Aus dem Zentrum Anatomie der Universität zu Köln
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Distribution of the nicotinic acetylcholine receptor subunits alpha4 and alpha7 in the human foetal brain

Inaugural-Dissertation zur Erlangung der Doktorwürde
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vorgelegt von
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Weitere Personen waren an der geistigen Herstellung der vorliegenden Arbeit nicht beteiligt. Insbesondere habe ich nicht die Hilfe eines Promotionsberaters in Anspruch genommen. Dritte haben von mir weder unmittelbar noch mittelbar geldwerte Leistungen für Arbeiten erhalten, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen.

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Basel, den 10. Juni 2008

Das in dieser Arbeit verwendete Hirngewebe wurde von Herrn Dr. G. van Noort und Herrn Dr. R.A.I de Vos vom Laboratorium Pathologie Oost Nederland entnommen, fixiert und in Paraffin eingebettet.

Die in dieser Arbeit angegebenen Experimente sind nach entsprechender Anleitung durch Frau Kirsten Pilz von mir selbst ausgeführt worden.

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Meinen Eltern

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ABBREVIATIONS

ABC	avidin-biotin-complex
α-BTX	α-bungarotoxin
ACh	acetylcholine
AChE	acetylcholinesterase
ADNFLE	autosomal dominant nocturnal frontal lobe epilepsy
AT	acetyltransferase
BSA	bovine serum albumen
ChAT	cholinacetyltransferase
ChE	cholinesterase
CNS	central nervous system
CP	cortical plate
CPsup	superficial part of the cortical plate
CPinf	cortical plate underneath the CPsup
D	density
DAB	diaminobenzidine
E	embryonic day
FCS	foetal calf serum
GABA	γ-aminobutyric acid
GFAP	glial fibrillary acidic protein
g.w.	gestational week
H_2O_2	hydrogen peroxide
I	intensity
Ig	immunglobulin
IZ	intermediate zone
L	cortical layers
mAb	monoclonal antibody
mRNA	messenger ribonucleic acid
MZ	marginal zone
nAChR	nicotinic acetylcholine receptor
PET	positron emission tomography
RT	room temperature
RT-PCR	reverse transcriptase-polymerase chain reaction
SIDS	sudden infant death syndrome

SP	subplate
SZ	subventricular zone
TBS	tris/NaCl buffer
VZ	ventricular zone
WM	white matter

1. INTRODUCTION

1.1 THE FOETAL HUMAN BRAIN

The central nervous system (CNS) is one of the most complex parts of the human organism. Although a lot of research has already been done, numerous pieces are still missing in the puzzle. In particular, the mechanisms of the brain's ontogenesis still remain to be solved.

On average, pregnancy in humans lasts 267 days from conception till birth. As the last menstruation is easier to determine, the gestation time is usually expressed in days post menstruationem or gestational weeks (g.w.), equalling 280 days or 40 weeks. The embryonic period lasts until g.w. ten; after that the foetal period starts. Histogenesis of the CNS continues until well after birth [52, for review cf. 38].

Kostovic et al. [51,52] described the ontogenesis of the human isocortex in detail. At the very beginning of the embryoblast's development three germinal layers develop from which all adult tissues originate: ectoderm, entoderm, and mesoderm. Part of the ectodermal cells later form the nervous system, beginning with the neural tube around day 22 of ontogenesis. Its rostral end swells and becomes the cerebrum, its caudal part transforms into the spinal cord and its cavity into the ventricular system. By the end of the third to fourth week the entire wall of the neural tube consists of a single layer of cells, the ventricular zone (VZ) [51,93]. These cells multiply and migrate along radial glia towards the pial surface where they grow axons and dendrites and form characteristic layers. Having reached their final destination, neurons are then thought to differentiate into different types. Shaping of synapses already takes place shortly after g.w. nine. The appearance of dendritic spines occurs in the second half of gestation. Synaptogenesis, cell proliferation, migration, and outgrowing of axons thus are overlapping processes for five to six months. Another major factor in brain development, in particular concerning the three-dimensional structure with its gyri and sulci, is apoptosis. The morphogenetic cell death already occurs at the beginning of telencephalic growth, when low levels of proliferation and high levels of apoptosis in the dorsal midline result in relative invagination and subdivision of the forebrain into two hemispheres [32]. Later in development it leads to a gradual dissolution of the subplate (SP), starting at the bottom of the sulci and resulting in cortical folding [69].

Kostovic et al. [51,52] established a subdivision into six periods (phase I to phase VI) each of which is characterised by typical cytoarchitectonic changes. Figure 1 shows examples of cresylviolet stainings of middle and late foetal cortices. Table 1 provides an overall view of the formation of the cerebrocortical layers.

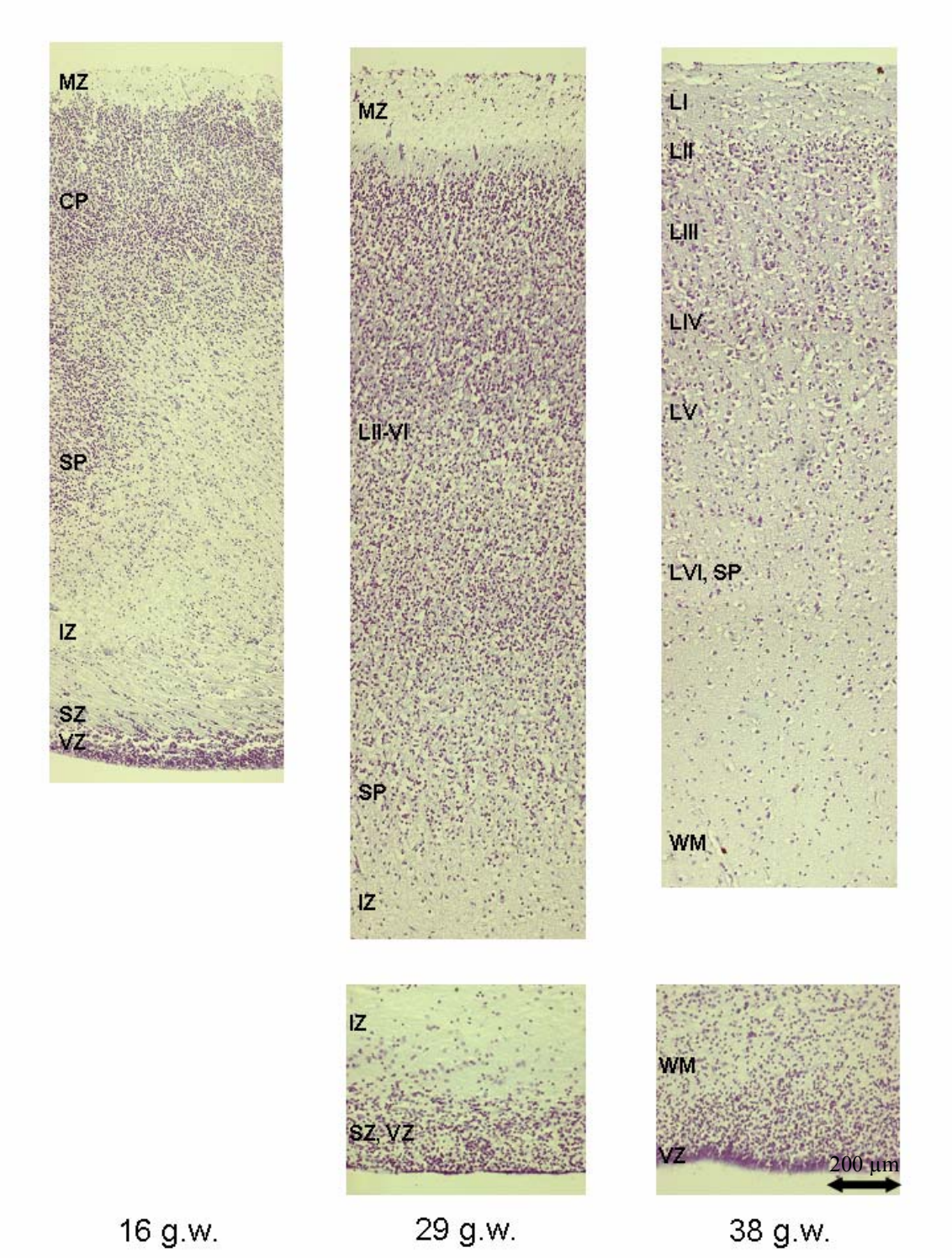

Fig.1: At 16 g.w. the subplate (SP) is the broadest layer in the developing brain. During the following weeks the cortical plate (CP) becomes more prominent and the cortical layers start to differentiate. The SP decreases in size. At 38 weeks the layers can be clearly distinguished. The cell-poor white matter (WM) expands.

Tab.1: Histogenesis of the human cortex [51]

Phase	Gest. Week	Stage	Ventricle → → → → → → → → Pial surface					
I	4-7	embryonic	VZ	SZ	IZ	MZ		
II	8-12	early foetal	VZ	SZ	IZ	CP	MZ	
III	13-15	middle foetal	VZ	SZ	IZ	SP	CP	MZ
IV	16-24	middle foetal	VZ	SZ	IZ	SP	CP	MZ
V	25-37	late foetal	VZ	SZ	IZ	SP	LII-VI	MZ
VI	38-42	newborn	VZ	WM	SP	LII-VI	LII-VI	LI

VZ=ventricular zone, SZ=subventricular zone, IZ=intermediate zone, MZ=marginal zone, CP=cortical plate, SP=subplate, L=cortical layers, WM=white matter

Phase I: During g.w. five the neuroepithelium of the VZ grows centrifugal cytoplasmic processes that reach a preformed basal membrane around the prosencephalon and form the second layer, the hypocellular MZ. Between these two layers the IZ, representing the major migration zone, appears during g.w. six. The IZ also contains bundles of axons which belong to neurons located in the basal forebrain and thalamus that project to the cortex. This is why the IZ is often called the "foetal white substance". Long processes of radial glial cells provide a pathway for the movement of cells into other layers [for review cf. 38]. Some cells also move tangentially to other brain regions or radially in the direction of the ventricle [71]. The proliferation of cells takes place in the VZ as well as in the surrounding SZ which appears at the end of the sixth g.w. Ventricular cells are precursors of both neurons and microglia [51].

Phase II: From g.w. eight onwards immature neurons that have moved through the IZ start to accumulate below the MZ, forming a very cell-dense layer called CP. The CP is a precursor of the major part of the cerebral cortex (LII-V). Studies have shown that the cell population of the CP follows an "inside-out" principle: The inner part of the CP develops from early generated cells whereas younger neurons migrate past them to form the superficial parts [10]. A smaller amount of well differentiated neurons, called Cajal-Retzius-cells, migrate into the MZ. Their axons run tangentially and can be found throughout the whole process of brain development [69].

Phase III/IV: A secondary cortical zone, the SP, forms during the middle foetal period below the CP. The SP becomes the most prominent layer, being four times as broad as the adjoining CP. It contains a diverse population of relatively few but comparatively mature cells [69,70] and lots of fibres and synapses. The afferents running from the basal forebrain (Ncl. basalis Meynert) and thalamus towards the cerebral cortex are delayed on their way through the SP. In that light the SP is seen as a "waiting compartment" [52,54]. MZ, CP, and part of the SP will later form the neocortical layers. At the end of phase IV neuronal migration culminates in the lamination of the CP and the chemical differentiation of cortical neurons begins [51]. Regarding the outer structure of the brain

this is the time when the morphological appearance of the brain changes rapidly. Number and depth of the sulci increase resulting in an enlargement of the cortical surface [55].
Phase V: Gliogenesis and ingrowing of fibres are the major causes for the thickening of the cerebral wall towards the end of the foetal period [53]. As a result of a decreasing neuronal proliferation rate and migration VZ and SZ gradually thin out. The VZ is replaced by ependyma which produces cerebrospinal fluid. In addition, the SP rapidly decreases in extent. Single interstitial cells in the adult WM probably represent a vestige of foetal SP neurons [53,69]. On the other side, neuronal differentiation and development of axons and synapses increase. Pyramidal cells get their characteristic shape and grow dendrites; thalamocortical axons arrive in layer IV. From g.w. 32 on the six typical cortical layers can be observed with variations in shaping and thickness according to the Brodmann areas [13]. The MZ is converted into layer I.
Phase VI: The definitive cortical outline is already constituted but is still immature at term. During the following weeks the maturation of the cerebral cortex proceeds further.

The two areas investigated during the course of this work were situated in the frontal and the occipital lobe. The Rolando fissure or central sulcus, which indicates the border between frontal and parietal lobe, appears around g.w. 24 and is constant within individuals. At 30 weeks secondary sulci begin to form; tertiary sulci appear around term [55]. In the mature frontal brain the precentral gyrus contains the motor cortex (area 4) which is the origin of the pyramidal or corticospinal tract. Area 4 has a somatotopic structure and the differentiation of layers is asynchronous in different regions: at 26 to 28 g.w. the areas corresponding to the trunk seem to be further advanced than those projecting to the lower limbs, which are in turn higher developed than the upper limb areas [55]. During phase V the appearance of Betz cells (large pyramidal neurons) indicates the primary motor area. Other areas begin to differentiate, too, but cannot be morphologically distinguished at that time [52].

The occipital lobe was our second focus of attention as it hosts the visual cortex which is rich in afferent neurons and has thus a high share of migrating cells [for review cf. 96]. From g.w. 19 onwards primary occipital sulci appear. On the medial side of the hemisphere the sulcus parietooccipitalis, the sulcus cinguli, and an outline of the calcarine fissure can be observed [51,55]. At around g.w. 24 the occipital lobe elongates and the calcarine sulcus forms its characteristic “T” junction with the parietooccipital sulcus. The mature visual pathway consists of four successive neurons originating in the retina and ending in the occipital primary visual cortex. The axons of the fourth neuron, called radiation of Gratiolet, approach the primary visual cortex (area 17) which is

situated in the walls of the calcarine sulcus. At approximately week 23 of gestation the first thalamocortical afferents appear in the prestriate visual cortex. Subsequently the six layers form and from g.w. 28 onwards layer IV broadens and splits into three sublayers containing the macroscopically visible stripe of Gennari [86] which is unique for area 17. It consists of myelinated axons of thalamocortical neurons because of which this part of the cortex has been named area striata. The primary visual cortex seems to be extremely vulnerable to disruption of its normal development as has been shown in studies on visual deprivation of kittens and monkeys [43,103]. The risk of damaging influences is especially high during the early postnatal period when the maximum growth of the visual cortex occurs [86].

1.2 Nicotinic Acetylcholine Receptors

The nicotinic acetylcholine receptors (nAChRs) belong to the superfamily of ligand-gated excitatory cation channels. The nAChRs can be found in tissues as diverse as e.g. skeletal muscle, neurons, lymphoid cells, fish electric organs, and keratinocytes and can be either pre- or postsynaptic. At presynaptic sites they can modulate the release of different neurotransmitters. At postsynaptic sites, as for example the neuromuscular junction, they mediate synaptic transmission [for reviews cf. 33a,49a,57,92].

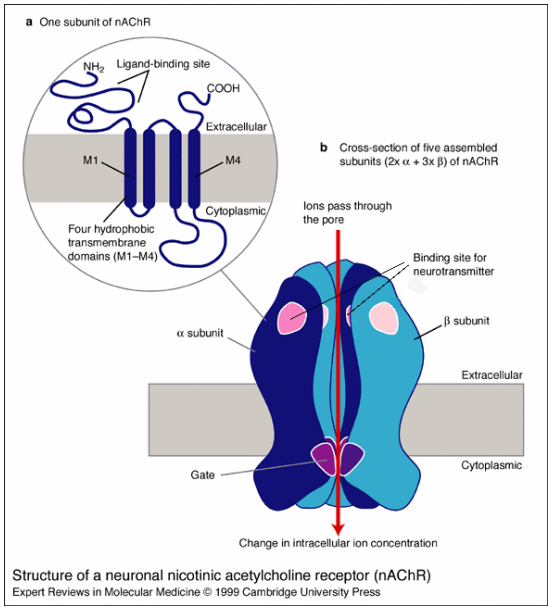

Fig.2: Schematic view of an α4β2 nAChR and its suspected pentamer structure: (a) A single subunit with its extracellular and transmembrane domains and the intracellular loop. (b) The channel is formed by five subunits which are inserted into the membrane.

The structure of the neuronal nAChRs is supposed to be pentameric (cf. fig.2) [18]. Different subunits assemble in various combinations as a group of five around a central channel. The nAChRs span the cell membrane protruding on both sides [49]. They consist of an extracellular, a transmembrane, and a cytoplasmatic region. The N-terminal extracellular domain includes the receptor binding site. There are four transmembrane domains (M1-M4) which show an extended cytoplasmatic loop between M3 and M4. This intracellular loop is unique in sequence and sometimes in size for each individual nAChR subunit. The M2 domains from each subunit are thought to form the lining of the ion channel [61,78]. The binding of acetylcholine (ACh) molecules to the receptor leads to a conformational change which allows cations such as calcium, sodium, and potassium to stream into the cell and depolarise the membrane [97].

In contrast to the skeletal muscle only comparatively small amounts of nAChRs can be found throughout the central and peripheral nervous system. Major cholinergic projections have been identified throughout various regions of the brain [61] e.g. originating in the basal nucleus of Meynert and the hippocampus [62]. While the role of nAChRs at the neuromuscular junction is well known it has not yet been completely clarified for the interneuronal transmission. Within the CNS nAChRs seem to have a mainly modulatory function [86]. Observations of the effects of nicotine indicate an influence of nAChRs on different physiological and endocrinological functions [34]. They act in the mediation of excitatory neurotransmission at some sites in the CNS where they contribute to nicotine-sensitive processes involved in emotion, sensory processing, cognition, and attention [61]. Cholinergic neurons modulate sleep and arousal at thalamic and cortical levels. ACh and the α7 nAChRs have been implicated to regulate the outgrowth of neuronal processes [81,105,for review cf. 58].

The most popular function, though, is the central effect of nicotine which causes addiction to tobacco. Smoking still causes thousands of premature deaths each year. Maternal smoking has adverse effects on foetal development. These include increased risk for sudden infant death syndrome (SIDS), behavioural abnormalities, and cognitive deficits. Being exposed to nicotine before birth might also influence the probability of a nicotine addiction later in life [13a,94]. Also in this respect more detailed data on foetal nAChR distribution are of great importance.

NAChRs also play an important role in several diseases of the nervous system. Within central neurons loss of high-affinity nicotine binding sites is associated with Alzheimer's and Parkinson's disease [for review cf. 49a]. Nicotinic AChRs are also involved in Tourette's syndrome, anxiety,

and analgesia [85, for review cf. 57]. The nicotinic α7 subunit may be involved in the auditory gating deficit observed in schizophrenia [20, for review cf. 49a]. A missense mutation of a gene on chromosome 20q, which maps to the α4 subunit, appears in patients with a form of partial epilepsy, the autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE). The mutation affects the cation channel function in α4/β2 nAChRs and thus causes frequent violent nightly seizures which usually begin during childhood [95]. A positron emission tomography (PET) study showed a change in the α4β2 nAChR density in ADNFLE patients [79]. Polymorphisms of this gene are also linked to childhood and juvenile absence epilepsies and juvenile myoclonic epilepsy [72].

1.3 nAChRs in the Human Foetal Brain

The development of neuronal connections comprises growth of presynaptic axons and postsynaptic dendrites which together form a synapse. Early afferent axons originate in brainstem, basal forebrain, and thalamus neurons. Outgrowing axons may contain synaptic vesicles even before establishing contact to dendrites [52]. Early immature axo-dendritic synapses in humans have less synaptic vesicles and a shorter synaptic membrane than those in the adult brain [51]. The first synapses have been observed by electron microscopy shortly after g.w. nine above and below the CP [52]. The earliest immature synapses found *within* the CP appear at g.w. 23 [54,66].

Cholinergic neurons are among the first to differentiate in the CNS [106]. Nicotinic AChR mRNA and protein are produced in more differentiated human neuronal cells at least from four to five g.w. onwards when they can be detected in the human spinal cord, medulla oblongata, pons, and subcortical forebrain [41]. In the cortex, by contrast, nAChR mRNA is not found until g.w. eight [41,42]. A previous study showed that [^{3}H]nicotine binding sites appear in the foetal human cortex at around 14-16 weeks [78], in another study they were found even at 12 weeks [14]. Their number rises and reaches a steady level at 22 weeks. Cholinacetyltransferase (ChAT) activity, however, is present at adult levels at eight weeks of gestation [78]. Thus innervation of the cortex by cholinergic axons precedes nAChR synthesis during ontogenesis. At the beginning of phase V intensive synaptogenesis occurs in the CP along with a transient high acetylcholinesterase (AChE) reactivity [52] which indicates a major period for cholinergic development.

It has been suggested that nAChRs play a morphogenetic role during early CNS development, i.e. in pathfinding and target direction [for reviews cf. 49a,58]. Some nAChR subtypes seem to be

involved in regulating gene expression, establishing and maintaining neuronal connections, as well as affecting synaptogenesis and neuronal migration [81, for reviews cf. 49a,82]. This view is supported by the discovery of nAChR at some sites before synapse maturation occurs and their presence on dendrites and axon terminals. Regulation of axonal growth cone or dendrite extension and retraction represents yet another way by that ACh, or exogenous nicotine, can influence neuronal circuitry and nervous system activity [61,105].

1.4 AIM OF THE THESIS

Uncovering the development of different neurotransmitter systems and their interactions in the foetal CNS is the basis for understanding the relationship between cortical morphology and function. It also serves to ascertain their role in response to neurotoxic agents, e.g. nicotine, alcohol or drugs, which potentially alter the neuronal development of the foetal brain and may cause behavioural abnormalities. Knowledge of brain development would aid the ever increasing possibilities of antenatal diagnosis and allow for early treatment or prevention of possible damages. The early presence of nAChRs during brain development is of major importance and considerable interest especially with regard to the effects of maternal smoking on foetal development.

Although proof of the existence of nAChRs during human foetal development has been established, an accurate assignment to specific areas or cell layers with regard to the corresponding morphological developmental phases still remains to be made. Previous studies have used brain cell homogenates following abortion by vacuum aspiration [27,41,42] which may have allowed qualitative and quantitative statements, but did not give evidence regarding the exact neuroanatomical distribution. Using cortex samples [59] made it possible to study the cortical distribution but only in a very limited area. Very few studies have yet used whole coronary slices to localise cellular nAChRs [2,28,89].

The whole coronary slices used in this study will facilitate the description of the neuroanatomical distribution of two important nAChR subunits. Studying the α4 and α7 subunit includes the two most abundant nicotinic receptor subtypes in the CNS, α4/β2 and the homomeric α7. It enables a comparison between two architectonically and functionally defined areas within the occipital and the frontal lobe. The use of immunohistochemistry will allow a subtype-specific cellular demonstration of the actual receptor subunit protein.

This leads to the following questions:

1. Within which layers and at what age do the nAChR subunits α4 and α7 appear during human cortical development?
2. Is there a difference between the development of the visual and the frontal cortex regarding the distribution of α4 and α7 nAChRs?
3. Concerning their regional distribution and time of appearance, can a distinction be made between the two subunits?

2. MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 TISSUE

In the course of this work two different areas of eight human foetal brain hemispheres of intermediate (g.w. 16, 17, 19, and 22) and late foetal age (g.w. 29, 33, 38, and 40) were examined (cf. tab.2). The areas chosen for this work were located in the precentral region and in the area around the presumptive calcarine sulcus.

The fixed and paraplast embedded brains were obtained through a collaboration with the Laboratorium Pathologie Oost Nederland in Enschede, Netherlands. The brains had been collected at autopsy following legal abortion or stillbirth. The determination of the gestational age was based on the crown-heel-length, the crown-rump-length, the foot length, the femoral length, and the weight of the foetus as well as the period of amenorrhea (cf. tab.2). Not exceeding a post mortem delay of 48 hours the foetal brains were fixed in 4% (v/v) buffered paraformaldehyde at 4°C for 2-4 days. The hemispheres were cut into coronary slices of 3-4 mm thickness, dehydrated in a graded series of ethanol dilutions (50%, 70%, 90%, and 100% (v/v)), and embedded in paraplast.

2.1.2 SUBSTANCES

BUFFERS, SOLUTIONS, AND CHEMICALS

0.01% (v/v) poly-L-lysine-solution

0.1 % poly-L-lysine, Sigma, Taufkirchen

distilled water

XEM-200

Vogel, Gießen

Isopropanol 50%, 70%, 90%, 100% (v/v)

isopropanol, Hofmann, Düsseldorf

distilled water

0.03% (w/v) cresyl-violet

sodium acetate trihydrate, Merck, Darmstadt

99%-100% acetic acid, Merck-Schuchardt, Hohenbrunn

cresyl-violet, Chroma, Köngen

Tab.2: Features of the human brain tissue samples used in this study

Gest. age [weeks]	Gender	Body [g]	Brain [g]	Crown-heel [cm]	Crown-rump [cm]	Foot [cm]	Femur [cm]	Cause of death
16	♂	175	24.3	19	13.5	2.3	2.2	Prune belly sequence, interruption of pregnancy
17	♂	111	19.4	17.5	12.3	2.2	2.0	Acute chorioamnionitis, immature delivery
19	♂	179	32	21.5	14.5	2.8	2.8	Acute chorioamnionitis, immature delivery
22	♂	562	70	30	21	4.4	3.9	Retroplacental haematoma, immature delivery
29	♀	1294	173	40	27	5.9	5.7	Solutio placentae
33	♀	1558	285	43	30	6.6	6.5	Complication of parenteral nutrition
38	♂	2868	330	50.5	34.5	8.2	8.2	Intrapartum asphyxia
40	♂	3508	375	51.5	36.3	8.0	8.1	Intrapartum asphyxia

DePeX

Serva, Heidelberg

Bodian-staining

Solution for impregnation

6% (w/v) copper filing (copper plate), Merck

1% (w/v) albumose silver, Merck

distilled water

Solution for reduction

1% (w/v) hydrochinon, Merck

1.75% (v/v) formaldehyde, Merck

distilled water

Gold precipitation: 1% (w/v) aqueous gold(III)chloride hydrogen acid solution

tetrachlorogold(III)acid yellow, Merck

acetic acid, Merck-Schuchhardt

distilled water

Differentiation: 2% (w/v) aqueous oxalic acid solution

oxalic acid (Oxalsäure z.A.), Merck

distilled water

Fixation: 5% (w/v) aqueous sodium thiosulphate solution

sodium thiosulphate (Natriumthiosulfat-5hydrat), Merck

distilled water

Immunohistochemistry

Tris/NaCl buffer (TBS: 0.05 M Tris, 0.15 M NaCl)

0.05 M tris(hydroxymethyl)-aminomethane z.A., Merck

0.15 M sodium chloride, Merck

adjusted to pH 7.6 with hydrochloric acid, Merck

distilled water

0.05% (w/v) trypsin in TBS

trypsin, Serva

0.1% (w/v) calcium chloride dihydrated, Fluka, Taufkirchen

adjusted to pH 7.8 with 1N sodium hydroxide (NaOH), Merck

1% and 30% (v/v) foetal calf serum (FCS) in TBS

foetal calf serum, Gibco BRL Life Technologies, Eggenstein

0.15% (v/v) hydrogen peroxide (H_2O_2)

methanol, Baker, Darmstadt

hydrogen peroxide liquid, Merck

1% (w/v) BSA/TBS

bovine serum albumen (BSA), Sigma

Glycerine gelatine

Merck

ANTIBODIES AND DETECTION SYSTEM

The primary monoclonal antibodies (mAb) directed to the nAChR subunits α4 and α7 were kindly provided by Dr. J. Lindstrom, University of Pennsylvania School of Medicine, Dept. of Neurosciences, Philadelphia, USA. For both mAb 299 and mAb 306 a secondary biotinylated antibody and the detection system were employed according to the streptavidin-biotinylated horseradish-peroxidase complex method, using 3,3'-diaminobenzidine (DAB) as a chromogen for visualisation. In order to determine an optimum in primary antibody concentration, a dilution series was carried out in a pilot experiment. The best results, showing intense labelling along with a minimum of background staining, were gained with the dilutions indicated in table 3. This concentration was therefore used throughout all experiments.

Tab.3: Antibodies and detection system

nAChR subunit	Primary antibody	Secondary antibody	Peroxidase complex	Detection
α4	mAb299 IgG1 (rat) (charge 3/1/95) 25µg/ml in 1% BSA/TBS	biotinylated sheep anti-rat Ig (Amersham, Freiburg) 20µg/ml in 1% BSA/TBS	streptavidin-biotinylated horseradish peroxidase complex (Amersham) 25µg/ml in 1% BSA/TBS	0.03% (w/v) DAB 3,3'-diaminobenzidine tetra hydrochloride tablets (Sigma, Taufkirchen) in TBS and 30% (v/v) H_2O_2
α7	mAb306 IgG1 (mouse) (charge 8/6/92) 10µg/ml in 1% BSA/TBS	biotinylated goat anti-mouse Ig (Amersham) 25µg/ml in 1% BSA/TBS		

2.1.3 Technical Devices

Microtome: Rotationmicrotome HM 350, Microm-Laborgeräte, Walldorf

Tetrander: Jung, Heidelberg

Water bath: Histo Bath HIS-2, Axel Johnson Lab System, kunz instruments aps, Denmark

Stretch-bank: Medax Nagel GmbH, Kiel

Slides: 75x25x1 mm: Shandon Histoslides (adhesive), Life Sciences International, Frankfurt

75x50x1 mm: special model (uncoated), Menzel, Braunschweig

Coverslips: Menzel

PAP-Pen: Dako, Hamburg

Filter paper: Schleicher & Schüll, Dassel

Microscope: Olympus BX-50, Tokio, Japan

Digital documentation with Olympus Vanox photomicroscope AHBT3, Hamburg

2.2 Methods

2.2.1 Tissue Preparation and Coating of Slides

The embedded tissue samples were cut into 7µm thin coronary slices using a microtome, smoothed out in a 50°C water bath and mounted on 25x75mm adhesive slides. Two blocks (frontal cortex, g.w. 38 and 40) were too large to fit into the microtome. They had to be cut using a tetrander and were then mounted on specially made oversized (50x75mm) slides. In order to enhance adhesion of these uncoated slides, they had to be coated by incubation in 0.01% poly-L-lysine for one hour. Then they were washed in distilled water for three times, dried on filter paper at room temperature (RT) over night and covered to keep dust particles off. After mounting the tissue the slides were left to dry on a stretch bank at 40°C for at least one night to improve adhesion.

2.2.2 Nissl and Bodian Staining

Nissl-staining [83] was carried out on every 100^{th} section of the frontal as well as of the occipital brain slices of each individual in order to check the quality of the specimens and to find corresponding and therefore comparable regions. Two out of ten individuals - not shown in table 2 - were excluded from the study because of a genetic defect in one case and badly preserved tissue in the other. In all remaining eight cases representative sections were sent to Prof. Dr. I. Kostovic and Dr. Z. Petanjek at the Croatian Institute for Brain Research, University of Zagreb, Croatia. They assisted us with choosing appropriate slices that were suitable for interindividual comparisons. An

additional Bodian-stained section was enclosed for all specimens of the visual cortex. This supplementary staining of the fibers located in the stripe of Gennari within layer IV after g.w. 22, was performed to facilitate the identification of the calcarine sulcus. For the evaluation later on the cresyl-violet samples were helpful in order to distinguish the different cortical layers. Furthermore, they served as a reference to estimate the total number of cells during the semiquantitative assessment of the immunohistochemical staining.

2.2.3 Immunohistochemical Incubation

Using the immunohistochemical avidin-biotin-complex-(ABC-)method the relevant antigens are detected in a three step technique. First the primary antibodies that bind to the protein (in this case the nAChR subunits α4 and α7, respectively) are applied. Then follows incubation with the secondary antibodies which are conjugated with the vitamin biotin and are directed against the immunoglobulines of the species the primary antibody was raised in. In a third step a peroxidase-conjugated biotin-avidin complex is applied which binds to the biotin of the secondary antibody. The enzyme horseradish-peroxidase forms a complex with its substrate H_2O_2 and catalyses a reaction in which the chromogen DAB donates one of its electrons. The visible product is the brown colour of the oxidised DAB.

In order to be able to compare the neuroanatomical distribution of the nAChR α4 and α7 subunits, two successive coronary sections of each region were mounted on adhesive slides to be incubated with mAb 299 and mAb 306, respectively. The slices were deparaffinated in XEM-200 (2x5 min), rehydrated in a declining series of isopropanol dilutions (100% (v/v) (3x2 min), 90% (2 min), 70% (5 min), 50% (5 min)), immersed in distilled water and washed in TBS at RT for 10 minutes. As a formaline fixation can cause the cross-linking of aldehydes and thus lead to a masking of antigens, the tissue was made more receptive to the primary antibodies by pretreatment with trypsin.

10 min	at 37°C	washing in TBS
20 min	at 37°C	incubation with 0.05% (w/v) trypsin in prewarmed TBS
10 min		rinsing under cold tap water to stop proteolysis

Next, unspecific binding sites in the tissue were blocked by incubating the sections with FCS. After antigen retrieval the sections had to be treated with hydrogen peroxide in order to block the endogenous peroxidase. This enzyme is mainly present in the white and red blood cells [12] and can induce unspecific background staining.

2x10 min	at RT	washing in 1% (v/v) FCS solution
20 min	at RT	incubation in 0.15% (v/v) H_2O_2

2x10 min	at RT	washing in 1% FCS (v/v) solution

The sections were then circled with a PAP-Pen to ensure a regular distribution of the antibodies on the tissue sections and to prevent leakage of antibody solution during the incubation process. All incubations with antibodies and detection system, respectively, were carried out in a humid chamber where all slides could be kept for a longer period of time without the danger of dehydration.

20 min	at RT	incubation with 30% (v/v) FCS
over night	at 4°C	incubation with primary antibody
2x10 min	at RT	washing in 1% FCS (v/v) solution
40 min	at RT	incubation with secondary antibody
2x10 min	at RT	washing in 1% FCS (v/v) solution
40 min	at RT	incubation with biotin-avidin peroxidase complex
3x10 min	at RT	washing in 1% FCS (v/v) solution
17 min	at RT	incubation with 0.03 % (w/v) DAB solution (filter before use)
10 min	at RT	washing in TBS

After rinsing in distilled water the sections were mounted using glycerine gelatine and a Menzel coverslip to protect them from dehydration.

2.2.4 Controls

Each series of experiments was repeated twice to be sure that the results were reproducible. In order to prove the specific binding of the biotinylated secondary antibody and the detection system, a control section was carried through with each immunohistochemical incubation series. In this negative control section the primary antibodies were omitted and replaced by 1% (w/v) BSA in TBS. All remaining steps were carried out following the protocol described above.

2.2.5 Evaluation

The intensity of the immunohistochemical staining as well as the density of the labelled cells in relation to the total density of cells was analysed at magnifications ranged from 10 to 40 fold using an Olympus microscope.

First the Nissl-staining was examined to get an overview of the structure of the section and an impression of how the cortical layers were composed. Then the corresponding immunolabelled sections were evaluated. In the frontal lobe the whole section was assessed; in the occipital lobe the cortex lining the calcarine sulcus was judged. The evaluation of the middle foetal stages included

MZ, CP, SP, IZ, SZ, and VZ. A further subdivision of the cortex into a superficial and a lower part was necessary in some cases (cf. chapter 3). In the late foetal group the cortical layers I to VI were analysed as well as the IZ, SZ, and VZ. Layer VI and SP were subsumed in the evaluation of the late foetal stages as they could not be clearly distinguished. Only cells showing a darker rim of cytoplasm omitting the nucleus were documented as labelled throughout the semiquantitative evaluation.

For the intensity of the staining as compared to the strongest signal in the series the following classification was established:

+	weak intensity
++	medium intensity
+++	maximum intensity

The density of cells which were immunoreactive for the nAChR α4 and α7 subunit was given in relation to the total number of cells in the appropriate layer as observed in the Nissl-stained specimen:

+	moderate density (approx. 25% of all cells)
++	middle density (about 50%)
+++	high density (more than 75%).

If no definite assignment could be made, intermediate classifications such as +-++ and ++-+++ were used.

2.2.6 DOCUMENTATION

The results of three different gestational ages (17, 29, and 38 weeks) were selected for documentation using an Olympus Vanox photomicroscope equipped with a digital camera. From the frontal and visual cortex adjacent sections showing α4 and α7 immunohistochemical staining as well as the corresponding Nissl-staining were digitally photographed. To get an impression of the whole coronary section each slice was first photographed at two fold magnification. The cortex and underlying layers towards to the ventricle were then magnified up to 40 fold. For each case an enlargement of the control slide was photographed as well.

3. RESULTS

In the present study the distribution of the α4 and α7 subunits of the nAChR was analysed in two different areas of the developing neocortex of human foetuses: the frontal lobe and the prospective calcarine sulcus. According to the description by Kostovic [51, cf. 1.1] each layer of the developing human brain was individually examined. The development of the distributional pattern was observed in eight gestational stages from 16 to 40 weeks.

3.1 HISTOLOGICAL FINDINGS

Nissl-staining with cresylviolet showed a good preservation of tissue in all eight cases used in this study. It also allowed a differentiation of the layers as well as an assessment of the cell density. Four specimens were assigned to a middle foetal stage, or period IV, as described before [51]. Another four foetal hemispheres belonged to period V, the late foetal stage.

3.1.1 FRONTAL LOBE

At mid-gestation the frontal lobe comprised six layers. The thin MZ, below the pial surface, showed only few, small and plump cells. Single larger Cajal-Retzius-cells could also be found. Below the MZ the CP was tightly packed with small cells. These were particularly dense in the upper parts of the CP and therefore a further distinction between the superficial part (CP sup) and the inferior part of the CP (CP inf) was made in the evaluation. At the earliest stage examined (16 g.w.) the SP had already formed and made up approximately half of the total cortical thickness. Its width decreased in the older stages whereas the thickness of the CP and that of the total cortex steadily increased. The cell density in the SP was clearly lower than in the CP. The IZ contained fewer cells than the SP whereas the germinal layers SZ and VZ were full of small as well as larger plump or oval cells. Within these two layers the VZ showed a higher cell density while the cells in the SZ were slightly bigger.

At g.w. 29, the earliest of the late foetal stages, the SP had become relatively thin but the cells had a similar shape compared to those in the SP of the younger stages. The CP had at least doubled in size and accumulations of small round cells below the MZ and in deeper layers of the CP already faintly resembled cortical lamination. Within the less dense areas singular larger cells, spindle- to triangular-shaped, appeared. Yet the layers could not be clearly distinguished until g.w. 38.

16 g.w.

	Frontal Cortex				Occipital Cortex			
	α4		α7		α4		α7	
	D	I	D	I	D	I	D	I
MZ	+	+	+-++	++	0	0	+	+
CPsup	+++	++	+++	++-+++	++-+++	+-++	+++	++-+++
CPinf	++-+++	+-++	++	+-++	++-+++	+	++-+++	++
SP	0-+	0-+	+	+	+-++	+	++-+++	+-++
IZ	+	0-+	+-++	+-++	0-+	0-+	+-++	+-++
SZ	++-+++	+	+	+	+-++	+	+-++	+-++
VZ	+++	+	++	+-++	+++	+	++-+++	+-++

	Frontal Cortex				Occipital Cortex			
	α4		α7		α4		α7	
	D	I	D	I	D	I	D	I
MZ	+	+	+-++	+-++	-	-	-	-
CPsup	+++	++	+++	++-+++	+++	+	+++	++-+++
CPinf	++-+++	+	++	+-++	++	+	++-+++	++
SP	++	+	++	+-++	+	+	++-+++	+-++
IZ	+-++	+	+-++	+-++	0-+	0-+	++-+++	+-++
SZ	++	+	+-++	+-++	+-++	+	++	++
VZ	+++	+	++	+-++	++-+++	++	+++	++

19 g.w.

	Frontal Cortex				Occipital Cortex			
	α4		α7		α4		α7	
	D	I	D	I	D	I	D	I
MZ	+-++	+-++	+-++	++	0	0-+	+	+
CPsup	+++	++-+++	++-+++	++	++-+++	++	+++	++-+++
CPinf	++-+++	+-++	++	+	++	+-++	++-+++	+-++
SP	++	+-++	+-++	++	+-++	+-++	++-+++	++
IZ	++	+-++	+-++	++	+	+	+-++	+-++
SZ	++	+-++	++	+-++	+	+	+-++	+-++
VZ	++-+++	+-++	++-+++	++	++	++	++	+-++

22 g.w.

	Frontal Cortex				Occipital Cortex			
	α4		α7		α4		α7	
	D	I	D	I	D	I	D	I
MZ	+	+-++	++	++	+	0-+	++	+-++
CPsup	+++	++	++-+++	++-+++	+++	+-++	+++	++-+++
CPinf	++	+-++	++	+-++	++	+-++	++	+-++
SP	++	+	++	++	+-++	+-++	++	++
IZ	0-+	0-+	+-++	+-++	+-++	+	+-++	+-++
SZ	++-+++	0-+	+	+-++	+-++	+	+-++	+-++
VZ	++	+	++	+-++	+++	+-++	++-+++	+-++

Tab.4: Semiquantitative evaluation of the early foetal stages
I = intensity + weak intensity ++ medium intensity +++ maximum intensity
D = density + moderate density (approx. 25% of all cells) ++ middle density (about 50%) +++ high density (more than 75%)

29 g.w.

	Frontal Cortex				Occipital Cortex			
	α4		α7		α4		α7	
	D	I	D	I	D	I	D	I
CP I	++	+-++	+-++	+-++	+-++	+-++	++	+-++
II	+++	+-++	++	+-++	++-+++	+-++	++	++-+++
III	++-+++	+-++	+	+	+-++	+	++	+-++
IV					+	+	0-+	0-+
V					+-++	+-++	+-++	+-++
VI/SZ	++	++	+-++	0-+	++	+-++	++	+-++
IZ	0-+	0-+	0	0	+-++	+-++	+-++	+-++
SZ	++-	+-++	0-+	0-+	-	-	-	-
VZ	++-	+-++	0-+	0-+	-	-	-	-

33 g.w.

	Frontal Cortex				Occipital Cortex			
	α4		α7		α4		α7	
	D	I	D	I	D	I	D	I
CP I	0-+	+	+-++	++	+	+	++-+++	++
II	++-+++	+-++	++-+++	+-++	++-+++	+-++	++-+++	++
III			++	+	++	+-++	++	+-++
IV			+	+	+	+	+	+
V			++-+++	+-++	+-++	+-++	++-+++	+-++
VI/SZ	++-+++	+-++	++	+-++	+-++	+-++	++	+-++
IZ	+	+	++	+-++	+	+-++	+-++	++
SZ	++	+	+	+	+	+	+-++	++
VZ	++-	+	0	0	+++	+-++	+++	++

38 g.w.

	Frontal Cortex				Occipital Cortex			
	α4		α7		α4		α7	
	D	I	D	I	D	I	D	I
CP I	+-++	+-++	++-+++	++-+++	+-++	+-++	++	++
II	++	+-++	++-+++	++	++	+-++	++-+++	++
III	+++	++	+++	++	+-++	+	++	+-++
IV	++	+-++	+-++	+-++	+-++	+	+-++	+
V	++	++	++-+++	+-++	++-+++	+-++	++-+++	++
VI/SZ	+-++	+-++	++-+++	++	++-+++	+-++	++-+++	++
IZ	++	+-++	++-+++	++	++	+-++	+-++	++
SZ	+	+	+	+-++	-	-	-	-
VZ	+++	+-++	+++	+-++	-	-	-	-

40 g.w.

	Frontal Cortex				Occipital Cortex			
	α4		α7		α4		α7	
	D	I	D	I	D	I	D	I
CP I	+-++	+-++	++-+++	++	+-++	+	++	+-++
II	++	+-++	++	++	++	+	++-+++	+-++
III	++-+++	++	+++	++	+-++	+-++	++-+++	++-+++
IV	+-++	+-++	++	+-++	+-++	+	++	+-++
V	++	+-++	+++	++	+-++	+-++	+++	++
VI/SZ	++	+-++	+++	++	++	+-++	++-+++	++-+++
IZ	+	+-++	++-+++	++	++	+-++	++	++
SZ	++	+-++	+-++	+-++	-	-	-	-
VZ	+++	+-++	+++	++	-	-	-	-

Tab.5: Semiquantitative evaluation of the late foetal stages
I = intensity + weak intensity ++ medium intensity +++ maximum intensity
D = density + moderate density (approx. 25% of all cells) ++ middle density (about 50%) +++ high density (more than 75%)

Layer I was about the same size as the mid-foetal MZ, containing more cells than the MZ but still being hypocellular in relation to the other layers. Within layer II and the thin layer IV lots of small round cells could be observed. The broader layers III and V showed fewer, polymorphous cells, many of which were pyramidal cells. The cells in layer VI were less dense and also comprised polymorphic cells with large nuclei. The SP could no longer be delineated from layer VI. With the appearance of more fibres surrounding the few but fairly large cells the IZ more and more resembled the adult WM. There was a thin and cell-dense SZ and a very fine hypocellular VZ with the adjoining ependyma.

3.1.2 Visual Cortex

In the older foetuses the calcarine sulcus already showed the characteristic lamination of Area 17 on microscopic inspection whereas in the younger individuals the first forming sulcus on the medial side was considered to be the prospective primary visual cortex [98].

The histological findings within the presumptive visual cortex roughly resembled those described above for the frontal cortex. The CP and SZ appeared to be slightly thicker than in the frontal cortex whereas SP and IZ were somewhat thinner. There were less pyramidal neurons in layers III and V of the late foetal brains. Layer IV was particularly well developed. The existence of the typical feature of area 17, the stripe of Gennari, could only be suspected through a slight reduction of cell density within layer IV at 38 and 40 g.w. Otherwise the distribution of cells was similar to that observed in the frontal cortex. In the sections of the two eldest individuals the lateral ventricle was not cut.

3.1.3 Bodian-Staining

This staining was carried out in order to identify area 17. The staining visualises fibres which in this case accumulate within the cell-poor layer IVb of the visual cortex. Fibres were hardly present before week 22 and in the older samples the difference to the neighbouring layers was not strong enough to make the Gennari stripe clearly visible.

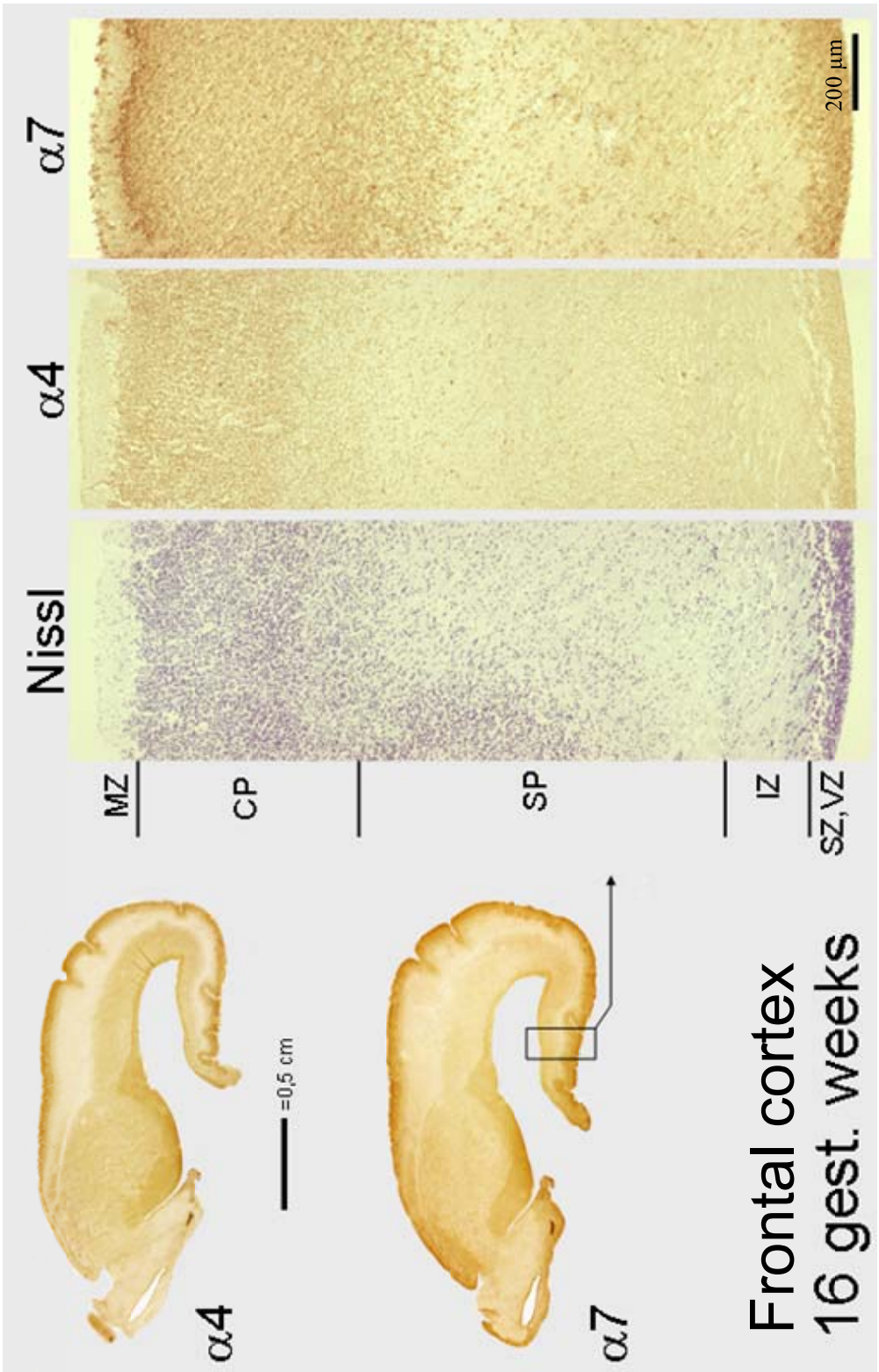

Fig.3: Frontal cortex at 16 g.w. Left: whole foetal hemispheres after immunostaining with α4 and α7. The rectangle shows the area that is magnified on the right for Nissl, α4 and α7 immunohistochemical staining.

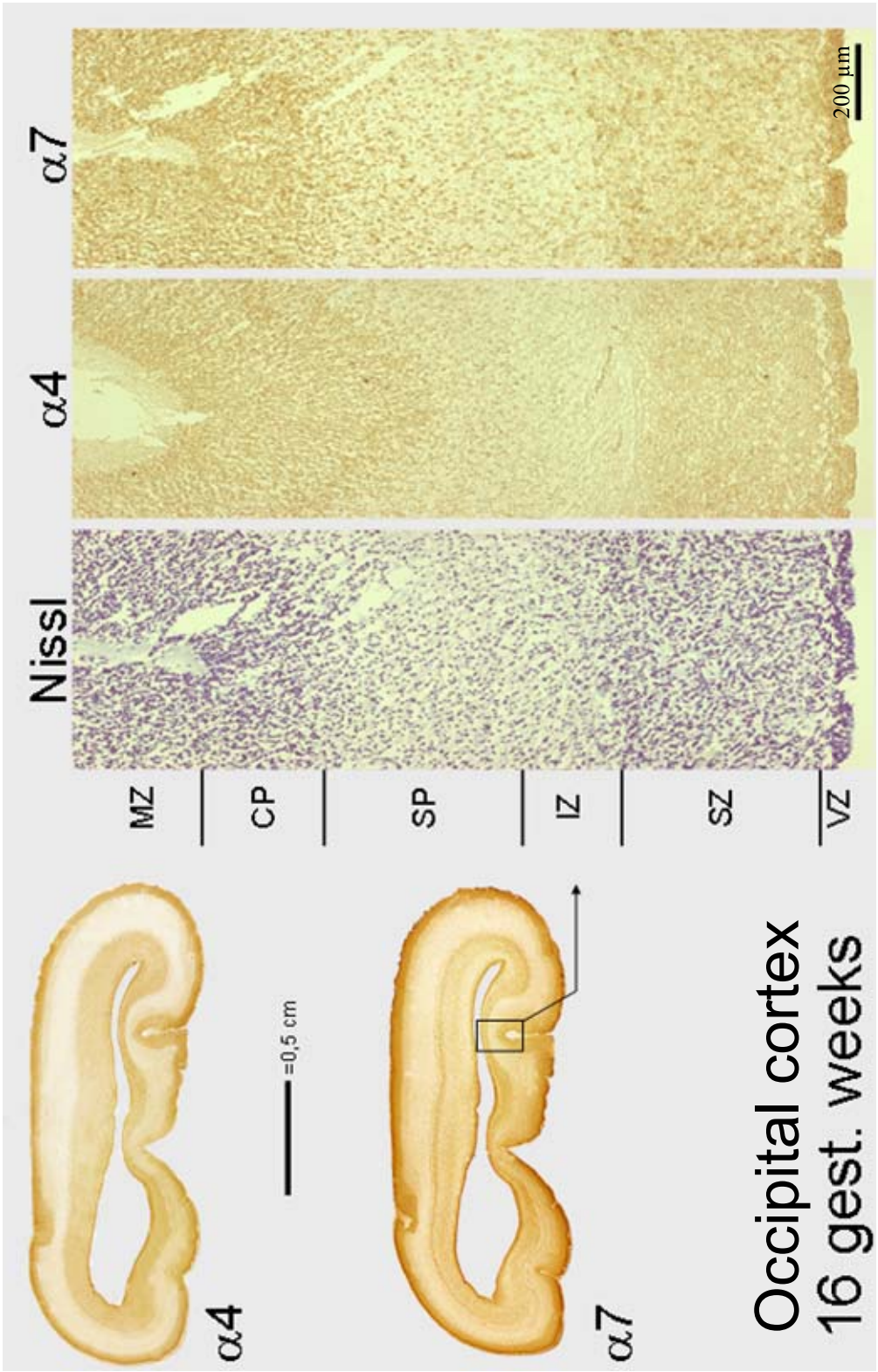

Fig.4: Occipital cortex at 16 g.w.

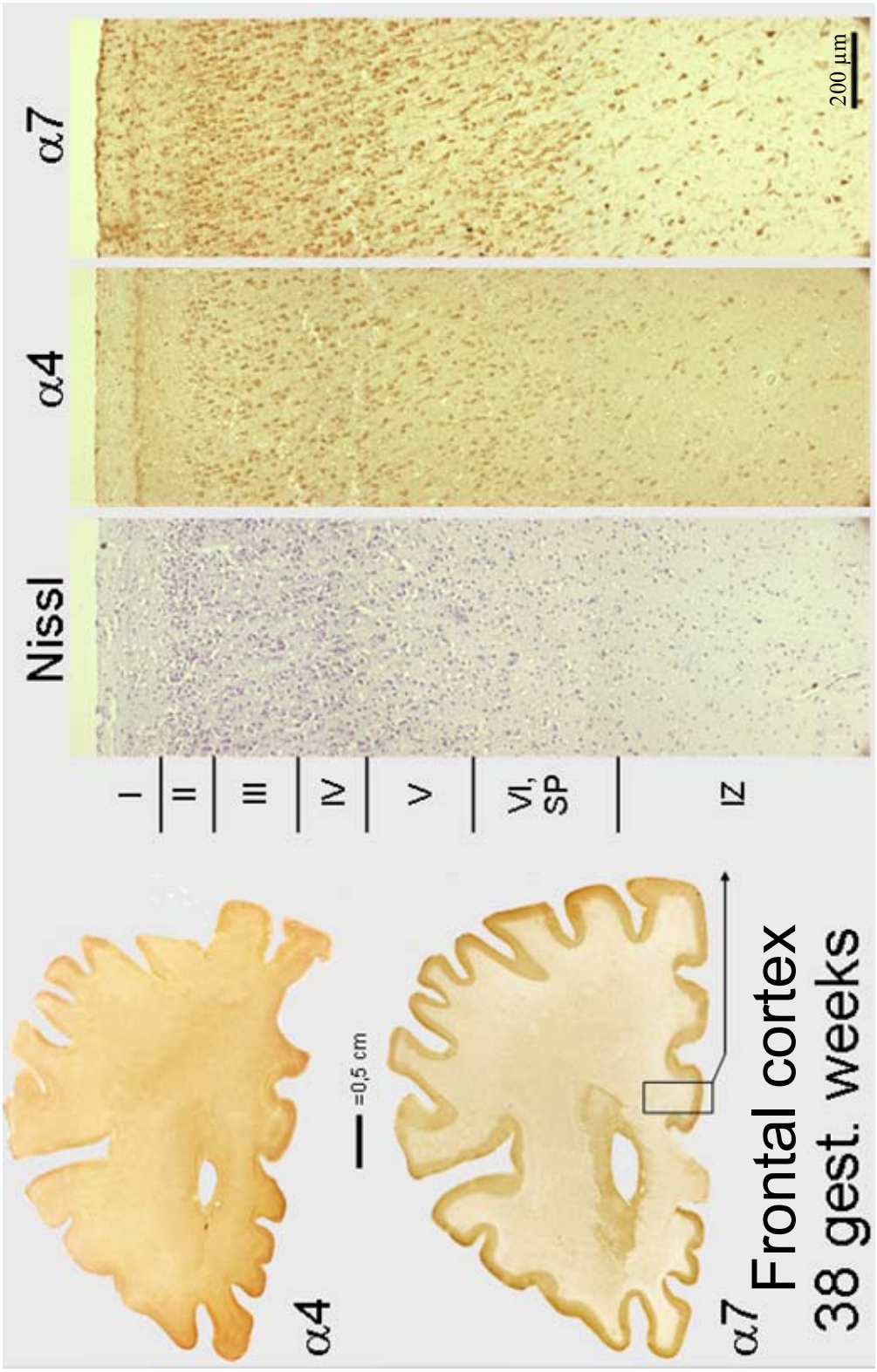

Fig.5: Frontal cortex at 38 g.w.

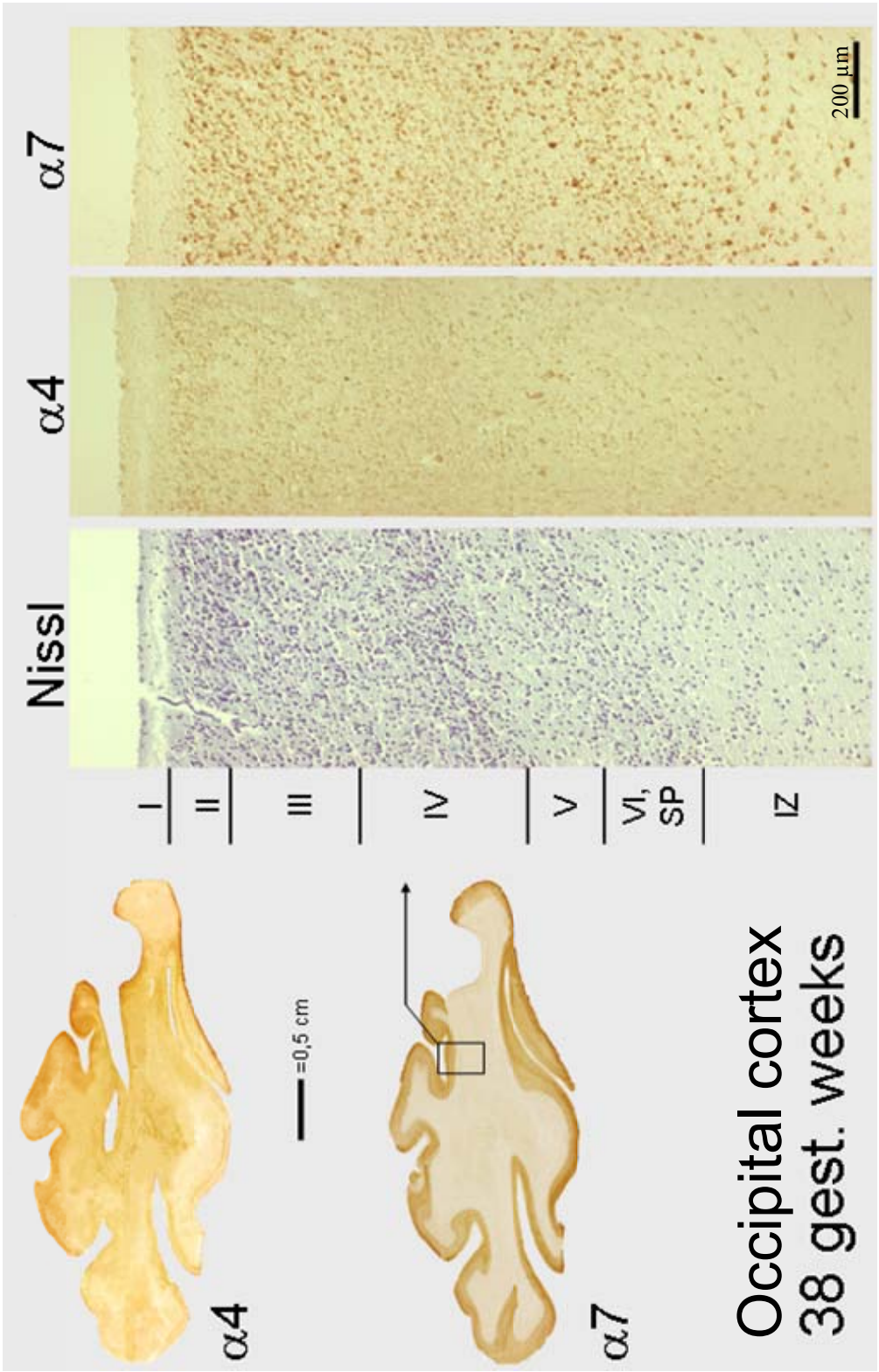

Fig.6: Occipital cortex at 38 g.w.

3.2 NAChRs in the Frontal Lobe

3.2.1 Middle Foetal Stages

The chemical reaction produced a brown staining which dyed the perikarya of the neurons as well as their processes. The labelling omitted the nucleus and only coloured the surrounding cytoplasm. In all four cases studied the expression of the α4 subunit protein was strongest in the CP sup and most of the tightly packed cells were marked. Because of the high nucleus-plasma ratio in the young neurons only a thin stained rim around the nucleus appeared. Hardly any signal could be detected in the MZ of all middle foetal brains. There was a very weak staining of individual medium-sized cells in the SP at 16 g.w. which gradually intensified during development. The IZ was mostly devoid of any signal. Within SZ and VZ a moderate staining in the majority of cells could be observed (cf. fig.7).

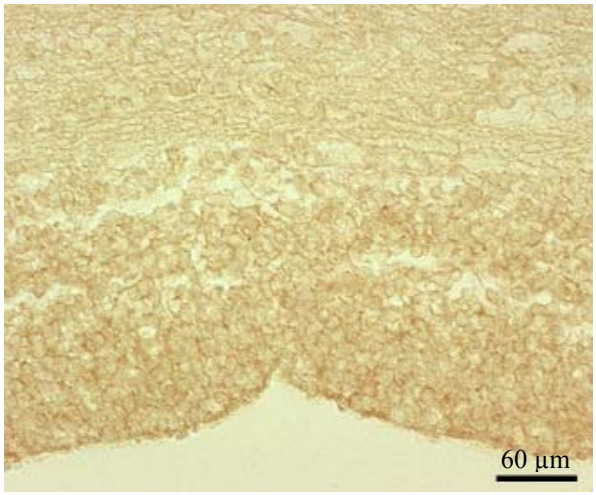

Fig.7: VZ at 16 g.w. (α4 immuno-staining, occipital cortex)
In the germinal layers cells were tightly packed showing a high density of only weakly labelled neurons. At this middle foetal stage the nucleus-cytoplasm ratio was still rather high, so immunoreactivity was reduced to a thin rim around the nucleus.

The α7 staining produced a more distinct signal than that of α4. Again the maximum intensity could be detected in CP sup, but there were also several marked cells in the MZ, SP, and IZ. As with the α4 staining, immunoreactive neurons within the SP became more numerous and also more intensely stained with increasing gestational age (cf. fig.8). At 22 weeks of gestation several large, heavily stained, oval cells appeared in the SP. Their labelled dendrites pointed into different directions. The α7 expression within SZ and VZ was similar to that of α4 with a somewhat darker staining.

3.2.2 Late Foetal Stages

With the onset of cortical lamination the most intense staining of α4-expressing neurons concentrated within layers III and V and particularly the pyramidal cells. While at 29 and 33 weeks the labelling was still strong in the former CP sup, i.e. the presumptive layers II and III, maximum

staining gradually shifted to layers III and V at 38 and 40 g.w. Most of the stained cells were pyramidal neurons whereas smaller round or oval cells had been stained much less distinctly. Layer I showed few but more heavily stained neurons than at mid-gestation. In the layer VI/SP area the labelling decreased from medium to weak intensity and density. The weakest staining was found in the IZ. SZ and VZ showed a high percentage of moderately stained cells.

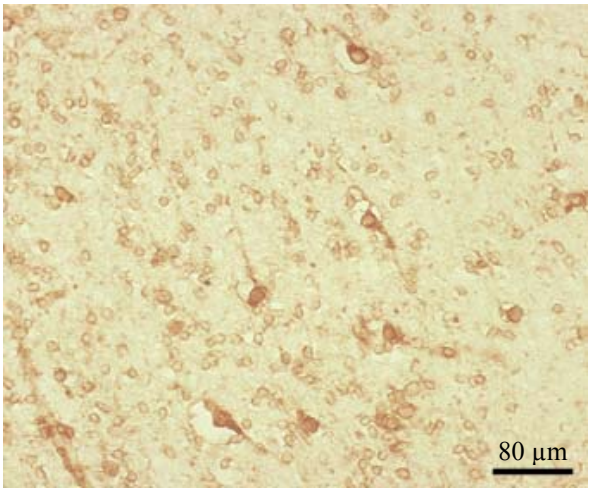

Fig.8: SP at 22 g.w. (α7 immuno-staining, frontal cortex)
Within the SP the number of immunoreactive neurons increased with the gestational age. Labelled dendrites pointed into different directions.

Again immunostaining of the α7 subunit produced a more distinguished signal than the α4 subunit. At g.w. 29 labelling in the CP was similar to that of α4-protein expressing neurons but very weak in the underlying layers. In g.w. 33 large neurons in the diminishing SP sent long dendrites to the CP (cf. fig.9). Smaller, round cells within the SP were stained as well though the signal was less strong. Staining in all layers intensified until in week 38 and 40 all cortical layers showed extremely well stained neurons, especially in layer I. The percentage of stained neurons in relation to the total number of cells also increased significantly. Single, large, heavily labelled neurons could be detected in the WM, especially in the part underlying the cortex.

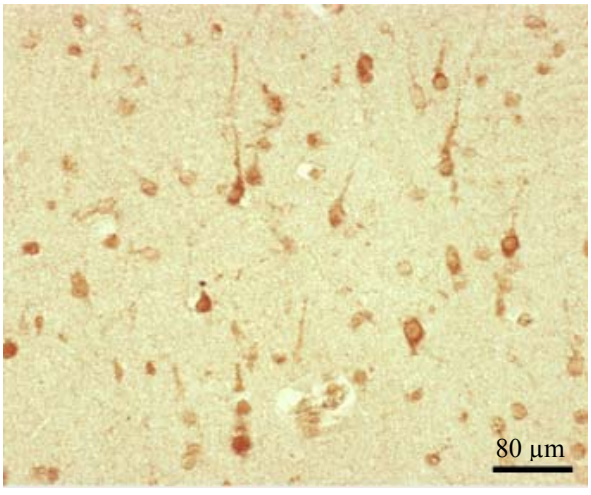

Fig.9: SP/WM at 33 g.w. (α7 immu-nostaining, frontal cortex)
Large neurons in the SP sent long dendrites to the CP. Smaller, round cells were less intensely stained.

3.3 NACHRS IN THE VISUAL CORTEX

3.3.1 MIDDLE FOETAL STAGES

In the prospective visual cortex the most intensely labelled cells immunoreactive for α4 could also be found in the superficial CP. The stained neurons were shaped as in the CP of the frontal cortex; the labelling though was discretely less intense than in the corresponding frontal layers of the same time of development. The MZ was almost totally devoid of stained cells as was the IZ at 16 and 17 weeks of gestation. More immunoreactive neurons became visible in the SP as the gestational age advanced. Cells were packed tightly in the VZ, showing a high density of increasingly stronger labelled α4 expressing neurons.

The existence of α7 subunit protein appeared again to be concentrated in the CP sup. But also in the CPinf as well as in parts of the SP most cells were strongly labelled. Hardly any marked neurons could be observed within MZ and IZ. The majority of the medium-sized plump germinal cells in the broad SZ and VZ were also quite intensely stained.

3.3.2 LATE FOETAL STAGES

In the older foetal visual cortex mostly large triangular cells in layers II as well as V and VI/SP were marked for α4. Labelling extended into the apical dendrites. Hardly any signal could be observed in the area of sensory input, layer IV. A smaller percentage of cells were marked and the staining was also significantly less intense than in the adjoining layers. Singular large neurons with apical dendrites were observed in layer V (cf. fig.10). Smaller neurons here were less intensely stained. The few neurons of the IZ showed weak labelling.

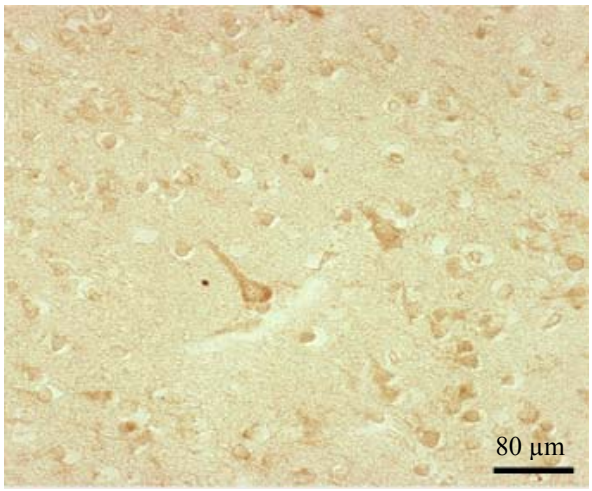

Fig.10: Layer V at 40 g.w. (α4 immunostaining, occipital cortex) Individual large neurons with apical dendrites appeared in layer V. Smaller neurons were less intensely stained.

The α7 immunostaining produced a stronger signal than that of α4 but the distribution was similar to the one on the α4 stained sections. The lack of strong labelling in layer IV proved to be even more noticeable, especially at 29 and 33 weeks. Numerous pyramidal neurons with intensely stained perikarya and long apical dendrites showed up in layer III (cf. fig.11). The nucleus was devoid of signal. In the 40 g.w. old brain the most distinct layers were III and VI. Here and at 38 weeks a particularly high number of cells was marked. Approximately half of the few cells in the WM were stained with moderate intensity.

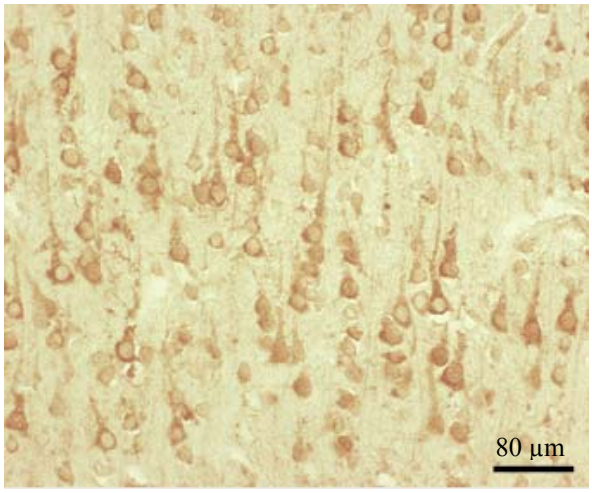

Fig.11: Layer III at 40 g.w. (α7 immunostaining, occipital cortex) Numerous, intensely stained pyramidal neurons with long apical dendrites could be found in layer III. The nucleus was devoid of signal.

3.4 CONTROLS

As described in chapter 2, a control slide was added to each run on which the application of the primary antibody had been replaced by BSA/TBS. In all cases these test sections showed no staining at all.

3.5 GENERAL SURVEY AND SUMMARY OF RESULTS

To prove that the results were reproducible each experiment was repeated twice. The triple tests produced similar results with slight variations in the intensity of the staining. The α4 and α7 protein were both existent at 16 weeks of gestation in the frontal as well as in the occipital brain regions. The expression of both proteins was similar in the middle foetal brains, also with regard to the two brain regions. Most marked cells were small and plump with a large nucleus and few cytoplasm. The strongest signal at this stage could be detected in the CPsup where stained cells were tightly

packed. MZ and IZ had a lower relative density and intensity of labelled neurons after α4 immunostaining in contrast to α7. The SP gradually showed more and larger marked neurons throughout development. Most of the SZ and VZ cells showed moderate staining.

Reaching an older foetal age the occurrence of α4- and α7-immunoreactivity varied, in particular in the cortical layers. Especially in the later stages the immunochemical response of the α7 subunit was stronger than that of α4. In the frontal cortex the most prominent α4 staining changed to layers III and V and here mainly to the big pyramidal-shaped neurons. The labelling of the cells in the SP decreased with its reduction of thickness. In contrast to these findings, α7 protein could also be detected in layer I and within large SP neurons with long vertical dendrites. Processes were usually strongly stained in the older brains. The α7 subunit was also present in the WM where it appeared within large cells that were close to the cortex. The visual area showed the most intense α4 labelling in layers II and V, α7 could mainly be found in layers III and VI. In both cases the granular cells of layer IV, the area of visual input, were hardly marked at all. The fibre-rich WM contained several weakly stained α4- and moderately to strongly stained α7-comprising cells.

Using macroscopic observation or a low magnification the prominent labelling of the CPsup appeared to be less intense in the depth of the sulci compared to the gyri, concerning both the α4 and the α7 subunit. This observation could be made in the middle as well as in the late foetal stages and in both areas investigated.

4. DISCUSSION

4.1 MATERIALS AND METHODS

4.1.1 ACQUISITION AND PREPARATION OF TISSUE

The present study is the first to examine the exact anatomical distribution of the nAChR subunits α4 and α7 on whole slices through the hemispheres of human foetuses of different gestational stages. This is due to the fact that the availability of human brain tissue is limited by methodological and ethical constraints. Tissue samples have to be taken post mortem so there will always be a certain delay until the brains can be removed and fixed. Human foetal brains can only be obtained from aborted or stillborn children who are often affected by severe conditions. A lack of oxygen before or during delivery can damage the CNS as neuronal tissue is particularly sensitive to hypoxia. This implies that the cause of death and the time span until the fixation of the brain may influence the quality of the tissue and thus the outcome of experiments. It is not known for exactly how long the nAChR protein remains stable, but a post mortem delay of up to 89 hours [21,28] did not influence the results in autoradiography studies. The choice of the specimens was made taking into account the cause of death and state of preservation of the tissue. Consequently one case of trisomy 21 was excluded as well as another one showing poor tissue preservation in the Nissl-staining.

4.1.2 DEFINING COMPARABLE REGIONS

At early developmental stages the neocortical areas proved to be remarkably similar to each other regarding cellular density as well as cortical thickness and architecture. The areas defined by Brodmann [13] do not develop until the late foetal stage [51]. This made it difficult to distinguish different areas by morphological criteria especially in the younger stages.

Area 17 was relatively easy to determine in late foetal stages because of the existence of the calcarine fissure located at the medial side of the hemisphere. In younger foetuses one could merely observe a slight indentation as a first sign of the sulcus. Unfortunately, the Bodian-staining (not shown) was not very helpful as fibres in the cortex usually do not abundantly appear until g.w. 22 and are sparse in the visual area in comparison to other brain regions [54]. In the older foetuses the fibres were distributed equally within the CP, only in the two oldest individuals a slight increase of fibres in layer IVb was observed.

Within the frontal cortex the precentral region was examined. Because the central sulcus is difficult to identify before g.w. 23 [51] we collaborated with Prof. I. Kostovic and Dr. Z. Petanjek of the Institute for Anatomy, University of Zagreb, who helped us with the development-dependent identification of the regions under investigation.

4.1.3 Primary Antibodies

The mAb 299 (α4) and 306 (α7) were used as primary antibodies. The mAb299 is directed against an extracellular epitope of the α4 subunit [76] whereas the mAb306 binds to an intracellular epitope located between the M3 and M4 domain of the α7 subunit [88]. Both antibodies were used in many studies before and were kindly provided by Dr. J. Lindstrom (cf. 2.1.2) within the framework of a long-standing collaboration. A series of pilot experiments revealed an optimum dilution of 1:200 regarding mAb299 and 1:500 for mAb306. These concentrations showed a good staining intensity of immunoreactive cells with little unspecific background staining.

The specificity of the antibodies was tested in oocyte expression systems as well as in subunit-transfected and non-transfected cells. In addition the antibodies were tested against cross-reactivity to other hitherto known subunits [102]. However, the use of these and other nAChR antisera in α7 and α4 knockout mice cast some doubts on the specificity of the antibodies. Data showing immunopositive signals in α7 knockout mice were presented by Herber et al. [44]. In this study two monoclonal (mAb 306 and 319) and two polyclonal antibodies (pAb 87 and pAb H-302) were used. The unlikely possibility of a faulty knockout mouse could be ruled out by PCR and α-bungarotoxin (α-BTX) binding. The publication of these results quickly revealed that similar findings had been obtained by several groups working with nAChR antibodies. Moser et al. [68] recently tested antibodies raised against α3, α4, α7, β2 and β4 nAChRs on brain tissue of the respective knock-out mice. In each case immunoreactivity was the same in wild-type and knock-out mice. These findings suggest that the tested antibodies share target epitopes with endogenous proteins. In the case of the mAb 306 a possible cross-reactivity with a mitochondrial protein was discovered [26]. It is remarkable, however, that immunohistochemical demonstration of the α7 subunit in foetal rat as well as human brain using mAb306 correlates with previous findings of α7 mRNA [36,59,89].

Until now it is not known if the findings in the mouse described above are relevant for human nAChRs, too. As long as the issue is not clarified the results described in the present study have to be interpreted with caution. The problems discussed above became public only after the experimental part of this study had been finished and after the findings had been presented at

scientific meetings. In order to confirm the results, more comparisons with mRNA analysis should be made. To further complicate matters, even binding assays have to be interpreted with great care as a recent study [63] proved that in addition to the α7 subunit α-BTX also binds to a subset of γ-aminobutyric acid (GABA) (A) receptors.

4.1.4 Evaluation

As can be clearly seen on Nissl-stained sections the cell density varies quite strongly from layer to layer. This is why throughout the evaluation the density of labelled neurons was expressed as a semiquantitative estimate of the subpopulation in a given layer and not as stained cells per area. It is possible that very strongly stained cells contain more nAChR subunit protein than cells that hardly show any labelling, but it was not possible to rule out if there was a linear relation between the absolute quantity of subunit protein and the intensity of the staining. It would therefore be interesting to measure subunit proteins by quantitative methods, i.e. Western blot analysis.

Because of the use of a semiquantitative evaluation method the assessment is influenced by the qualification and experience of the evaluator. A more objective means of evaluation would be the use of the CAST(computer assisted stereology tool)-grid system where the connected computer system makes a projection of an exemplary counting of stained cells in a defined area. This method relies on the existence of single, well distinguishable cells which makes it less suitable for studies on foetal tissue as the cells, especially in the younger stages, can be very tightly packed and overlapping.

4.2 Interpretation of Results

4.2.1 General Aspects

While in later stages neuronal cells develop their characteristic shape (cf. 3.1), at a middle foetal age even the discrimination of glioblasts versus neuroblasts can be difficult because of their similar form. Glial fibrillary acidic protein (GFAP), which can be found in glial cells such as astrocytes, is present in the human foetal brain from g.w. 9 onwards [104]. A colocalisation study showed that α4 and α7 mRNA and GFAP could not be found together in the same cells in adult human cortex [99]. These findings support the fact that the α4 and α7 subunits are not expressed in astrocytes and that the stained cells in this study were neuroblasts.

There was a widespread neuronal expression of both the α4 and the α7 subunit in the foetal cortex from g.w. 16 onwards. This supports the theory that cholinergic receptor subunits appear early during development and play a role in neuronal migration (cf. 4.4). The fact that the α4 as well as the α7 subunits could in some parts of the frontal and occipital cortex be found in more than 75% of neurons might indicate that both subunits can be simultaneously expressed by one neuron. This was already suggested after autoradiographic studies on the rat cortex which showed an overlapping distribution of α4 and α3 subunits [22].

The cells showed a darker staining following incubation with mAb306 than upon labelling with mAb299. These findings were consistent throughout all incubation series carried out but were more prominent in the older foetal stages. It is possible that there is indeed a higher concentration of α7 protein and a wider distribution of α7 nAChRs in all layers, but with regard to the yet unsolved problem of antibody specificity the interpretation of this fact is rather delicate. A different expression rate of the subunits among different neuronal populations might be a reason for slighter variations in the intensity of the immunoreaction within one layer.

4.2.2 Cortical Plate

At middle foetal stages (g.w. 16-22) the CPsup showed the most intense staining for α4 and α7 in both areas examined. The labelling was just a little fainter in the calcarine fissure. While electron microscopical studies have shown that immature synapses exist above and below the CP from g.w. 9 onwards, the earliest synapses *within* the CP do not appear before week 23 [66]. It seems thus reasonable to assume that both subunits are expressed by young neurons even before the first synapses in the CP form.

The high nAChR reactivity in the CPsup as compared to the rest of the CP cannot simply be explained by a higher density of cells as the Nissl-staining shows the same density throughout the whole CP. Mesulam et al. [65] described a similar phenomenon in adult human brain. In fact they observed that cholinergic axons and nicotinic binding sites are predominant in the superficial compared to the deeper layers of adult human cortex. At very low magnifications (cf. fig.3-6) the intense immunostaining of the CPsup appeared as a dark line surrounding the whole contour of the hemisphere. This line seemed to get much less distinct in the depth of the sulci. This fact could be observed after α4 as well as after α7 immunostaining in both areas and all stages examined. Whether it indicates that nAChRs play a role in the formation of a gyrus is speculative at this point. This theory will have to be investigated in further studies.

In the frontal cortex the maximum of α4- and α7-immunostaining gradually changed from the CPsup to the two pyramidal layers III and V during the following weeks of gestation. Large pyramidal neurons, including their long apical dendrites which extended right into layer I, were heavily labelled. According to the general inside-out rule of neuronal maturation and also regarding Golgi studies on dendritic development [69] pyramidal neurons in layer V differentiate earlier than those in layer III. In the present study the immunostaining of the two examined subunits showed no difference between layer III and layer V.

A clearly weaker signal could be observed in the granular layers II and IV where the cortical afferents terminate. Smaller round and oval cells in all layers were hardly marked at all. In the visual cortex the main α4 labelling could be seen in layers II and V and the α7 subunit existed mainly in layers III and VI. There was hardly any signal for both subunits in the layer of visual input, layer IV. On the α7 slides this contrast to the well stained layers below and above was even more obvious. A lower concentration of α4-immunostained cells in layer IV of human foetal brains has also been described by Schröder et al. [89]. Also in the adult human brain layer IV contained less α7-mRNA, which was shown through in situ hybridisation by Wevers et al. [100]. According to a different publication [51] thalamocortical neurons that project to layer IV show an intensive temporary AChE activity during period V that disappears later. It might be possible that the nAChRs in layer IV consist of other subtypes than the ones examined in this study where only few α4 and α7 subunits were found in this layer.

4.2.3 SUBPLATE

The number of α4 positive neurons in the SP increased with time while α7 subunits were already present in higher amounts at the earliest stages examined. This indicates that neurons expressing α7 subunits reach the SP some weeks earlier than those carrying α4 subunit proteins. Some of the SP neurons showed long vertical, immunostained dendrites. Golgi studies [69] have shown that SP neurons possess more dendrites than those in the CP which was interpreted as an indication of earlier differentiation.

The SP has largely been seen as a “waiting compartment” for afferents arriving from the nucleus basalis Meynert and the thalamus [54]. The afferent fibres make contact with SP neurons and form early synapses on their way to the neocortex [53,69]. SP neurons probably are a “guide” for ascending afferents [for review cf. 4]. The abundant presence of the nAChR subunits α4 and α7 in

the SP suggests an involvement of nAChRs in immature synapses and again leads back to the question to what extent nAChRs are involved in neuronal migration (cf. 4.5).

Together with the degeneration of the SP in the last weeks of gestation, number and staining of nAChR positive cells also decreased, except for some large neurons with long apical dendrites which reacted strongly to mAb306 and could be found in the SP around week 33 and during the following weeks in the WM adjoining the cortex. The characteristics of these interstitial neurons were very similar to those of the non-pyramidal neurons of layer VI which made it difficult to clearly distinguish between the two layers in late gestation. Several studies [53,69,70] show that most SP neurons, displaying a polymorphic structure, are located subjacent to the CP. Kostovic and Rakic [53,54] and also Mrzljak et al. [69,70] observed that these cells are still present in the newborn and can even be seen in the adult brain.

4.2.4 Intermediate Zone

In the IZ no nAChRs were detected in the younger foetuses and only single cells were positive for nAChRs within the older brains. In the preterm stages the WM contained hardly any cells and only less than 50% of them were weakly to moderately stained. A high percentage of neurons in the IZ are migrating [69]. In a previous study on adult human brain AChE histochemistry showed that about 20% of interstitial WM neurons are cholinergic [53]. They are possibly components of a diffuse cholinergic system which is distributed throughout the telencephalic WM.

4.2.5 Germinal Layers

The VZ and SZ contain a heterogeneous cell population. Immunostaining for GFAP indicates that also precursors of glial cells exist within the early VZ [35]. The existence of moderately nAChR-stained cells in the germinal layers at mid gestation which then faded towards end of term suggests that neurons which have not yet started their way to the neocortex also express α4 and α7 subunits.

4.3 The Role of Animal Models

Developmental processes in the CNS have been investigated in several species. Two reasons for the preference of animal studies are the better availability of animal tissue and the use of experimental techniques which cannot be applied on humans. To what extent findings in animal studies can be transferred to humans has not yet been completely clarified.

In comparison to humans, the CNS of rodents is smaller and the period of gestation shorter, so that maturational changes happen almost simultaneously and are less easy to discern than in humans. In preterm rats between E18 and E21, for example, each day of development roughly equals two to three weeks of human ontogenesis [8]. The distribution of nAChRs in adult mouse brain in comparison to rat brain shows slightly different results and binding levels are lower in the rat neocortex [22]. In conclusion, if disparities in the distribution of nAChRs already exist in closely related species such as mouse and rat, one has to be cautious with transferring findings from rodents to human beings.

So far it seems that the expression pattern of nAChRs in rat neocortex is similar to the one in humans but cannot indiscriminately be transferred [2,22,36,75, for review cf. 33]. Immunoreactivity for α7 was ubiquitously distributed in adult rat telencephalon [23,36] with the major staining occuring in layer V. Neurons in layer I were hardly stained, cells in layers II to IV showed a weak level of immunoreactivity and those in layer VI a moderate level. These results are similar to our results in the late gestational human brain.

Few scientists have worked with primates in this field and hardly any studies are available covering foetal development in either humans or primates.

4.4 Development of the Cholinergic System

There are various methods to study the different components of the cholinergic system at their various times of appearance. The detection of nAChR protein by immunohistochemistry, as in the present study, does not reflect their functionality at the examined stages. Nothing is known so far about the time span between the occurence of the subunits and their assembly into pentamers within the cell membrane and thus their binding capacity. It would therefore be interesting to study the receptor activity itself. A major obstacle is the lack of an appropriate animal model which would help to overcome the obvious limits to research on human subjects. The only non-invasive method so far which allows to study nAChRs in vivo in humans uses nicotinic ligands and PET. Because of the exposure to radiation the procedure is contraindicated during pregnancy. Additionally it is still very expensive and time-costly and has to be further improved [73].

In electron microscopical studies on the distribution of synapses these could be identified above and below the human CP from g.w. 8.5 on [66]. Vesicles exist in the presynaptic axons but are fewer than in mature synapses. The first detection of synapses within the CP succeeded in a foetus of 23 weeks [66] which seems to be in contradiction to our findings where both of the examined subunits were present at g.w. 16. It has been observed before that nicotine binding, which requires nAChRs, often seems to be uncorrelated with cholinergic innervation during the first half of gestation [19]. In rat cortex α4 gene expression precedes the ingrowth of cholinergic fibres [75]. The discrepancy between the early existence of nAChRs proven in this study and the relatively late appearance of synapses in the CP could be an indication that nicotinic receptors exist in the neocortex even before cholinergic afferents arrive and form synapses. Kostovic and Judas [52] suggested that functional activity of cholinergic synapses is very likely at 15 and particularly after 24 g.w. when thalamocortical and basal forebrain input is established in the cortex. Synapses at that stage seem to be morphologically mature and the necessary prerequisites for transmission exist including, as we have shown, the nAChR subunits α4 and α7. Synaptogenesis and arrival of cholinergic fibres coincide with changes in bioelectrical activity which can be visualised in electroencephalograms of human foetuses by intermittent high-amplitude bursts [24]. In order to clarify the functional state of synapses at a certain time in gestation further electrophysiological studies, for example using patch clamp on primary cell cultures from human foetal brain, will be necessary.

Of major interest is the detection of nAChR subunits by in situ hybridisation. While immunohistochemistry shows the neuroanatomical distribution of the receptor itself, in situ hybridisation localises the corresponding mRNA and thus the production site of the receptor protein. The first α4 and α7 mRNA transcripts could be detected by RT-PCR at 9.5 weeks in human cortex homogenates [42]. During the first trimester gene transcripts of several nAChRs, including α4 and α7, were observed considerably earlier than the receptor proteins themselves [27,41]. Unfortunately this period could not be covered in the present study due to the lack of brain tissue of the appropriate stages. A wide distribution of α4 mRNA was found by Agulhon et al. [2] in coronary sections of 25 week old foetuses. High expression occurred in the cerebral cortex with strong labelling in the cell-dense inner and outer layers of frontal and occipital cortex. No signal was detected in the periventricular cells which is in contrast to our observations. Apart from that the findings match our own described above. Another study providing a direct comparison between the detection of nAChR protein by immunohistochemistry and the corresponding mRNA by in situ hybridisation achieved correlating results with both techniques [89]. In the adult human cortex α4 and α7 transcripts were found in various neurons in all cortical layers. While α4 mRNA expression was

dominant in giant pyramidal neurons, α7 mRNA mainly appeared in pyramidal-shaped neurons in layers II/III, V, and VI as well as in layer I cells [100].

Autoradiography studies with labelled nicotinic agonists help to trace functional nAChRs. Unfortunately, no unequivocally subtype-specific ligands are available until now so that the method is sensitive but not completely specific. Application of [^{3}H]-labelled nicotine can detect functional receptors, in particular α4/β2 which appears to be the most abundant nAChR subtype in the brain [30,102]. In the foetal human cortex [^{3}H]nicotine binding could be detected at the earliest at 14 to 16 weeks. The maximum activity was reached at 22 weeks but was still substantially below adult values [14,77]. These findings suggest that the α4 subunits may assemble to functional receptors at an early stage. [^{3}H]nicotine binding in adult human somatosensory and visual cortex was highest in the upper and lower layers but sparse in layer IV, the layer of sensory input. This corroborates our own findings in antenatal brain that showed only low to moderate staining in the mentioned layer. In the motor cortex the outer half of the cortex was strongly labelled by [^{3}H]nicotine [22]. [^{3}H]nicotine labelling showed a wider distribution in the occipital than in the frontal cortex in adult human brain [1], a finding not supported by our observations concerning the foetal brain.

The α7 subunits form homomeric nAChR subtypes and bind α-BTX with high affinity. In the light of the recent findings by McCann et al. [63] the interpretation of co-distributions of the α7 subunit and α-BTX-binding sites will have to be handled with caution. Furthermore, α-BTX binds only to assembled α7 nAChRs which means that early single subunits cannot be detected. There is a strong correlation between the distribution of the α7 subunit and the detection of α-BTX in deep cortical layers of the adult rat brain [23]. A linear relationship between α-BTX binding and α7 immunoreactivity could not be established as in some cases weak levels of α-BTX binding corresponded with intense immunostaining. Falk et al. [27,28] demonstrated the existence of α-BTX binding sites in the early foetal human cortex as early as nine g.w., a period that was not covered in the present study.

The major source of cholinergic innervation for the cerebral cortex are large neurons in the nucleus basalis Meynert and other magnocellular nuclei of the basal forebrain which contain AChE and ChAT, indicating the production of ACh [50]. Presynaptic acetyltransferase (AT) activity is fully present at eight weeks [78], the first cholinesterase (ChE) and AChE reactivity has been shown in the neuropil of the nucleus basalis complex anlage at g.w. nine [50]. A week later ChE and at 15 weeks also AChE and ChAT-positive fibres emerge and approach the cortical anlage through the

external capsule [15,50]. It has been observed that they do not invade the SP until 15 to 18 g.w. [50,54]. Intensive synaptogenesis takes place along with a strong AChE reactivity in the neuropil of the CP at 24 to 30 g.w. [50,52]. Against this background our findings were somewhat surprising as they distinctly detected nAChR protein in the CP at 16 weeks. It seems reasonable to assume that in the cholinergic system receptors are generated before cholinergic fibers and their specific enzymes occur. Lukas [61] suggests that gene promotor sequences are likely to dictate which AChR subunits are expressed in particular cells at specific times during development and respond to signals targeting the nucleus to control AChR expression. Existence of nAChRs has been considered as equivalent to the maturation of the cholinergic system. Yet, the coexistence of cholinergic axons and receptors does not necessarily imply a functional synapse. All components for the building of a cholinergic synapse, including its receptors, are there long before the synapse is actually formed.

4.5 Significance of nAChRs in Cortical Development

The formation of cortical areas has been discussed extensively and the debate has not yet come to an end. Apparently, a relationship exists between the arrival of afferent fibres, the organisation of the cortical layers and the maturation of cortical neurons. On one hand it has been suggested that afferent inputs affect the cellular architecture and thus shape the cortex [for review cf. 96]. The other theory is that “intrinsic” patterning information is encoded in the cortical cells and is independent from arriving afferents [for review cf. 67].

It has previously been proposed that besides their role in neurotransmission nAChRs may also be regarded as trophic factors during the early stages of pre- and postnatal life and in the development of cortical synapses. ACh, for example, facilitates synaptic modification in the striate cortex [9]. In vivo manipulations of the cholinergic system and nAChRs decrease cell numbers, disorganise lamination of the cortex and retard the maturation of the cholinergic sytem [46,72]. That means that nAChRs are likely to be involved in the shaping of the three-dimensional structure of the foetal brain.

One of the major questions in neuroembryology is what enables growing axons to find their cellular targets. Many mechanisms seem to be involved. In a study on the migration of neurons from the human ganglionic eminence to the dorsal thalamus it was suggested that cellular mechanisms and diffusible guidance cues are part of neuronal migration pathways [56]. The Cajal-Retzius cells of

layer I, for example, express reelin which is thought to repulse migrating neurons and thus governs the patterning of cortical neurons into layers [for review cf. 38]. Also nAChRs seem to be involved in navigating outgrowing axons. Pugh and Berg [81] and Zheng et al. [105] have shown that activation of nAChRs can alter neurite outgrowth and make growth cones turn. The early appearance of α4 and α7 nAChR shown in this study and their abundance within the SP, where afferents are delayed, and within the CP, where synapses form, fits in with the theory that both subunits play a role in neuronal migration.

Regulation of neurotransmitter systems has been found to be mainly activity-dependent. Previous studies have shown that transmitter and receptor immunoreactivity change after inhibition of sensory input. For example the expression of α7 nAChRs was reduced in the somatosensory cortex of neonatal mice after vibrissal follicle lesions [11]. Visual deprivation in monkeys was followed by a reduction of GABAergic neurons in Area 17 [43]. In tests carried out on postnatal mice removal of whiskers on one side of the muzzle resulted in an increase of α7 nAChRs in layer IV of the contralateral cortex [11]. Although such invasive experiments cannot be carried out on humans for obvious reasons, it is likely that also in humans activity-dependent regulation of neurotransmitters plays a role in development and plasticity.

4.6 Clinical Relevance

Meanwhile it has been accepted that the major factor leading to regular smoking is an addiction to nicotine. This implies that nicotine has a strong effect on the CNS. Nicotine can pass the placental barrier and thus affect foetal development, especially as the foetus is exposed to much higher nicotine concentrations than the mother [60]. There has been a lot of research going on during the last years in order to determine to what extent repeated stimulation of nAChRs through maternal smoking affects their expression in the foetus.

Studies have come up with several suggestions. A publication by Falk et al. [29] revealed that binding of the nAChR subunit α4 and the human foetal gene expression pattern of the α4 and α7 subunit changed in various regions of the brain after maternal smoking. Treatment of human prenatal brain cell cultures with nicotine led to increased [^{3}H]epibatidine and [^{3}H]cytisine binding and α3 and α7 mRNA expression in cortical cells [42]. In another study on rat pups which were treated postnatally with nicotine heteromeric nAChRs were significantly upregulated [48]. The

reversible effect did not include homomeric nAChRs such as α7 and the mRNA expression of α4, β2, and α7 subunits was not altered. The results show that exposure to nicotine during brain development influences the expession of nAChRs and their mRNA and that the effects are characteristic of both the brain region and the developmental stage. Higher cognitive functions depend on the appropriate number, organisation and connectivity of neurons in the neocortex. It was speculated that due to the fact that nicotine can mimic the effect of ACh it might influence the course of brain development.

One major risk factor for the occurrence of SIDS is exposure of the foetus to nicotine during pregnancy. Several studies brought an impairment of the developing cholinergic system by nicotine up for discussion. Frank et al. [31] reported an upregulation of nicotinic and muscarinic cholinergic receptor mRNA as well as an alteration of the sleep/wake cylce ontogenesis in neonatal rats after application of nicotine to the mother during pregnancy. The authors suggested an interference with the regulation of vigilance states which causes a predisposition to SIDS. Another work concentrated on the premature activation of nAChRs on adrenal chromaffin cells which were thought to disturb the neonatal catecholamine stress response following hypoxia [94].

An increased risk of nicotine addiction in adolescents whose mothers had smoked during pregnancy was suggested by Chen et al. [16]. He found that in rats that had received nicotine during gestation, gene transcripts for the α3, α4, α5, and β4 nAChR subunits and ^{125}I-epibatidine binding sites were reduced in dopaminergic brain regions. These data support the hypothesis that gestational nicotine exposure reduces the sensitivity of the dopamine system for nicotine and that more frequent smoking is needed in adult life in order to activate the mesolimbic dopamine system.

The effects of nicotine during development are not restricted to the CNS. The fact that infants born to mothers who smoke are more liable to respiratory ailments and compromised lung function might be due to the existence of the nAChR subunit α7 in pulmonary fibroblasts. Gestational stimulation of these receptors led to the synthesis and accumulation of collagen type I and III in airway walls and alveolar compartments of fetal monkey lung and resulted in an increased airway thickness [90].

In this study the smoking habits of the mothers were not assessed. Further studies should take this into account and focus on possible variations in the distribution of cerebral nAChRs between foetuses with smoking and non-smoking mothers. It is of great importance to further investigate

how changes in nAChR expression can be linked to postnatal impairments such as SIDS, respiratory dysfunction or later nicotine addiction.

4.7 CONCLUSIONS

As the cholinergic system develops rather early in gestation, it reaches a high complexity before birth. In the course of this work we could show that the nAChR subunits α4 and α7 are widely spread throughout the human foetal brain between g.w. 16 and 40 and that quantity and distribution increase until birth. The variation in the appearance of the subunits within frontal and visual cortex, in particular in the later foetal stages, might be due to the fact that the development and differentiation of the visual cortex precedes that of most other parts of the cerebrum. While eyesight matures quickly after birth and the infant has the ability to see within some weeks, other abilities such as movement develop during the course of months and years. The comparison to other methods detecting various components of the cholinergic system showed that nAChRs are among the first components of the cholinergic synapses that can be detected in the developing human brain.

Animal models are a necessary tool to study the developing neuroanatomical structures but it is not yet clear to what extent the findings in animals can be transferred to humans. The distribution of the nAChR subunits α4 and α7 in human foetuses seems to be similar to that previously described in rodents.

The present study supplements previous investigations carried out on the subject. Although some light could be shed on the neuroanatomical distribution of the nAChR subunits α4 and α7 in two different developing human brain areas the observations are still incomplete. Due to the limited acquisition of appropriate tissue only exemplary stages could be examined and no specimens of the early gestational stages were available. As this is a general problem in research on human foetal brain the highly interesting question regarding the exact allocation of the very first receptor subunits still has to be answered. It will probably take some time before a complete and comprehensive view on the development of the cholinergic system will be possible.

5. SUMMARY

In addition to the role nicotinic acetylcholine receptors play in neuronal signal transduction they are also likely to be involved in CNS morphogenesis and neuronal migration during the development of the human brain. Nevertheless, only few data on the neuroanatomical distribution of nAChRs in the human foetal brain are available.

Using immunohistochemistry the distribution of neurons expressing the subunits α4 and α7 has been systematically investigated on coronary sections through the frontal cortex and the area around the calcarine fissure. Eight whole foetal human hemispheres of different middle and late gestational stages (16 to 40 weeks) were examined.

Both α4 and α7 receptor subunits were present at gestational age 16 weeks in the frontal as well as in the occipital cortex. In most cases the immunochemical signal for the α7 subunit was stronger than that for the α4 subunit. The expression of both proteins was similar in the middle foetal period also with regard to the two brain regions. The highest density and intensity of labelled cells at this stage, most of them being small and plump with a large nucleus, could be detected in the superficial part of the cortical plate. Marginal and intermediate zone hosted few and moderately stained neurons. More immunoreactive neurons became visible in the subplate as the gestational age increased. Most of the cells in the subventricular and ventricular zones showed moderate staining. At more advanced stages occurrence of α4 and α7 immunoreactivity varied. In the frontal cortex the most prominent α4 staining changed to layers III and V and here mainly to big pyramidal-shaped neurons. The intensity of the labelling of the subplate cells decreased with the reduction of thickness in this layer. The α7 protein could mainly be detected in layer I and within large subplate neurons with long vertical dendrites. It was also present within large cells in the superficial part of the white matter. The visual area showed the most intense α4 labelling in layers II and V, α7 could mainly be found in layers III and VI. In both cases the granular cells of layer IV, the stratum of visual input, were hardly marked at all. The fibre-rich white matter contained a medium share of weakly stained α4- and moderately to strongly stained α7-expressing cells.

In conclusion nAChRs are present at an early developmental stage within different layers of the cortical plate and to a lesser extent also in the underlying layers. This may indicate an involvement in the differentiation of cortical circuitry elements. Our findings match those obtained with in situ hybridisation and receptor autoradiography and are similar to the observations made in the rat.

6. ZUSAMMENFASSUNG

Nikotinische Acetylcholinrezeptoren (nAChR) scheinen die Morphogenese des Zentralnervensystems und die Wanderung von Nervenzellen während der menschlichen Entwicklung zu beeinflussen. Dennoch liegen bis jetzt nur wenige Daten zur neuroanatomischen Verteilung von nAChR im menschlichen fetalen Gehirn vor.

Die Verteilung der Rezeptoruntereinheiten α4 und α7 wurde immunhistochemisch an Koronarschnitten durch den frontalen Kortex und den Sulcus calcarinus von acht menschlichen fetalen Gehirnen dargestellt. Dabei handelte es sich um vier Individuen aus dem mittleren Gestationsstadium (16, 17, 19 und 22 Wochen) und vier weitere aus dem spätfetalen Stadium (29, 33, 38 und 40 Wochen). Die Auswertung erfolgte semiquantitativ bei 10- bis 40facher Vergrößerung.

Sowohl die α4- als auch die α7-Untereinheit konnte nach 16 Gestationswochen in der frontalen und in der okzipitalen Region nachgewiesen werden. In den meisten Fällen, besonders bei den älteren Feten, war die Färbung für α7 stärker als diejenige für α4.

In den mittleren fetalen Stadien gab es bezüglich der Verteilung der Untereinheiten in den beiden Regionen nur geringe Unterschiede. Die meisten der immunhistochemisch gefärbten Zellen waren klein und rundlich mit einem großen Zellkern und wenig Zytoplasma. Die stärkste Färbung wurde in der oberflächlichen kortikalen Platte beobachtet, wo die Zellen dicht gepackt lagen. Die wenigen Neurone in der Marginalzone und in der Intermediärzone waren nach der Inkubation mit dem α4-Antikörper schwächer angefärbt als nach der Detektion durch den α7-Antikörper. Die Subplate wies mit zunehmender Entwicklungsdauer mehr und größere immunpositive Zellen auf. Der größte Teil der Ventrikulär- und Subventrikulärzellen war nur mäßig gefärbt.

Mit zunehmendem Gestationsalter war die stärkste Färbung für die Untereinheit α4 im frontalen Kortex nun in den Laminae III und V zu finden, vor allem in großen, pyramidenförmigen Neuronen. Die Subplate-Zellen waren weniger intensiv markiert. Im Gegensatz dazu konnte das α7-Protein in Lamina I und in großen Subplate-Neuronen mit langen vertikalen Dendriten nachgewiesen werden. Die α7-Untereinheit war auch in Ansammlungen großer Zellen in der weißen Substanz unterhalb des Kortex zu beobachten.

Im Bereich der Sehrinde war die intensivste Färbung für das α4-Protein in den Laminae II und V, die für das α7-Protein in den Laminae III und VI zu finden. Die Körnerzellen von Lamina IV, der Schicht der visuellen Afferenzen, waren kaum markiert. Die faserreiche weiße Substanz beinhaltete nur mäßig viele Zellen, diese waren schwach positiv für α4 und mäßig bis stark gefärbt für α7.

Mit kleiner Vergrößerung fiel in der Übersicht auf, dass die Färbung der oberflächlichen kortikalen Platte in allen Stadien und in beiden Regionen insgesamt in den Sulci weniger intensiv zu sein schien als auf den Gyri.

Zusammengefasst können nAChR im menschlichen Gehirn bereits früh in der Entwicklung nachgewiesen werden und sind während der gesamten Fetalphase vorhanden. Sie befinden sich in verschiedenen kortikalen Schichten, dabei gibt es vor allem in der späteren Fetalzeit Unterschiede, sowohl in der Verteilung der beiden Untereinheiten α4 und α7, als auch in Bezug auf die beiden untersuchten Regionen. Die Ergebnisse können als weiterer Hinweis darauf interpretiert werden, dass nAChR in die Differenzierung kortikaler Schaltkreise involviert sind. Unsere Beobachtungen stimmen mit denen der in situ-Hybridisierung und Rezeptor-Autoradiographie weitgehend überein und ähneln den Daten, die bei der Ratte erhoben wurden.

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8. PRELIMINARY PUBLICATIONS

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