subtype (Berger et al., 1998). Neuronal cultures in our study were more affected by low concentrations of ethanol than the mixed cultures. The moderate effect of nicotine alone observed in this thesis may be due to the relatively small number of progenitor cells that are present after 7-8 days in culture and/or the low density of nAChRs in the immature first trimester cortex (Hellström-Lindahl et al., 1998; Falk et al., 2002).

In addition, a tendency to protective effects of increasing nicotine concentrations was observed in combination with a physiologically relevant ethanol concentration (10 mM) in the mixed cultures. Neuroprotective effects of nicotinic agonists against ethanol induced cytotoxicity have been reported in studies on cultures of prenatal rat cortex, hippocampus, and cerebellum as well as in PC 12 cells (Li et al., 1999; de Fiebre and de Fiebre, 2003; Tizabi et al., 2003; Tizabi et al., 2004) and in vivo (de Fiebre and de Fiebre, 2005). Several pathways mediating these neuroprotective effects of nAChRs have been identified, including increased expression of Ca^{2+} buffering proteins and neurotrophic factors, and activation of phosphatase calcineurin (Jonnala and Buccafusco, 2001; Prendergast et al., 2001; Qiao et al., 2003; Stevens et al., 2003).

Cell replication is partly regulated by neurotransmitters (Ernst et al., 2001). ACh, acting through mAChRs, stimulates the proliferation of neural stem and progenitor cells and astrocytes (Ma et al., 2000; Guizzetti et al., 2003). A decreased carbachol-induced proliferation of astrocytes after exposure to 25 and 50 mM ethanol have been observed; this was probably due to interference with the phosphoinositide metabolism (Guizzetti et al., 2003). Inhibition of ethanol on the basal proliferation is found after exposure to high ethanol concentrations (100 mM and above) (Costa and Guizzetti, 1999). In our study we investigated the effects of nicotine and ethanol alone or in combination on the basal proliferation in the primary cell cultures with both neurons and astrocytes. No effects were seen after exposure to only one drug, but there was a reduction in cell replication after combined treatment. Possibly this is due to the fact that the majority of cells in our mixed cultures have started to differentiate and lost their ability to

proliferate after 7-8 days in culture and also that basal proliferation is less affected by ethanol than proliferation induced by ACh and carbachol. Early ethanol exposure influences the neuronal generation. Several studies have shown that replication of neural stem and progenitor cells is susceptible to ethanol exposure during neurogenesis - but not prior to or after this period - in both humans and rats (Miller, 1995; Crews et al., 2003; Hao et al., 2003).

The moderate effect of nicotine and ethanol on proliferation seen in this thesis could not explain the observed decrease in cell viability. Further studies investigating the ability of nicotine and ethanol to induce apoptosis are needed to better understand the mechanisms behind the cell death. A more accurate characterization of the cell cultures used in this thesis is required, investigating the co-expression of GFAP or β-tubulin with neural stem cell markers such as Sox-2 and nestin as well as co-expression with nAChR subunits.

In electrophysiological studies, physiologically relevant concentrations of ethanol can either inhibit or enhance the ACh-evoked currents, depending on the nAChR subtype involved (Aistrup et al., 1999) and a dose-related decrease in the number of nAChRs has been observed after ethanol exposure (Gorbounova et al., 1998; Dohrman and Reiter, 2003). The activated open nAChR is supposed to be in a phosphorylated state and phosphokinase C is believed to be involved (Wang and Sun, 2005). Acute exposure to ethanol is suggested to activate phosphokinase C, leading to a larger proportion of nAChRs being in an active, phosphorylated state, a situation that necessitates a decrease in the number of nAChRs to maintain homeostasis (Dohrman and Reiter, 2003).

CONCLUDING REMARKS

Nicotine exposure in the form of maternal smoking in developed countries is likely to represent the single most important cause of pre-and postnatal morbidity and mortality (Pidoplichko et al., 1997). Pre- and postnatal nicotine exposure is also associated with long-term effects such as cognitive disturbances, behavioural problems and childhood respiratory disorders (Higgins, 2002). Once the supine sleeping model has become established, the maternal smoking took over as the predominant cause of SIDS (Anderson et al., 2005).

The studies presented in this thesis demonstrate the early presence of cholinergic receptors in the human prenatal brain and the ability of nicotine exposure in utero to influence the expression of gene transcripts and the receptor proteins of both nAChRs and mAChRs. Changes of these receptors in brain stem by nicotine may be of importance since several nuclei regulating cardiac activity, respiration, sleep and arousal are located in the brain stem. In addition, nicotine in combination with ethanol enhances the toxic effects of these substances in primary cultures of human prenatal cortex. The presence of functional nAChRs on mouse stem and progenitor cells (Atluri et al., 2001; Schneider et al., 2002) raises questions concerning their functional role prior to development of CNS and the influence of nicotine exposure on phenotype of the cells.

Beside the transmitter and the receptor, also the developmental context in which the stimulation occurs determines the net effect on the fate of the target cell. Is the developing brain more vulnerable to the effects of nicotine during a certain stage of development? Since ACh and cholinergic signalling play a critical role in virtually all phases of brain maturation, from before the neural tube stage to late phases including adolescence (Slotkin, 2004), it is hardly likely that any safe periods exist. Smoking will provide excessive cholinergic stimulation throughout fetal life. Nicotine from maternal smoking or environmental tobacco smoke can discoordinate the numerous events in cell replication, differentiation, and synaptic development that are necessary for the proper assembly of the mammalian brain.

The adverse effects of nicotine also extend to other transmitter systems. Changes in both the noradrenergic and dopaminergic systems after prenatal nicotine exposure persist into adulthood (Navarro et al., 1988; Seidler et al., 1992; Oliff and Gallardo, 1999). Effects of nicotine on both the transmitter and receptors in the serotonergic system have been reported (Kenny et al., 2001; Muneoka et al., 2001).

Drugs of abuse that modify the signalling of neurotransmitter systems and the intracellular messengers are likely to affect the development of the CNS. Cocaine, like nicotine, affects cell replication and compromises brain development, leading to an increased risk of spontaneous abortion and to cognitive deficits that manifest during childhood (Slotkin, 1998; Ness et al., 1999; Harvey, 2004). Prenatal exposure to amphetamine and 3,4-methylenedioxymethamphetamine (ecstasy) induce persistent

neurochemical and behavioural alterations in the offspring (Nasif et al., 1999; Koprich et al., 2003).

The ability of the nervous system to repair and compensate for these disturbances may depend on where and when they occur. Despite the profound alterations in cell morphology observed, caused by prenatal nicotine exposure, a substantial recovery can occur, so that the brain structures are not grossly abnormal when examined in adolescence or adulthood (Roy et al., 2002).

Although the number of women who smokes during pregnancy is decreasing in the Western world - less than 10 % of Swedish women continue to smoke while pregnant – tobacco use has great impact on health and the economy in our society. The same is true of environmental tobacco smoke. Since the neurobehavioral disturbances observed contribute to school and career failure and increased risk of criminal behaviour, the advantage to be gained from reducing maternal smoking are obvious. Pregnant women should be advised to totally refrain from smoking and ethanol intake during gestation. Nicotine substitution may not be a safe alternative.

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