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NEONATAL NORADRENALINE DEPLETION
IN RAT BRAIN: EFFECTS ON THE DEVELOPMENT
OF CEREHELLUM AND GABA_A RECEPTORS

ACADEMIC DISSERTATION

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LIST OF ORIGINAL COMMUNICATIONS

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals I-V. Some unpublished results are also presented.

- I Podkletnova I, Raevsky V and Alho H (1996) Reduction of GABAergic transmission and alterations in behavior after 6-OHDA treatment of rats. *Dev. Brain Res.* 94:197-204.

- II Podkletnova I and Alho H. (1998) Neonatal noradrenalin depletion prevents the transition of Bergmann glia in the developing cerebellum. *J Chem. Neuroanat.* 14: 167-173.

- III Podkletnova I, Alho H, Mäkelä R, Lüddens H, Helén P and Korpi E R (2000) Neonatal 6-hydroxydopamine treatment affects GABA_A receptor subunit expression during postnatal development of the rat cerebellum. *Int. J Dev. Neurosci.* (in press).

- IV Podkletnova I, Mäkelä R, Korpi E R, Lüddens H, Helén P and H. Alho (2000) Neonatal 6-hydroxydopamine treatment affects GABA_A receptor subunit expression in the frontal cortex but not the hippocampus of rats during postnatal development. *Dev. Neurosci.* (in press).

- V Podkletnova I, Rothstein JD, Helén P and Alho H . Activation of cerebellar microglia after 6-OHDA induced oxidative stress (submitted).

ABBREVIATIONS

6-OHDA	6-hydroxydopamine
AChE	acetylcholinesterase
BZ	benzodiazepine
CNS	central nervous system
DA	dopamine
DBH	dopamine-beta-hydroxylase
DBI	diazepam binding inhibitor
EGL	external granule cell layer
GABA	γ -aminobutyrate
GABA _A	γ -aminobutyrate type A receptor
GABA _B	γ -aminobutyrate type B receptor
GAD	glutamate decarboxylase
GLAST	glial glutamate transporter
IGL	internal granule cell layer
IR	immunoreactivity
NA	noradrenaline
PBS	phosphate buffered saline
PCP	phencyclidine
PD	postnatal day
SSC	standard sodium citrate buffer
TBPS	<i>tert</i> -[³⁵ S]bitylbicyclophosphorothionate
TH	tyrosine hydroxylase
Tris	tris(hydroxymethyl)aminomethane

ABSTRACT

One of the primary means for studying neuronal function and development in the central nervous system has been the destruction of noradrenergic (NA) innervation by 6-hydroxydopamine (6-OHDA). 6-OHDA is an isomer of noradrenaline. Systemic neonatal application causes degeneration of glutamatergic granule cells in the neocerebellum (lobules V-VII) – the part of the brain that appears postnatally and that receives strong noradrenergic neurotrophic input. The exact mechanism behind this destruction, however, is not known, and little attention has been paid to the role of glial cells and GABA. Therefore, the aim of this research was to study in detail the postnatal period of brain maturation after 6-OHDA treatment, focusing especially on Bergmann glial cells and GABA_A receptors.

The methods employed were immunohistochemistry, in situ hybridization, ligand binding, and behavioral tests. The results revealed, for the first time, that after 6-OHDA treatment, Bergmann glial cells were altered and the expression GABA_A receptor subunits $\alpha 1$ and $\alpha 6$ were reduced especially in granule cells. The Bergmann glial cells were abnormally located and structurally different, with no intimate associations with Purkinje cells. Significant microglial activation was also observed. The animals showed changes in behavior, especially in their orientation to novel environment. Interestingly, all these changes were transient.

Elimination of the NA terminals should prevent activation of adrenoceptors on the Bergmann glia; this in turn could abolish Ca²⁺ release from the glial cells and thus perhaps disrupt the normal migration of granule cells. Degenerated granule and

Bergmann glial cells may possibly have induced the invasion of microglial from the cerebellar medulla to the cerebellar cortex, but microglia may also protect the surviving cells against the toxic environment. The temporary suppression of the expression of $\alpha 1$ and $\alpha 6$ subunits may cause the dysfunction of cerebellar GABA_A receptors during the first postnatal month, which in turn may partially result in the spatial disorientation and other behavioral abnormalities that have been observed in these animals. It is concluded that noradrenergic input has an important trophic role in the development of cerebellar granule cells, and that the depletion of this input causes transient destructive alterations in cells that are important for normal granule cell development.

INTRODUCTION

Noradrenaline is an important factor for the development of nervous system. It has been discovered recently that noradrenaline supports target cell replication and initiates postsynaptic receptor development (Duncan et al. 1990, Rowe et al. 1993, Wagner et al. 1995). The frontal cortex and cerebellum are two brain structures that undergo their peak of cell replication/ differentiation postnatally (Altman 1969, Altman and Winfree 1977, van Eden and Uylings 1985). These structures are the targets to alteration in neuronal development after neonatal noradrenergic lesion (Lovell 1982, Seidler et al. 1995).

Neurotoxic drug 6-hydroxydopamine (6-OHDA) is used to selectively and permanently destroy NA terminals in the rat brain (Gustafson and Moore 1987, Sachs and Jonsson 1975). Neonatal 6-OHDA treatment is known to reduce the dendritic branching of pyramidal neurons in the rat cerebral cortex so, that apical dendrites do not reach layers I-II of the cortex but remain located in layer III (Felten et al. 1982). The elimination of NA terminals by 6-OHDA affects the foliation in cerebellum. The neocerebellum is the most vulnerable to postnatal toxic influences, since at birth all types of neocerebellar cells are in the proliferative stage (Robain et al. 1985, Jacobson 1978).. However, the mechanisms behind these changes are not known, and the possible involvement of Bergmann glia and GABAergic system has not been studied in details.

The present study is focused on the development of the rat brain during the first postnatal month with special attention to the alterations in the frontal cortex and the cerebellum after neurotoxic elimination of noradrenergic (NA) terminals.

REVIEW OF THE LITERATURE

1. DEVELOPMENT OF THE RAT FRONTAL CORTEX AND CEREBELLUM

1.1. Postnatal development of the rat frontal cortex

The cerebral cortex is organized normally into five layers. They are numbered I to V, starting with the outermost layer. The last neurons to develop in the cerebral cortex are those in the outer layers, with one exception: layer I is one of the first two to develop, along with layer V (Marin-Padilla 1970, Bayer and Altman 1990). The outer layers are also the most recent to have appeared during evolution. Two layers of older neurons, the marginal and subplate zones, are believed to possess unique roles in cortical development. While subplate cells have been found to be essential for the establishment of thalamocortical relationships, the Cajal-Retzius neurons of layer I provide a tonic excitatory input that is essential for the maturation of cortical neurons. From postnatal day 8 (PD8) onward, Cajal-Retzius cells have disappeared from the neocortex (del Rio et al 1995, Geula et al 1995).

The neurogenesis in the frontal cortex is completed by the 21st embryonic day, and it continues only in the dorsal parts of the cortex where the cells of the second layer are being formed. The formation of neuroglia is very active (Bisconte and Marty 1975). The penetration of fibers containing catecholamines and serotonin through the intermediate layer is increasing. The cortical plate reaches 300 μm of thickness. Pyramidal neuroblasts situated deep inside reach considerable size and tend develop branches. The neuroblasts of the upper parts of the cortical plate are more primitive (Derer 1974). Nearly all of the types of cells of the different layers of the cortex are already present in the newborn rat

brain, but morphologically it is not yet possible to distinguish the six cortical layers seen in the adult. The cortical plate becomes thicker, and the intermediate zone becomes thinner (Altman 1969). Many bipolar cells are found in the surface area. The pyramidal neuroblasts have apical dendrites, but the dendrites seldom have branches. This stage is characterized by the neurons having direct contacts with each other, as proved by electron microscope studies (König et al 1975). The cells with the Nissley substance are not yet found in the cortex by PD3 - PD5 when the synapses begin to develop. The pyramidal neuroblasts of the inner layers differentiate earlier than those elsewhere. They synthesize RNA, but the activity of their respiratory and oxidizing enzymes is still very low (Derer 1974). All the layers that are typically seen in the brain of an adult rat can be found by PD6. At this stage the thickness of the layers increases unevenly with time. Thus, layer V grows 2.6 times thicker from PD2 to PD7, but the growth of layers II-IV occurs later (3 times thicker for the 7-11 days old rats). Also by PD6 the pyramidal cells contain Nissley substance, and their dendrites have small spines. By PD10 - PD15 the glial membrane of neurons and the vascular capillary system are developed. Rhythmic electrical activity can be detected beginning from PD10. In layer IV the active development occurs on PD7 - PD10. Apical dendrites of the pyramidal cells of layer V reach layer I, and many basal dendrites appear. By PD12 the average number of the main dendrites per cell is practically the same as in the adult animal, but the secondary branching and the formation of synapses occurs only by PD24. While pyramidal cells can be found in the brain of the newborn rats, the astrocytes are generated only by PD9 - PD11. In layers II and III the neuronal differentiation can be observed by PD8 - PD12. The maximum increase of connectivity in the rat cortex occurs between PD12 - PD20, as evidenced by the increase

in the density of cortical axons and by the increase in the number of synapses in the cortex (Aghajanian and Bloom 1967).

1.2. Postnatal development of the rat cerebellum

The stage of the cerebellar development at birth is correlated with the locomotor and motor coordination abilities of newborn animals. In precocial animals that are able to walk soon after birth (such as the chick, guinea pig, and ungulates), the cerebellum is well developed at birth; whereas in altricial animals (such as the mouse, rat, and man) that are helpless at birth, the cerebellum is in a corresponding state of immaturity, and its histogenesis and morphogenesis occur mainly after birth. (Jacobson 1978).

The rat cerebellum undergoes its peak of development postnatally (Altman and Winfree 1977). The maximum activity of lactate dehydrogenase and succinate dehydrogenase is observed in the Purkinje cells on PD2; in the Golgi cells on PD9; in the basket cells and astocytes on PD12; and in the granular cells on PD16 (Woodward et al 1971). The formation of granule cells in the embryonic outer layer occurs during the first postnatal week (Altman and Winfree 1977). Anatomical specialization in the cerebellum of the 5 day old rats is almost complete. The embryonic outer germinal layer is considerably increased and often contains mitoses. The molecular layer is not yet developed. The Purkinje cells, in groups of two or three, are in the phase of astrocyte-like cells with non-oriented dendrites (Dadoune 1969). The thickness of the embryonic outer germinal layer reaches its maximum when the rat is 9 days old. At this stage the mitotic activity in the layer begins to decrease, and by PD11 mitotic bodies become rather rare. In the molecular zone it is possible to find a small number of basket cells and astrocytes. The inside granular layer continues to grow. The Purkinje cells have pear-like bodies and

dendrites with secondary and tertiary branches that are in the phase of orientation. The activity of glutamate dehydrogenase in those cells is at maximum and later decreases (Dadoune 1969).

By PD11 the surface germinal layer begins to disappear, but it still exists inside the fissures of the cerebellum. By PD14, in addition to the existing fissures, secondary fissures appear: centralis, culmen, uvula, nodulus. By PD16 typical dendrite branches of the basket cells appear. By PD20 the embryonic outer granule layer disappears, and the activity of acetylcholinesterase (AChE) in the Purkinje archicerebellum cells vanishes. AChE exists in the Purkinje cells at the stage of the neuroblast (in the brain of newborns) and disappears during their differentiation to neurons (Csillik et al 1964).

The cerebellar cortex has three cortical layers - named in order from the outer surface: molecular layer, Purkinje cell layer, and granule cell layer. Purkinje cells are the principal neurons and provide the sole pathway out of the cerebellar cortex. Impulses are conducted into the cortex by the climbing fibers which originate in the inferior olive, and by mossy fibers which are mostly the terminals of spinocerebellar and pontocerebellar fibers. There are also axons containing monoamines that originate in the locus coeruleus, raphé nuclei, and substantia nigra (reviewed in Voogd and Glickstein 1998). The climbing fibers terminate on thorns on the large dendritic branches of Purkinje cells, and the mossy fibers terminate in structures called glomeruli in which they form synapses with the dendrites of granule cells and the axons of Golgi type II cells. The granule cells receive an input from the mossy fibers. The granule cell axons, called the parallel fibers because they have a trajectory parallel to the axis of the folium, form synaptic connections on the dendritic spines a single row of Purkinje cells.

2. THE FORMATION OF THE NORADRENERGIC SYSTEM

The main criterion for the formation of any modulatory system is the setting of synaptic contacts. In case of noradrenergic innervation, this process includes the differentiation and development of the noradrenergic neurons of the locus coeruleus, the growth of their axons to the upper parts of brain, and the formation of fully developed synaptic contacts between the terminals of these axons and the innervated neurons. The published information concerning this question has been obtained mainly from ontogenetic studies of rats.

The differentiation of neurons of the locus coeruleus in rats brain begins on the 10th-13th day of embryogenesis, which is earlier than in all the other parts of the brain (Lauder and Bloom 1974). Several days later it is possible to find the presence of noradrenaline in these neurons.

2.1. Development of the noradrenergic system in the frontal cortex

The noradrenaline-containing axons grow in the rostral direction, innervate the upper parts of brain, and reaching the core plate during the period of active proliferation and migration of cells to the neocortex (the 16th-17th day of embryogenesis) (Olson and Geiger 1973, Levitt and Moore 1979). It is possible that fibers growing from locus coeruleus initiate the differentiation of the innervated cells because it begins in the cortex and in the hippocampus only after the appearance of the noradrenergic axons. It has been also found that the target cells may influence the speed of growing noradrenergic fibers at early stages of prenatal development (di Porzio and Estenoz 1982).

Thus, we have grounds to conclude that noradrenergic neurons of the locus coeruleus complete their development early and grow into the frontal parts of the brain, reaching neocortex during the embryonic period. Synaptic contacts of noradrenergic fibers with target cells are already formed at birth.

2.2. Development of the noradrenergic system in the cerebellum

The invasion of noradrenergic terminals in the cerebellum can be revealed using dopamine- β -hydroxylase (DBH, the final enzyme of the synthetic pathway for noradrenaline) as an antigenic marker. (Verney et al 1982). The arrival of DBH-containing fibers in the cerebellar cortex of the vermis occurs progressively in a gradient fashion, starting in the anterior lobules (I-II-III-IV-V) and the uvula (IX) earlier than in the posterior (VI-VII-VIII) and the nodular (X) ones.

Except in the case of unusually thick, highly-fluorescent fibers noticed in the external granule cell layer (EGL) during the first 2 weeks after birth, the arrival of DBH-containing fibers through the cerebellar cortex follows an inside-out gradient: at birth sparse fluorescent axons are observed in the album of the cerebellum. These axons invade the internal granule cell layer (IGL) at PD2 and reach the Purkinje cell layer from PD5 to PD15. The density of fibers increases in this layer, which becomes the most innervated layer of the cerebellar cortex, as it is in the adult brain. When the external granule cells migrate, DBH-containing fibers extend progressively from the interior to the surface in molecular layer and show an adult pattern at PD30. The transient presence of fibers containing a large amount of DBH in the intense proliferation area (EGL) could suggest a relationship between NA and cell division (Lovell 1982).

After removal of the granule cells in neonatal mammals, the immature pattern of Purkinje cell dendritic branching persists in the adult: the dendritic spines survive but are reduced in size, and the Purkinje cell dendrites are not oriented toward the outer surface of the cerebellum but are deflected laterally (Jacobson 1978).

3. DEVELOPMENT OF GABA_A RECEPTORS

GABA_A receptors, encoded by at least 19 subunit genes (Davies et al 1997), are GABA-gated ion channels mediating the major inhibitory neurotransmission in the adult mammalian central nervous system (CNS). Most receptors are assembled from α and β subunit variants in combination with the $\gamma 2$ subunit. Coexpression of α and β subunits is sufficient to form GABA-gated ion channels (as shown with recombinant receptors), but the addition of the $\gamma 2$ subunit is required for the expression of GABA_A receptors containing benzodiazepine (BZ) binding sites (Günter et al 1995). Prenatal brain development appears to be unaffected in $\gamma 2$ -deficient mice. This is evidenced by their normal brain cytoarchitecture at birth, by the normal emergence of modular brain structures such as the barrelettes in the brain stem, and by the unaltered spatio-temporal expression patterns of GABA_A receptor subunits, including the developmental switch from $\alpha 2$ to $\alpha 1$ subunit expression at about the time of birth (Meinecke and Rakic 1990).

3.1 Ontogeny of receptor subunits

There are 19 GABA_A receptor mRNAs. Each of them exhibits a specific regional and temporal pattern of expression in the developing brain. The most marked change in the expression occurs for the α subunit mRNAs. Fetal and neonatal GABA_A receptors contain mainly $\alpha 2$, $\alpha 3$, or $\alpha 5$ subunits, while most adult ones contain the $\alpha 1$ subunit

(Laurie et al 1992). The $\beta 2$ and $\gamma 2$ transcripts are present in the embryo, and their expression increases after birth. The early postnatal peak of expression is apparent for $\beta 1$ (PD6-PD12), $\beta 3$ (PD12), $\gamma 1$ (PD0-PD6), and $\gamma 3$ (PD6) subunits. The $\alpha 6$ and δ mRNAs appear postnatally, and their expression increases markedly with age (Laurie et al 1992).

3.2. GABA_A receptor subunits in cerebral cortex, hippocampus, and cerebellum

Considerable changes in the principal subunit mRNAs occur in the developing rat cerebral cortex during perinatal and postnatal maturation. During the last prenatal week, the $\alpha 1$, $\alpha 5$, $\beta 2$, and $\beta 3$ mRNAs are the most abundant in the outer cortical layer (layer I); the $\alpha 3$ and $\gamma 2$ transcripts are found in both outer and inner (subplate) layers; and the $\alpha 2$, $\alpha 4$, $\beta 1$, and $\gamma 1$ are mainly located in the lower intermediate or ventricular zones. The changes in the predominance of GABA_A subunits coincide with the stratification of cortical neurons (Jacobson 1978, Miller 1988). From PD6 to adulthood, $\alpha 1$, three β , and $\gamma 2$ mRNAs are mainly expressed in the superficial and deep cortical layers, while $\alpha 5$ mRNA is predominant in the middle layers until PD12 but remains in the deep cortical layers in the adult brain (Van Eden et al 1995).

In contrast to this changing predominance of GABA_A subunit mRNAs in the cerebral cortex, mature spatial mRNA patterns in the hippocampal pyramidal cells and dentate gyrus granule cells dominate already in newborn rats. Thus, $\alpha 1$, $\alpha 2$, $\beta 1$, $\beta 2$, $\beta 3$, $\gamma 1$, $\gamma 2$, and $\gamma 3$ transcripts are expressed homogeneously; but $\alpha 3$ and $\alpha 5$ mRNAs are concentrated in CA3; and $\alpha 4$ and δ mRNAs are concentrated in the dentate gyrus (Wisden et al 1992).

In the cerebellum, most GABA_A receptor mRNA expression is restricted to the period of postnatal development. The adult pattern of $\alpha 1$, $\beta 2$, $\beta 3$, and $\gamma 2$ subunits is present at

PD0 only in the Purkinje cells. The transcripts for $\alpha 2$, $\alpha 3$, $\beta 3$, and $\gamma 1$ mRNAs are present in EGL at PD0, and decline thereafter. In postmigratory granule cells, $\alpha 1$, $\alpha 6$, $\beta 2$, $\beta 3$, $\gamma 2$, and δ mRNAs exhibit a very pronounced increase between PD6-PD12 to the adult levels of expression. The “halo” of hybridization for $\alpha 2$ and $\gamma 2$ mRNAs in Bergmann glia is apparent after PD12. Labeling of stellate/basket cells in the molecular layer by probes for $\alpha 1$, $\beta 2$, and $\gamma 2$ mRNAs is observed only in the adult cerebellum (Muller et al 1994).

3.3. Maturation of the GABAergic transmission

The development of GABAergic neurons is defined by the content of glutamate decarboxylase (GAD) which provides the necessary conditions for their growth. Ontogenetic studies of this enzyme prove that during the first week of postnatal development the GAD level in the brain is rather low. The primary increase in GAD activity occurs during the time period from PD8 to PD24 (as shown on rats by MacDonald et al 1987). The content of the enzyme at this time reaches a stable level in all the layers of the cerebral cortex with the exception of layer IV where the level of GAD continues to increase at the older ages.

GAD, which participates in the synthesis of GABA, is first found in embryogenesis simultaneously with the appearance of GABA. In spite of the 6-fold increase during prenatal period, the activity of this enzyme in newborn rats is much lower than in adults (Coyle and Enna 1976). While the endogenous GABA and the uptake capacity reach the mature level by the moment of birth, the activity of enzymes providing the synthesis and the reuptake of the neurotransmitter become developed only by the end of the first month

of life (Coyle et al 1982). During the first 10 postnatal days the activity of GAD does not change considerably, and it starts to increase rapidly by the 4th week of life.

4. SENSITIVE PERIOD AND THE NEUROTROPHIC FUNCTION OF NEUROTRANSMITTERS IN THE DEVELOPING BRAIN

One period in early ontogenesis – from the first to third postnatal weeks in rats – is particularly important. Behaviorally this is the period for the process called "imprinting" (Lemke and Lange 1999). This is the initial period when the brain synaptic network is forming as a reflection of outer stimuli. Adult behavior in general and the ability to select an important signal from the sensory flow is based on the imprinted information. It is suggested that imprinting is based on catecholaminergic neurotransmission, while the real selection is provided by GABAergic inhibition (Reiser et al 1999).

During the critical period, exposure of target cells to catecholamines provides two promotional signals for brain cell development. At first, low synaptic levels of neurotransmitter associated with early phases of synaptogenesis support target cell replication ((Lauder 1985, Duncan et al 1990). Subsequently, they provide the necessary "pioneer" signals that initiate postsynaptic receptor development and function (Seidler et al 1995). Inhibition of DBH, the enzyme catalyzing the conversion of dopamine to noradrenaline, or application of α -adrenergic receptor antagonists substantially reduces subsequent embryonic neuronal differentiation, while exogenous noradrenaline (10^{-6} M) or dopamine (10^{-6} M) increases the number of neurons that subsequently differentiate in culture (Rowe et al 1993).

The composition and presumably the properties of embryonic/early postnatal rat GABA_A receptors differ markedly from those expressed in the adult brain. Activation of neonatal GABA_A receptors induces depolarization and a rise in intracellular calcium concentration in both cerebellar and cortical neurons (Connor et al 1987, Yuste and Katz 1991). Elevated intracellular Ca²⁺ is an important factor in neuronal growth and differentiation (Spitzer 1991). In primary culture of embryonic and neonatal brain tissues, GABA promotes neurite extension, synaptogenesis, and the synthesis of its own receptors (Hansen et al 1987, Wolff et al 1987).

Genesis of mature-type synapses accelerates after PD12 (Aghajanian and Bloom 1967). This is the stage at which GABA is likely to become an inhibitory transmitter and induce membrane hyperpolarization by the activation of chloride channels (for review see Cherubini et al 1991).

5. GLIAL CELLS

5.1. Bergmann glia

Bergmann glia proliferate earlier than do neurons in the cerebellum. Postnatal proliferation of Bergmann glial cells occurs mainly between PD6 and PD9. The origin of Bergmann glia has been under discussion for a long period of time. Initially precursors of Bergmann cells were considered to be located in EGL (Das 1976). Later it has been proven that dividing progenitors in the white matter migrate as immature cells into the cerebellar cortex before differentiating into Bergmann glia, astrocytes, oligodendrocytes, and interneurons in their appropriate layers. EGL cells do not produce glia but only granule cells (Zang and Goldman 1996). The spatial and temporal patterns in postnatal

proliferation of Bergmann glial cells have been shown by [^3H]thymidine incorporation. The labeling index in the paleocerebellum (lobules I- VIa, VIII-X) reaches its peak on PD 6-7 and in neocerebellum (lobules VIb-VII) on PD 8-9 (Shiga et al 1983). Lesioning the noradrenergic projections or blocking neurotransmission with a receptor antagonist prevents astrogliosis and glial cell proliferation (Rowe et al 1993, Hodges-Savola et al 1996).

The Bergmann glia of the rodent cerebellum is an example of a glial cell population that sequentially performs different functions in early and later phases of tissue maturation. During the first 2 weeks after birth, a major function of the Bergmann glia is to guide the migration of granule cells from the proliferative zone on the surface of the cerebellar cortex to their ultimate resting place deep in the Purkinje cell layer (Das 1976, Muller et al 1996). By the third postnatal week, the Bergmann glia becomes increasingly intimately associated with the rapidly maturing Purkinje cells, whose somata and dendritic trees they embrace (Fisher et al 1993, Reichenbach et al 1995).

5.2. Neuron-glia interactions in the cerebellum

Bergmann glia plays an important role in NA modulation of neuronal activity in the cerebellum. The glial responses to NA and KCl exposure are not dependent upon neuronal action potentials. NA acts directly on $\alpha 1$ -adrenergic receptors and triggers the release of Ca^{2+} from internal stores in Bergmann glia (Shao and McCarthy, 1997). Glia modulate NMDA-mediated signaling in primary cultures of cerebellar granule cells (Beaman-Hall et al 1998). Recently it has been found that the release of noradrenaline from afferent fibers activates a glial α -adrenoceptor that promotes Ca^{2+} release from intracellular stores. An increase in glial Ca^{2+} is known to stimulate a diversity of

processes such as transmitter release, energy metabolism, and cell proliferation. Thus, the adrenoceptor-mediated mechanism described here is well suited for a feedback modulation of neuronal function that is independent of glutamate (Kulik et al 1999).

5.3. Microglia

Microglial activation is often used as a marker of brain injury. Microglia in the normal mature brain do not proliferate and show a downregulated immunophenotype adapted to the specialized microenvironment of the CNS. Such microglial cells are called resting cells (Davis et al 1994). Microglia in the embryonic and early postnatal cerebellum are present with round and amoeboid shapes in the region of the future cerebellar medulla until PD10. After this time only resting microglia are found in the cerebellum (Ashwell 1990).

During various pathological processes (including degenerative neurological diseases, neurotoxic neuronal death, and ischemia-induced neuronal death) microglia actively proliferate, change their ramified shape into an amoeboid one, and begin to phagocytose degenerating cells (Kreutzberg 1996). These cells in the pathological brain are called activated microglia. Microglial reactivity in the early postnatal brain usually peaks at 3 days postlesion and returns to the control level at 7-10 postlesion days (Acarin et al 1997). The activated microglia can secrete neurotoxic as well as potentially neuroprotective substances (Fujita et al 1998, Young et al 1999) .

6. 6-HYDROXYDOPAMINE - A NEUROTOXIC DRUG

In 1967 Tranzer and Thoenen discovered “chemical sympathectomy” – the destruction of peripheral sympathetic (noradrenergic) neurons by an isomer of NA,

namely, 6-OHDA. The toxic effect of 6-OHDA on different brain regions and peripheral neurons has been intensively studied ever since (Kostrzewa and Jacobowitz 1974). In the CNS and peripheral nervous system of mature animals, NA terminals are able to regenerate after 6-OHDA administration. In newborn rodents, doses of 6-OHDA higher than 100 mg/kg cause the destruction of sympathetic postganglionic neurons. Central noradrenergic neurons are also damaged, since 6-OHDA has the ability to pass through the still not fully developed blood-brain barrier of neonates (Sachs and Jonsson 1975).

6.1. Mechanism of toxicological action

6-OHDA leads to cell death by the oxidative stress of the metabolites from the auto-oxidized 6-OHDA (Super et al 1997). The spontaneous autooxidation of 6-OHDA proceeds by a free radical chain reaction involving the superoxide anion radical and produces the corresponding chromogen (6-OHDA quinon) and hydrogen peroxide. The rate of this reaction is reduced by natural antioxidants: superoxide dismutase, catalase, and NADH quinone oxidoreductase (Padiglia et al 1997). Another proposed mechanism is that the 6-OHDA inhibits the activity of NADH quinone reductase in mitochondrial complex I (Glinka et al 1998), which in turn reduces the cellular energy supply and may lead to cell death.

6.2. Influence on the development of cerebellum

A reduction in the noradrenaline content is achieved by neonatal 6-OHDA injections. Systemic 6-OHDA treatment produces the depletion of NA terminals in the cerebellum, reduction in the vermis area, alterations in foliation, and changes in the schedule of EGL development (Lovell 1982). Similar alterations in the vermis of cerebellum are observed after all types of 6-OHDA application (infusion directly into the locus coeruleus nuclei,

or injection directly into the 4th ventricle). This means that the elimination of noradrenergic terminals by itself is sufficient to reduce the growth of the cerebellum at certain periods of postnatal life (Robain et al 1985).

6.3. Behavioral alterations after 6-Hydroxydopamine treatment

Disturbance in the NA system and even total destruction of locus coeruleus in adult rats do not produce any effect on habituation, anxiety, or motor activity (Crow et al 1978, Hauber et al 1994). The acquisition of new locomotor tasks is, however, impaired proportionally to degree of cerebellar noradrenergic depletion (Watson and McElligott 1984).

On the other hand, the neonatal injection of 6-OHDA produces numerous behavioral alterations. The initial behavioral changes (such as the decreased food and water consumption, lowered body temperature, and decreased locomotion) are reversible and result from NA or DA release from central catecholaminergic neurons after 6-OHDA treatment (Kostrzewa and Jacobowitz 1974). More permanent behavioral alterations reflect the destruction of neurons in the CNS. Among them are a lack of self-grooming (Breese and Traylor 1970), increased aggressiveness, hypersensitivity to tactile stimulation (Evetts and Iversen 1970), and defects in sensory attention (Carli et al 1983, Fukuda et al. 1977).

AIMS OF THE STUDY

Developmental studies of the rat brain have demonstrated that extensive reorganization of the brain networks occurs during the early postnatal period. The noradrenergic trophic influence is found to be necessary to initiate the formation of the excitatory and inhibitory synaptic connections. Little is known, however, about the interactions between neurons and glial cells during the postnatal brain development.

The subject of this research was the development of rat brain after the elimination of NA terminals by 6-OHDA treatment. The study was designed to answer the following questions:

1. What kind of alterations in rat behavior can be observed during the first postnatal month ?
2. In which brain region is GABAergic transmission disturbed after systemic 6-OHDA application?
3. Are GABA_A receptors altered in any of the brain regions?
4. Which glial cells are involved in the cerebellar response to the toxic influence?
5. What is the possible role of the cell-to-cell interactions in the cerebellar development?

MATERIALS AND METHODS

1. EXPERIMENTAL ANIMALS

A total of 235 of new-born and 30 adult Sprague-Dawley rats were used in this study. 7- 8 pups of both sexes were maintained with every mother in a plastic cage with free access to food and water. The animals were kept on a constant 12/12 h light-dark cycle. The weight was measured, and the behavior was observed and recorded with a video-camera daily from the day of birth until PD 40. The Institutional Animal Care and Use Committee of the University of Tampere approved all experimental protocols.

2. 6-HYDROXYDOPAMINE TREATMENT (I -V)

Pups from each litter were randomly divided into 2 groups on the day of birth, designated as postnatal day 0 (PD0). One group of rats received the 6-OHDA (Sigma) injection (100 mg/kg) subcutaneously in a volume of 0.1 ml in 0.9% saline containing 0.04% L-ascorbic acid (Sigma) at PD1, PD2, and PD3. The second (control) group of rats received an equal volume of the saline. After injection, the pups were marked and returned to the lactating mothers.

3. PHENCYCLIDINE TREATMENT (V)

As a positive control for microglial activation, three control animals from the same litters received at PD3 intraperitoneal injections (50 mg/kg) of phencyclidine (PCP), (RBI Natick MA USA) in 0.9% saline and were sacrificed at PD 10.

4. BEHAVIOURAL TESTS (I)

The development of "open field" behavior was studied as described recently (Podkletnova et al 1995). Briefly, the animals were placed in the center of a square plastic box measuring 25 x 25 cm, with 20 cm high walls. The floor of the box was divided into

25 squares (5 x 5 cm). The box was placed so that no external landmark signs were visible to the animals. Motion activity in the box was recorded by the video-camera placed 1 meter above the box. Locomotor activity counts (crossing a dividing line in one of the squares) were performed automatically with the computer connected to the video-camera. Motion activity was estimated as the cumulative number of visits to each square during a 3-min test period.

Orientation was tested by using a 3 cm wide by 60 cm long board inclined at 45 degrees, and a 10 cm wide by 40 cm long staircase inclined at 60 degrees. The ability to orient was measured as the time spend on the board or on the staircase .

5. TISSUE PREPARATION AND IMMUNOHISTOCHEMISTRY (I-V)

5.1. Tissue preparation

Depending upon the further tissue preparation, rats at PD5, PD10, PD15, PD20, or PD40 were: a) sacrificed by decapitation and their brains rapidly removed, frozen on dry ice, and kept frozen at -70°C until sectioned (III-IV); or b) anaesthetized with chloral hydrate (350 mg/kg, i.p.) and the systemic circulation rinsed by cardiac perfusion of 0.9% saline at room temperature, followed by perfusion with fixatives, as specified below.

5.2. Localization of noradrenergic terminals (I-V).

In each experimental trial, a few control and 6-OHDA-treated rats were perfused with ice-cold 4% paraformaldehyde / 0.1% glutaraldehyde in 0.1 M phosphate buffered saline (PBS, pH 7.4) and postfixed with the same fixative for 2 h, washed in PBS, stored overnight in 15% sucrose in the same buffer, and cut into 10-12 µm thick cryosections with a MICROM cryostat (Heidelberg, Germany). NA neurons and terminals were

visualized by immunocytochemistry employing mouse monoclonal tyrosine hydroxylase antiserum (TH) (INGSTAR) diluted 1:1000.

5.3. Localization of GABA neurons and $\alpha 1$ and $\alpha 6$ subunits of the GABA_A receptors (I, III, IV)

In order to visualize GABA neurons and terminals, the rats were perfused with a 1% paraformaldehyde, 0.34% L-lysine, 0.05% sodium m-periodate in 0.1 M PBS, pH 7.4. The brains were postfixed with the same ice-cold fixative for 2 h, washed in PBS, stored in 15% sucrose in the same buffer overnight, and cut into 10-12 μ m thick cryosections. The antiserum used was mouse monoclonal glutamate decarboxylase (GAD-6) (Boehringer Mannheim) diluted 1:1000, which labels strongly synaptic buttons and fibers of GABAergic cells and less intensely their cell bodies (Chang and Gottlieb 1988).

To visualize rat $\alpha 1$ and $\alpha 6$ subunits of the GABA_A receptors, cryostat sections of unfixed frozen brains from 8 experimental and 8 control animals at each time point (PD10, PD15, PD20) were fixed with ice-cold 4 % paraformaldehyde (30 min), washed in PBS, and used for immunohistochemical staining. The antisera used were affinity-purified rabbit polyclonal antisera to rat $\alpha 1$ (1:100) and $\alpha 6$ (1:300) subunits of the GABA_A receptor (Killisch et al 1991).

5.4. Localization of microglial and Bergmann glial cells (II, V).

The cerebella were removed immediately after decapitation and fixed with Bouin's solution (Pudney and Anderson 1995) overnight, immersed sequentially for 24 h in 50% and then 70% ethanol to remove picric acid, dehydrated, embedded in paraffin, sectioned at 5 μ m, and mounted on glass slides coated with poly-L-lysine.

Microglial cells were visualized by lectin cytochemistry using peroxidase-labeled *Griffonia simplicifolia* agglutinin (Sigma). To label Bergmann glial cells, rabbit polyclonal antiserum to glutamate transporter GLAST (diluted 1:1000, Rothstein et al 1994) and rabbit polyclonal antiserum to diazepam binding inhibitor (DBI, diluted 1:4000, Alho et al 1990) were used.

5.5. Visualization of antigen-antibody complexes and verification of immunohistochemical specificity (I-V).

Immunohistochemical staining was conducted with the VECTASTAIN ABC Mouse IgG Kit. Antigen-antibody complexes were visualized by incubation with a 3,3'-diaminobenzidine (Sigma, 0.2 g/l in 0.1 M Tris-HCl, pH 7.4) and hydrogen peroxide (0.001%) for 5 min at room temperature. Immunohistochemical specificity was verified by incubating the sections with preimmune sera instead of primary antisera and by preabsorbing antisera with the corresponding peptides. No specific reaction was observed after these incubations.

5.6. Image analysis (I - V)

Brain sections were analyzed in a Nikon Microphot-fxa by “Image Pro Plus” analyzer. The mean gray value of the preimmune control sections was used as the background value. On the stained sections, the cells exceeding this value were considered immunopositive. The intensity of staining was estimated in arbitrary units of optical density. The number of labeled cells or terminals (density) was calculated from six control and six 6-OHDA-treated animals at every time point. For each animal, cells in the corresponding brain regions were counted separately from four different 1000 μm^2

areas in three consecutive sections (25 μm distance) and expressed as the mean of all these counts per 1000 μm^2 .

5.7. Morphometric analysis

Four cerebellar sections of each time point, consecutive to those used for immunohistochemistry, were stained with hematoxylin-eosin for morphometric analysis. The density of granule cells was calculated from 1000 μm^2 areas of lobules VII - X (paleocerebellum) and lobules V-VII (neocerebellum).

6. ELECTRON MICROSCOPY

The standard procedures were employed to prepare cerebella for electron microscopy. Briefly, the brains were fixed in 2.5% glutaraldehyde; declive (lobule VIb) were separated from the cerebellum, post-fixed with ice-cold 2% osmium tetroxide in 0.1 M PBS (pH 7.4) for 1 h, dehydrated in ethanol, embedded via propylene oxide in capsules with Epon 812 (Ladd Research Industries: Burlington, VT), and cut into ultra-thin sections with an Ultratome III (LKB; Rockville, MD). The sections were examined with a JEOL (JEM-1200 EX) electron microscope.

7. IN SITU HYBRIDIZATION AND BINDING STUDIES

7.1. Determination of GABA_A receptor subunit mRNAs (III, IV)

Antisense oligonucleotide probes were used for the determination of $\alpha 1$, $\alpha 2$, $\alpha 5$, $\beta 2$, $\beta 3$, and $\gamma 2$ subunit mRNA expression as specified and described by Wisden *et al.* (1992). To determine the mRNA expression of the $\alpha 6$ subunit, the oligonucleotide probe described in Korpi *et al.* (1994) was used. Probes were 3'-end labeled using a 30:1 molar ratio of α - ^{35}S -dATP (1136 TBq/mol; DuPont de Nemours) to an oligonucleotide (0.3 pmol) and terminal deoxynucleotidyl transferase (Boehringer Mannheim, Germany).

Hybridization was performed on the air-dried sections overnight at 42°C. Labeled probes were applied to the sections, diluted in hybridization buffer [50 % formamide, 4 × SSC (1 × SSC: 0.15 M NaCl, 0.015 M Na-citrate), 10 % dextran sulphate, and 1 mM dithiothreitol]. Hybridization was followed by washing of the sections in 1× SSC at room temperature,

1 × SSC at 55°C for 30 min, 1 × SSC and 0.1 × SSC at room temperature for 3 min. The sections were dehydrated and air-dried before exposing to plastic ¹⁴C-standards onto Hyperfilm-β_{max} (Amersham, Buckinghamshire, U.K.) for 4 weeks. Signal specificity was confirmed by competition experiment in the presence of a 100-fold excess of an unlabelled probe, resulting in no more than background labeling.

7.2. Binding studies (IV)

The regional localization of *tert*-[³⁵S]bitylbicyclophosphorothionate ([³⁵S]TPBS) binding was performed autoradiographically as previously described in detail (Olsen, 1990; Korpi et al 1996). Briefly, after preincubation in an ice-water bath for 15 minutes in 50 mM Tris-HCl buffer supplemented with 120 mM NaCl (pH 7.4), serial cryostat sections were incubated in 6 nM [³⁵S]TBPS (DuPont de Nemours-New England Nuclear, Dreieich, Germany) for 90 min at room temperature using 750 µl liquid bubbles over sections on object glasses in a humid chamber. Nonspecific binding was determined with 10 µM picrotoxinin (Sigma) in [³⁵S]TBPS autoradiography. The incubation was followed by washing of the sections three times for 15 s in ice-cold 10 mM Tris-HCl buffer, pH 7.4, and distilled water, air-drying at room temperature, and exposing to Hyperfilm-β_{max} (Amersham, Buckinghamshire, U.K.) together with plastic ¹⁴C-standards for 4 weeks.

7.3. Data analysis

The autoradiographic and *in situ* hybridization films were evaluated using MCID M4 image analysis devices and applications (Imaging Research, St. Catharines, Canada). The grain densities for studied brain regions of each animal were averaged from 32 measurements (8 measurements x 4 sections). The standards exposed simultaneously with brain sections were used as a reference with the resulting binding values converted to radioactivity levels estimated for gray matter areas (MBq/kg for ^{14}C).

8. STATISTICS

Original data from each experimental trial was obtained from groups separated according to age and treatment/control. Not less than 10 estimations were obtained from each experimental animal at every time point. All results are expressed as mean values \pm S.E.M. The group differences were tested for significance and for interactions (of treatment x age) using analysis of variance (ANOVA) with repeated measurements and Student's *t*-test.

RESULTS

1. BEHAVIOR

There was no significant difference between the experimental (6-OHDA treated) and the control rats either in body weight or locomotor activity in home cage at any observed time point (PD0, PD5, PD10, PD15, PD20, PD40).

1.1. “Open field” test (I)

Starting from PD10 the animals whose eyes were open, were tested daily in the "open field" box. The locomotor activity of the 6-OHDA treated rats did not differ from that of the control rats. The patterns of the open field exploration were, however, totally different. At PD10 the control rats moved around the central initial point, and began to move gradually out from the central point in bigger and bigger circles until they were covering the whole field (at PD15). At PD 20, they started randomly visiting every square in the open field. 6-OHDA treated animals escaped from the open area and moved to the corners or near the walls. At PD10 these animals moved in one direction from the central point; at PD15 they preferred to stay in a certain corner of the field; and finally at PD20 they usually ran only to locations near the walls. However, at PD40 the difference in movement between control and treated rats disappeared.

1.2. “Orientation” test (I)

The orientation was tested at PD20 by placing the rats on the inclined board or inclined staircase. When placed in the middle of the board, with head up, the control rats turned around and easily went down thus reaching the floor within 16 ± 5 s. 6-OHDA treated rats showed poor orientation ability and remained in the middle of the board throughout the period of observation (3 min) or slipped down after 62 ± 24 s. When

placed on the staircase, the control rats quickly turned around and came down after 24 ± 8 s. 6-OHDA treated rats remained motionless and did not find their way down during the 3 min observation period.

2. THE NORADRENERGIC INNERVATION OF THE CEREBRAL CORTEX AND CEREBELLUM (I-V)

In order to confirm the degree of degeneration for the NA terminals, in each experimental trial, the control and 6-OHDA-lesioned brains were stained with TH antiserum at PD10, PD15, and PD 20.

In control animals, the visual and somatosensory areas of the cortex and the hippocampus had the mature pattern for the distribution and the density of NA terminals at PD10. This pattern did not change significantly at PD40 (unpublished data). In the dorsal prefrontal cortex, the number of NA terminals increased by half from PD10 to PD15, and did not change in the older animals.

In 6-OHDA-treated animals, a 73-82% reduction in the number of NA terminals was observed in layers I-III of the dorsal prefrontal cortex at PD 15, and no recovery was observed at PD20 (I). In the visual and somatosensory areas of the cortex and the hippocampus of treated animals the number of NA terminals at PD 10 was reduced to 24-35% of the levels in the corresponding control area with no recovery at PD40 (unpublished data).

In control cerebellum, at PD10 the dense NA innervation was observed in the white matter, the granule cell layer, and the transient EGL (layer of immature granule cells). At PD15, the Purkinje cell layer had the densest NA innervation, which persisted at PD20-PD40. Along with granule cell migration, the density of NA innervation in the molecular

layer decreased, while in the granule cell layer there were no significant changes in NA terminal density at PD40 as compared to PD10.

In 6-OHDA treated cerebellum, few NA terminals were still labeled in the white matter at PD 10. There was no labeling at PD15 in the cerebellar cortex (Fig.1 B). NA terminals reappeared in the granule cell layer of paleocerebellum but not in neocerebellum at PD20. The normal pattern of NA terminals was observed in all areas of cerebellum at PD40 (unpublished data).

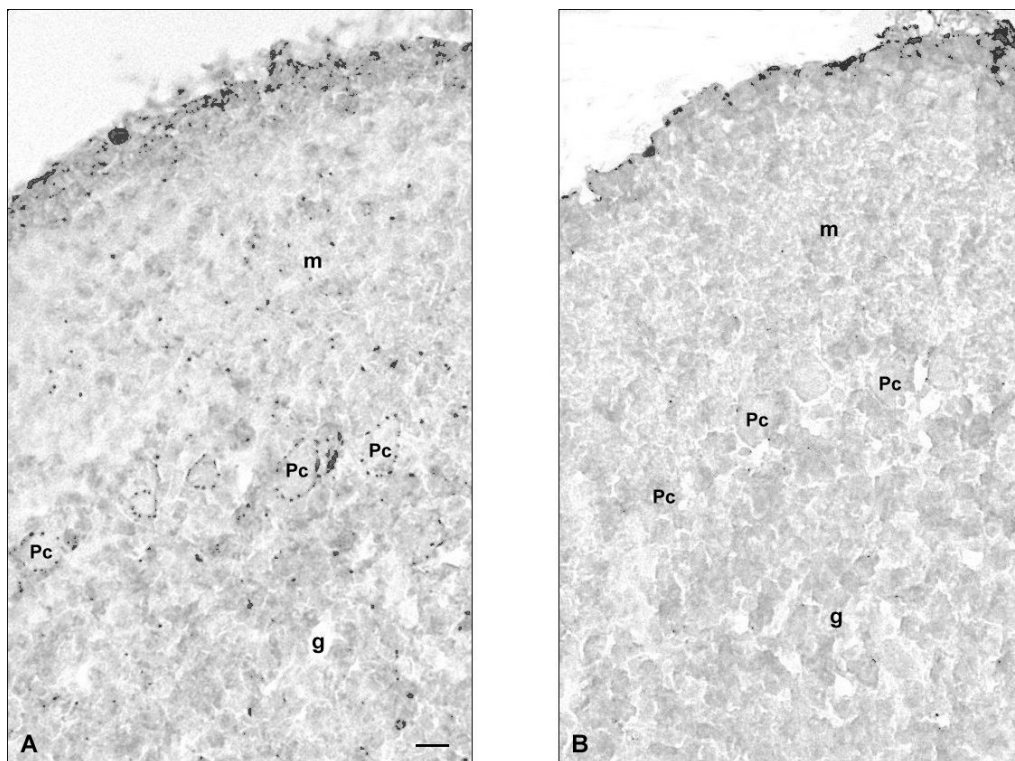


Fig.1. TH- LI in neocerebellum at PD 15 (Bar 20 nm)

A - In the control brain, the numerous TH-positive NA terminals are present in the molecular (m) and granule (g) layers and around Purkinje cells (Pc).

B - TH-positive NA terminals are absent in 6-OHDA treated cerebellum.

3. ALTERATIONS IN THE CEREBELLUM

3.1. General morphoanatomical structure (II, III)

The morphoanatomical structure of the cerebellum in 6-OHDA-treated rats (PD10, PD15, PD20, and PD40) was observed in hematoxylin-eosin stained semi-thin sections. The neocerebellum (lobuli VI, VII, VIII,) was smaller than that in the control animals, and the secondary foliation was deficient or absent (II). Morphometric analysis of sections showed that in the neocerebellum the total area of the granule cell layer of 6-OHDA-treated animals was $22\% \pm 6\%$ smaller than that in the control brains at all time points. However, there was no difference in the granule cell density.

3.2. Immunohistochemistry of Bergmann glial cells (II, V)

The Bergmann glial cells were identified immunochemically by GLAST and DBI polyclonal antisera. The reduction in the density of GLAST-LI and DBI-LI Bergmann cells in neocerebellum of 6-OHDA-treated animals was significant at PD 15 and PD20 (II). The Bergmann glial cells were shielding the Purkinje cells in the control cerebella. In lesioned neocerebellum, Purkinje cells were seldom surrounded by Bergmann glia, and Bergmann cells were often situated on the border between the granular and molecular cell layers (V). GLAST- and DBI-immunostaining of Bergmann cells in neocerebellum was significantly less intensive ($p < 0.001$) in lesioned cerebella than in control ones (V). There were no differences in paleocerebellum (lobulus II).

3.3. Electron microscopy of Bergmann glial cells (II)

Neocerebellum from 6-OHDA treated and control rats was examined in the electron microscope. The protoplasm of Bergmann glial cells covered the Purkinje cell bodies in

the control samples. The number of Bergmann glial cells was equal to 3 ± 1 cells in the cross-sections through the body of in the control brain.

Purkinje cells in the lesioned cerebella were not shielded by Bergmann glia protoplasm. The protoplasmic processes of Bergmann glia extended around neighboring cells in the control cerebella. The protoplasm of altered Bergmann glia was much less spread around the neighboring cells than in the control samples and the nuclei were not round but irregular in shape after neonatal 6-OHDA treatment. Bergmann glia cells in lesioned cerebellum often were found in the granular layer away from the Purkinje cells.

3.4. Immunohistochemistry of GABA_A receptors (III)

The immunostaining of $\alpha 1$ - and $\alpha 6$ -subunits of the GABA_A receptors was performed on the cerebella of 6-OHDA treated and control animals from PD10, PD15, PD20, and PD40. In the granular and molecular layers of the control cerebella, $\alpha 1$ - and $\alpha 6$ -subunit staining was weak at PD10. Positive immunoreactivity was not detected in 6-OHDA-treated cerebella. At PD15 intensive $\alpha 1$ -immunolabelling was observed in the Purkinje cell layer and in the granule cell layer of the control cerebella, but in the lesioned cerebella about 50% of the $\alpha 1$ -immunopositive cells were located in the molecular layer. The immunostaining with $\alpha 6$ subunit antiserum resulted in labeling of the cells only in the granule cell layer. The intensity of $\alpha 6$ -subunit immunostaining was three times less in 6-OHDA-treated animals than in the controls. At PD20, the intensity of immunostaining in the treated animals was one half of that in the control animals. At PD40 there was no difference between the control and lesioned neocerebella in the immunostaining for $\alpha 1$ - and $\alpha 6$ -subunits of GABA_A receptors.

3.5. The expression of GABA_A receptor subunit mRNAs (III)

In situ hybridization for the determination of $\alpha 1$, $\alpha 2$, $\alpha 5$, $\alpha 6$, $\beta 2$, $\beta 3$, and $\gamma 2$ subunit mRNA expression was performed with antisense oligonucleotide probes in the cerebella of 6-OHDA treated and control animals at PD5, PD10, PD15, PD20, and PD40. In the neocerebellum of treated rats, $\alpha 6$ subunit mRNA expression was reduced two-fold at PD10 and 11-fold at PD15 relative to levels in the corresponding controls. At older ages the difference became smaller: there was a 6-fold reduction at PD20 and only a 16% reduction at PD40. Significant reductions in $\alpha 1$ mRNA was detected at PD10, PD15 ($P < 0.0001$), and PD20 ($P < 0.005$). The reduction in $\gamma 2$ mRNA was significant at PD5 and PD10 ($P < 0.001$). $\beta 2$ subunit mRNA expression was not diminished (III).

3.6. Expression of lectin staining in 6-OHDA treated cerebellum (V)

The amount of small amoeboid shaped microglia was equal in the control and lesioned brains at PD5. At PD10, after 6-OHDA treatment, lectin-positive monocytes and activated microglia were found in the cerebellar cortex and medulla. The number of lectin-positive microglia was significantly increased in the cerebellar medulla and in the granule cell layer. The transformation of microglia from resting to activated cells occurred in the neocerebellum but not in the paleocerebellum. The intensity of lectin labeling in activated microglia was increased especially in the cells located in the granule cell layer. At PD20, the microglial cells in treated animals were mainly transformed back into resting ramified cells, and there was no difference from the control cerebella.

PCP treatment caused a marked activation of microglia in the perivascular area and in the cerebellar medulla. The lectin staining in the cerebellar medulla was three times more intense than in the corresponding controls.

4. ALTERATIONS IN THE FRONTAL CORTEX

4.1. Immunohistochemistry of GABAergic terminals (I)

GABAergic terminals were visualized using GAD antiserum. In the dorsal prefrontal area of the cortex, the density of terminals and the intensity of GAD in 6-OHDA treated animals at PD10 - PD20 decreased significantly (43-55%) relative to the control animals of the same age. In the visual and somatosensory areas, there were no differences between 6-OHDA treated and control animals.

4.2. Immunohistochemistry of GABA_A receptor subunits (IV)

In the frontal cortex of 6-OHDA treated rats at PD 10, the staining for $\alpha 1$ was negative, but a weak positive $\alpha 1$ -IR was observed in the majority of cells in control rats. At PD 15 the number of $\alpha 1$ -IR positive cells increased in the controls; it was lower in the treated animals, especially in cortical layers II-III where the number of $\alpha 1$ -IR positive cells was half of that in the controls. At PD 20 the difference between control and lesioned cortexes was diminished, and there were no differences at PD 40. Hematoxylin-eosin counterstaining of the same brain regions revealed no difference in the cell numbers between the control and lesioned brains.

There was no difference in $\alpha 1$ -IR between hippocampi from control and 6-OHDA treated animals at any time point.

4.3. The expression of GABA_A receptor subunits (IV)

The expression of $\alpha 1$ and $\gamma 2$ subunits in the frontal cortex of treated rats was significantly lower during PD5 - PD20 than in the controls ($P < 0.0001$). The $\alpha 1$ signal in the upper layers of the cortex in 6-OHDA lesioned brains was one half of that in the

controls. The expression of $\gamma 2$ mRNA was significantly less in 6-OHDA lesioned cortexes at PD5 and at PD10 ($P < 0.0001$), but there were no differences at PD15 and PD20. At PD40 the mRNA levels no longer differed between control and treated animals.

6-OHDA treatment did not change the expression of any studied subunits in the hippocampus.

4.4. Binding study (IV)

Picrotoxin-sensitive [^{35}S]TBPS binding in the frontal cortex of the control animals was present at PD5 at the level of 4.0 ± 0.5 MBq/kg. During the first postnatal month, the signal gradually increased up to 296 ± 15 MBq/kg at PD40. In 6-OHDA treated animals the signal was not detectable at PD5. [^{35}S]TBPS binding density in the frontal cortex of the treated animals at PD10 was three-fold less than that in the controls (23 ± 8 MBq/kg vs. 62 ± 17 MBq/kg). By PD 15 the signal in the treated brains was about 20% less than the signal in the control brains (156 ± 22 MBq/kg vs. 181 ± 11 MBq/kg). There was no difference at PD 20.

- α_1 - reduced by 1/3
- α_6 - reduced 11-fold
- γ_2 - reduced by 1/3

DISCUSSION

I. THE NEUROTOXIC ACTION OF 6-HYDROXYDOPAMINE

As shown before, the systemic administration of the catecholaminergic uptake blocker 6-OHDA to neonatal rats causes the degeneration of NA terminals in the neocortex and cerebellum (Sachs and Jonsson 1975, Lovell 1982). The mechanism behind this phenomenon is not clear. 6-OHDA inhibits the activity of mitochondrial complex I (Glinka et al 1998), which causes oxidative stress, and may lead to the cell death. In addition, 6-OHDA undergoes spontaneous autoxidation that proceeds by a free radical chain reaction involving the superoxide anion radical and produces the corresponding chromogen (6-hydroxydopamine quinone) and hydrogen peroxide (Padiglia et al 1997; Super et al 1997). The products of 6-OHDA autoxidation react nonspecifically with the neuronal structures and eventually destroy NA terminals.

Differences in uptake efficiency underlie the selectivity seen in the destruction of NA terminals. The efficiency is about five times higher in NA terminals compared to DA terminals (Molina-Holgado et al 1993). 6-OHDA uptake into target cells can be prevented by desipramine, a blocker of neuronal uptake-1 (Sachs and Jonsson 1995). This specific destruction of NA terminals has been used widely in the studies of the peripheral and cortical neuronal plasticity (Kostrzewa and Jacobowitz 1974, Felten et al 1982, Gustafson and Moore 1987), but little attention has been focused on the cerebellum and glial cells.

2. METHODOLOGICAL CONSIDERATIONS

2.1. Drug application.

In the present study, 6-OHDA application was performed without any pre-treatment with NA-uptake blockers (i.e. desipramine) or DA-uptake blockers (i.e. amfolenic acid) which might have produced preferential lesioning for NA terminals or DA terminals (Luthman et al 1989, 1997). For that reason, we can not exclude the involvement of DA-lesion in the observed behavioral alterations. However, previously published data confirm that systemic 6-OHDA application selectively affects NA-terminals of the locus coeruleus with minor or no effects on the DA-system. As far as the cerebellum is concerned, it does not receive DA innervation and only NA terminals are present there (Jacobson 1978).

2.2. Behavioral models.

Two space-orientation tests were developed and performed to estimate the behavioral changes of rat pups after 6-OHDA treatment. In the case of the adult rats, the behavior in the open field is traditionally analyzed by counting such behavioral elements as “scraping”, “self-grooming”, or “rearing” (Thiel et al 1998). This approach is impossible for the rats at PD10-PD15 because at this age the behavioral elements, listed above, are not yet present in the rat behavior (Geisler et al. 1996, Piggins and Merali 1992). In the present study, a new method for the evaluation of the open field behavior was designed as the calculation of the cumulative number of visits to each square during the test period. Another new tool for studying the behavior of young rats was to use a staircase and an inclined board in an “orientation” test. One methodological factor that can contribute to the gradual decrease of the differences between control and 6-OHDA treated rats is the

repetition of the tests every day. The repetition can cause either habituation and learning; thus, the task cannot be considered equivalent to that used in a novel situation. As it has been shown earlier, learning ability is not destroyed after 6-OHDA lesion (Carli et al 1983, Hauber et al 1994). Other sources of interference, such as uneven lighting of the “open field” or the influence of the experimenter, were carefully controlled.

2.3. Cytochemical methods.

In the present experiments, several approaches were used to study the development of the rat brain after 6-OHDA neurotoxic lesion. Immunocytochemical and *in situ* hybridization studies were conducted to reveal the alterations in neuronal and glial cells. Immunocytochemistry is a well-established technique that is extremely useful in the cellular localization of target proteins. However, artifactual false staining may mimic specific immunostaining. In this study, the specificity of the antiserum was tested with preabsorption controls. When the antibodies used were not previously well characterized (GABA_A subunits), the immunostaining was controlled by omitting the primary antiserum and by performing the control staining and the staining with specific antiserum simultaneously. Quantitative evaluation of the reaction product formed by antigen-antibody complexes is problematic. Therefore, the evaluation of immunocytochemistry can be considered to be only semi-quantitative both in the case of the number of immunoreactive cells and the staining intensity. The intensity of the reaction product can be measured in arbitrary units when the conditions of staining are identical for all the compared samples.

In situ hybridization histochemistry is a widely used technique for studying mRNA expression in tissue sections. It allows both visualization of mRNAs at the cellular level

and quantitative analysis of changes in the mRNA levels. Performing quantitative analyses of the mRNA expression in the samples that have variable fixation and storage times, or on sections with variable thickness, may give false results. To minimize the possibility of unspecific signals and to reduce the background of reaction, we modified the standard protocol by using unfixed frozen brains and performing hybridization on freshly fixed frozen slides (Korpi et al 1994), where the thickness of tissue was carefully controlled. The resolution of *in situ* hybridization films was too low to distinguish the difference between cerebellar layers in neocerebellum at PD5-PD15. However, this was not a major problem in the study because the main changes among the GABA_A receptor subunits after 6-OHDA treatment occurred in the granule cells.

2.4. Ligand autoradiography.

It is not known how well autoradiographic conditions mimic the *in vivo* conditions and to what degree the *post-mortem* changes occur in receptor compositions. The results obtained from [³⁵S]TPBS binding did not produce the same pharmacological profile, as *in situ* hybridization for GABA_A receptor subunits. However, ligand autoradiography has been shown to be useful in studying the effects and location of the effects of various pharmacological substances, or when combined with recombinant receptor studies where determined subunit combinations are used (Korpi et al 1996).

3. ALTERATIONS IN THE FRONTAL CORTEX

Of all cortical structures, the frontal cortex is the last to mature (Altman 1969). In the newborn rat, the cortical layers are not yet recognizable (Marin-Padilla, 1970). It has

been shown that NAergic transmission is vitally important for the transformation of the neuronal and glial precursors to mature cells (Rowe et al 1993).

During cortical maturation, GABAergic interneurons of the upper layers are the last to migrate to their final destination: the timing of their migration is PD 3-5 (Drerer 1974). That coincides with the period of 6-OHDA application and subsequent NA elimination in our study. In the absence of a trophic NA influence via α -adrenoceptors (Kawaguchi and Shindou 1998), GABAergic neurons do not mature normally. This can explain the lack of GAD-IR in the upper layers (I-II) of the frontal cortex (I). The hippocampus and the visual and motor cortexes are more mature in the newborn rats. That is why the elimination of NA terminals did not cause any disturbance in GABAergic transmission there (I and IV).

It is well documented now that the developmental switch from $\alpha 2$ to $\alpha 1$ subunit in GABA_A receptor coincides with the establishment of synaptic connections (Fritschy et al 1994). Obviously, the absence of GABAergic neurons in the upper layers of cortex arrested the release of GABA, which in turn may cause the delayed appearance of $\alpha 1$ subunit.

It has been shown that systemic 6-OHDA application reduces the dendritic branching of pyramidal neurons in the rat cortex to such an extent that apical dendrites do not reach layers I-II of the cortex but remain located in layer III (Felten et al 1982). In the present study, the temporal reduction in the $\gamma 2$ subunit expression was found in cortical layers I-II (IV). The alterations in GABA_A receptor subunits were transient, and the normal level of mRNA expression was observed by PD 40. This can be explained by the observations that thalamocerebral and pontocerebral afferents enter the cortex during the first postnatal

month and release other monoamines which are known to be responsible for neuronal plasticity (Coyle 1982, Osterheld-Haas and Hornung 1996, Hohmann and Berger-Sweeney 1998).

4. ALTERATIONS IN THE CEREBELLUM

In rodents, the maturation of the cerebellum occurs mainly postnatally (Altman and Winfree 1977). The first two postnatal weeks are the crucial time for the development of the cerebellar cells (Duncan et al 1990, Seidler et al 1995). The neocerebellum is the most vulnerable to postnatal toxic influence, since at birth all types of neocerebellar cells are in proliferative stage (Jacobson 1978). The agents able to alter the schedule of germinal cell proliferation (e.g., alcohols, glucocorticoids, opioids) cause alterations in the development of neocerebellum (Lovell, 1982; Moore et al., 1999).

The terminals of NA neurons invade the cerebellum on PD2, form a transient dense network in the submeningeal area of the cerebellum during first two postnatal weeks, and establish the intensive innervation of Purkinje cells and Bergmann glial cells on PD5-PD15 (Verney et al 1982). The schematic presentation of normal cellular organization in cerebellum is given in Fig.2 A.

The elimination of NA terminals by 6-OHDA disrupts the normal development of rat cerebellum. The present study confirmed the earlier observations that the total area of neocerebellum is reduced after systemic 6-OHDA administration, the secondary foliation is not developed, and the number of granule cells in the neocerebellum is diminished by one third (Lovell 1982). The results of the present study also demonstrated that granule

cells immunopositive for $\alpha 1$ subunit of GABA_A receptor were abnormally located at PD15 in molecular layer of the 6-OHDA treated neocerebellum (III).

GABAergic transmission in the survived granule cells was transiently altered, the expression of $\alpha 1$ subunit mRNA in 6-OHDA treated neocerebellum at PD15 was 60% lower than the control values, and the expression of $\alpha 6$ subunit mRNA was up to 9 times lower than in the corresponding control samples. In the absence of a NA influence to granule cells, the $\alpha 1$ subunit can replace the $\alpha 6$ subunit in GABA_A receptors (Thompson et al 1996), and this may explain the non-equal reduction of the two subunits. The reduction in $\gamma 2$ subunit mRNA was most obvious at PD5-PD10, probably because of the intensive signal from $\gamma 2$ subunit mRNA on Purkinje cell dendrites (Khan et al 1996)

The observed changes in GABA_A receptors were reversible. It is possible that noradrenergic sprouting and the regrowth of NA terminals to the cerebellum, which occurs after PD20 (Gustafson and Moore 1987), enable the granule neurons to recover normal $\alpha 6$ and $\alpha 1$ subunit expression by PD40.

4.1. Alterations in glial cells

This is the first study demonstrating that Bergmann glial cells are abnormal in their location and structure and do not form intimate association with Purkinje cells after 6-OHDA treatment (see Fig.2 B). In the normal cerebellum, Bergmann glial fibers guide granule cells during their migration from the transient EGL through the molecular and Purkinje cell layer to the developing granule cell layer (Muller et al 1996). Granule cell migration occurs after extracellular Ca^{2+} release. Ca^{2+} is released from Bergmann glia in response to the stimulation of the glial adrenergic receptors (Kulik et al 1999).

Elimination of NA terminals abolishes the possibility of Ca^{2+} release and thus disrupts the normal migration of granule cells.

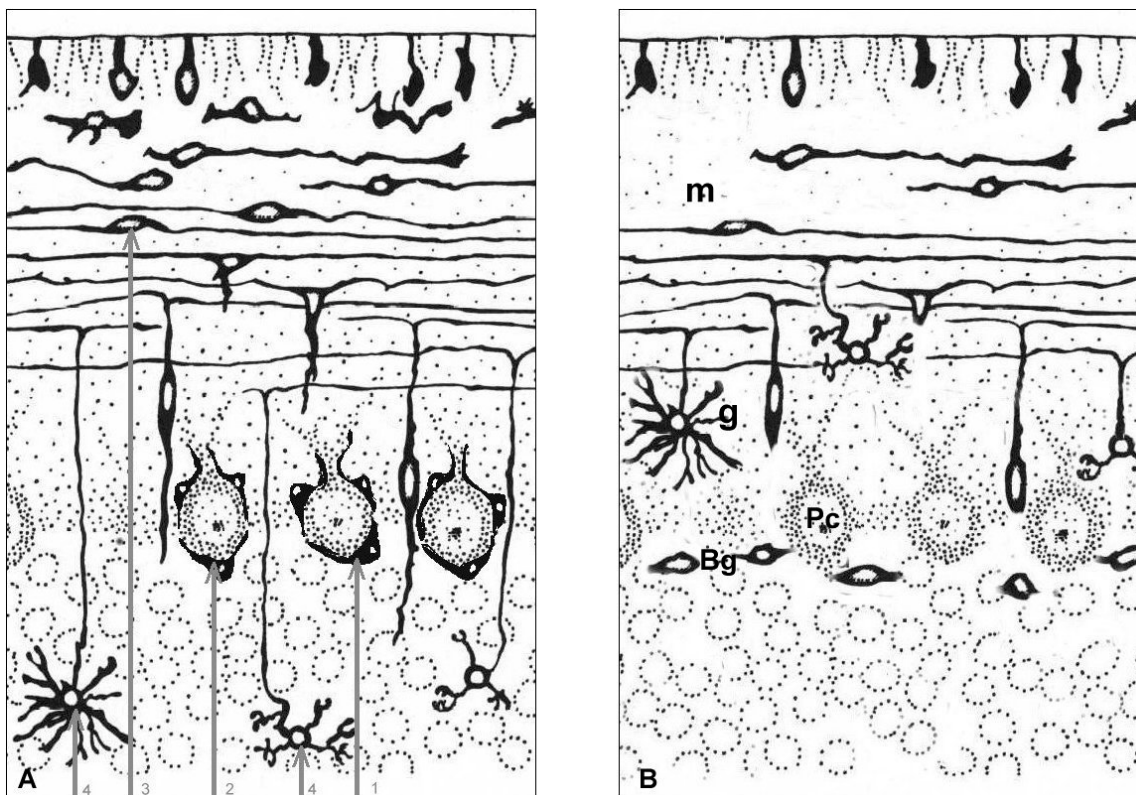


Fig. 2. Schematic presentation of the alterations in cerebellum after 6-OHDA treatment

A - In the control cerebellum NA terminals activate adrenergic receptors on Bergmann glial cells (1), Purkinje cells (2), premature granule cells (3) and mature granule cells (4).

B - In 6-OHDA treated cerebellum in the absence of NA terminals granule cells (g) do not pass Purkinje cell (Pc) layer, but remain in the molecular (m) layer; Bergmann glia (Bg) are diminished in their number and do not cover Purkinje cells.

After systemic application, 6-OHDA is transported to the brain by blood vessels. The presence of the neurotoxic substance in the blood vessels was shown by microglial activation. Activated microglial cells were found in the granule cell layer and in the

region of Bergmann glial cells. This microglial activation may be due to the degenerated granule cells and Bergmann glial cells. Interestingly, it has been proposed that this microglial activation after toxic free radical associated damage may also have cell protective action (Fujita et al 1998). This was not studied here, but requires further investigation.

5. ALTERATIONS IN BEHAVIOUR

The main alterations in behavior observed in 6-OHDA treated rats compared to their littermates were the avoidance of the open space in the “open field” test and the poor ability to orientate in the new environment. More detailed description of the locomotor activity for treated rats can be made in terms of the stereotyped behavior, when the animal constantly repeated the movement out of the corner and back there. Stereotypic behavior is typically observed in the situations of conflict or frustration. On the basis of the present knowledge, it is proposed that stereotyped activities gain strength because of the positive feedback effect of the sensory stimulation on their underlying control systems (Dantzer 1986). This feedback may provide the necessary tonic influence to the brain structures in the absence of NA terminals.

6-OHDA treated rats had disturbances in their ability to orient in three-dimensional space in a novel situation. The transient failure in the performance of “orientation tests” most probably occurred as a result of cerebellar malfunction. In the absence of Bergmann glial shield, unprotected Purkinje cells could be overloaded with synaptic connections (Castejon 1990). Insufficient functioning of GABAergic receptors in granule cells can contribute to the lack of spatial orientation in 6-OHDA treated rats.

SUMMARY AND CONCLUSIONS

Systemic administration of the catecholaminergic neurotoxin 6-OHDA to neonatal rats caused degeneration of NA terminals in the rat brain. Elimination of noradrenergic terminals by 6-OHDA disrupted normal development of the frontal cortex and cerebellum.

1. Compared to their littermates, treated rats had altered behavior demonstrated by poor ability in exploration and orienting in the new environment. This may be due to the dysfunction of GABA_A receptors during the first postnatal month.
2. Depletion of noradrenergic terminals resulted in a decreased number of GABAergic terminals and reduction in the expression of $\alpha 1$ and $\gamma 2$ mRNA of GABA_A receptor subunits in the frontal cortex.
3. Neonatal 6-OHDA administration temporarily suppressed the expression of $\alpha 1$ and $\alpha 6$ subunits of the GABA_A receptor in the granule cells of neocerebellum.
4. The Bergmann glial cells were abnormally located, structurally different, and free of intimate associations with Purkinje cells. Significant microglial activation was observed in the damaged areas.
5. It is possible that elimination of NA terminals disrupted the normal migration of granule cells. The invasion of microglial cells from the cerebellar medulla to the cerebellar cortex was most likely induced by the degenerating granule and Bergmann glial cells, but it may also protect the surviving cells against the toxic environment.

The unique possibility to selectively destroy noradrenergic neurons (preferentially nerve terminals) in the brain by systemic administration of 6-OHDA provides a valuable tool for studying psychopharmacological drugs. The data of the present study indicate that the neonatal elimination of trophic noradrenergic influences causes a delay in the development of GABAergic system in the frontal cortex and neocerebellum of the rat brain. Although these changes were transient, the results indicate that careful consideration should be given before using drugs causing changes in noradrenergic neurotransmission, such as clinically-used antidepressants (e.g., monoamine oxidase inhibitors), during pregnancy.

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ORIGINAL COMMUNICATIONS

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