NEURAL GROWTH - WITH SPECIAL EMPHASIS ON ADULT NEUROGENESIS AND THE EFFECT OF ANTIEPILEPTIC DRUGS

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To My Parents –
For your endless support
ABSTRACT

Neurons were for a long time thought to not renew themselves. In the 1960ies the phenomenon of neurogenesis was discovered, but it was not until 1998 that neurogenesis was demonstrated in humans. In this thesis neurogenesis was studied using a unique genetic mouse model (mceph/mceph), with postnatal epilepsy and excessive brain growth, due to a truncated Kv1.1 subunit. The model was used to learn more about how a channelopathy can disturb hippocampal neurogenesis, leading to hyperplasia, and how this can be treated. First, the expression and trafficking of the truncated potassium ion channel Kv1.1 was described to reveal its molecular nature. It was shown that the defective Kv1.1 does not form functional channels and moreover has the potential to render other potassium channel subunits non-functional. Even though lack of Kv1.1 is enough for excessive hippocampal growth, the defect Kv1.1 peptide worsens the epileptic condition by blocking additional Kv1 subunits. Cells have previously been shown to be enlarged in the hippocampus of this mouse. In this thesis a doubling in number of neurons and astrocytes was demonstrated by stereology. The increase in number of neurons was due to increased neurogenesis and altered apoptosis. To identify transcripts involved in the overgrowth of the mceph/mceph hippocampus a genome-wide screen for transcripts expressed at different levels in mceph/mceph versus wild type was performed. The following genes, involved in regulation of cell number, were verified as differentially regulated in mceph/mceph; NPY, Penk, Fjx1 and Vgf. Previously it was shown that oral treatment with the antiepileptic drug CBZ protect mceph/mceph mice from developing enlarged hippocampus. This thesis shows that all hippocampal regions studied were protected from overgrowth and that the number of both neurons and astrocytes were normalized despite ongoing severe seizures. Transcripts potentially involved in the protection against the hippocampal overgrowth and hyperplasia were identified based on different expression levels in a microarray analysis. Verified genes include Mlc1, Sstr4, ApoD, Ndn, Aatk and Rgs2. These transcripts have a proposed function in proliferation, differentiation and/or apoptosis. Finally, an analysis of the effect of AEDs in utero, with focus on the head size of the newborn, was conducted on a large population-based Swedish cohort. This study revealed that the use of CBZ and VPA is increasing despite reports of malformations and growth retardations of the baby. Furthermore, CBZ and VPA monotherapy significantly reduced the head circumference (HC) and AED polytherapy increase the rate of small HC (> 2 SD). The implications of a smaller head on the development of the child is uncertain but should be explored. CBZ mono- and polytherapy significantly reduced gestational age (GA) and there was a tendency for clonazepam and gabapentin monotherapy to reduce GA. The relevance of the reduced pregnancy duration is not clear but indicates a need for further studies in order to optimize treatment regimes for epileptic pregnant women.
LIST OF PUBLICATIONS


III. Almgren M, Persson B, Lavebratt C. Carbamazepine protects against neuronal hyperplasia and abnormal gene expression in the megencephaly mouse. Submitted to Neurobiology of Disease

IV. Almgren M, Källén B, Lavebratt C. Population-based study of antiepileptic drug exposure in utero - influence on head circumference in newborns. Submitted to Epilepsia

"Wisdom begins in wonder"
Socrates
ADDITIONAL PUBLICATIONS

I. **Almgren MA, Henriksson KC, Fujimoto J, Chang CL.**

II. Henriksson KC, **Almgren MA, Thurlow R, Varki NM, Chang CL.**

III. **Almgren M, Schalling M, Lavebratt C.**
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LIST OF ABBREVIATIONS

Aatk  apoptosis-associated tyrosine kinase
AED  Antiepileptic drug
ApoD  apolipoprotein D
BDNF  Brain derived neurotrophic factor
Bhlhb5  basic helix-loop-basic helix 5
BMI  Body mass index
BrdU  bromodeoxyuridine
CBZ  carbamazepine
CCK  cholecystokinin
CNS  central nervous system
CV  coefficient of variance
DCX  doublecortin
DG  dentate gyrus
EA1  episodic ataxia 1
ER  endoplasmic reticulum
Fjx1  four jointed box 1
GA  gestational age
GABA  gamma-aminobutyric acid
GBP  gabapentin
GCL  granular cell layer
GFAP  glial fibrillary acidic protein
Grin1  G protein-regulated inducer of neurite outgrowth 1
HC  head circumference
HEK293A  human embryonal kidney cell 293A
HRP  horseradish peroxidase
IGF-1  insulin-like growth factor-1
ISH  in situ hybridization
kDa  kilo Dalton
mTLE  medial temporal lobe epilepsy
MeOH  methanol
Mlc1  megalencephalic leukoencephalopathy with subcortical cysts 1
Ndn  necedin
NeuN  neuronal nuclei
NPY  neuropeptide Y
NMDA  N-methyl-d-aspartate
NSC  Neural stem cell
PBS  Phosphate buffered saline
PCR  Poly chain reaction
PSA-NCAM  polysialylated neural cell adhesion molecule
Rgs2  Regulator of G-protein signaling 2
SD  standard deviation
SDS  standard deviation score
SDS  sodium dodecyl sulfate
Neural growth – with special emphasis on adult neurogenesis and the effect of antiepileptic drugs

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<table>
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<tr>
<th>Abbreviation</th>
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<tr>
<td>SE</td>
<td>status epilepticus</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of mean</td>
</tr>
<tr>
<td>SGZ</td>
<td>subgranular zone</td>
</tr>
<tr>
<td>Sstr4</td>
<td>somatostatin receptor 4</td>
</tr>
<tr>
<td>SVZ</td>
<td>subventricular zone</td>
</tr>
<tr>
<td>TLE</td>
<td>temporal lobe epilepsy</td>
</tr>
<tr>
<td>TUNEL</td>
<td>terminal dUTP Nick End Labeling</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
<tr>
<td>Vgf</td>
<td>VGF nerve growth factor inducible</td>
</tr>
<tr>
<td>VPA</td>
<td>valporic acid/valporat</td>
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1 INTRODUCTION

The main focus of this thesis is on regulation of neural growth and brain size. It covers multiple fields and the project started with an analysis of a mouse model with enlarged brain; the megencephaly mouse. This mouse has a uniquely two-fold enlarged hippocampus and displays severe epilepsy due to a malfunctioning ion channel. To understand the mechanism of this ion-channelopathy causing epilepsy and brain enlargement it is important to know the nature of the basic molecular defect, which we explored first. Thereafter we investigated the number of cells in hippocampus, one of the structures most enlarged, in this mouse and soon realized it had an unusually high production of new neurons (neurogenesis). Neurogenesis is commonly enhanced in epilepsy, but the numbers of neurons are normally balanced by cell death (apoptosis) to not result in overgrowth. In an attempt to protect the brain from overgrowth we treated the mice with the antiepileptic drug carbamazepine – a drug also known to reduce head size in utero. Successfully the size of the brain and the number of neurons got completely normalized, despite remaining seizures! Curious of which genes that regulated the number of neurons before and after treatment, we carried out a whole genome expression analysis, which revealed a number of promising candidates. The process of neurogenesis is still relatively unknown and any key to its regulation are of high interest, one day possibly contributing to the development of treatment for neurodegenerative diseases or depression. Enlightened by the effect of carbamazepine on brain size in our mouse model we also speculate that it could be used to treat certain brain overgrowth syndromes in humans. Finally, we were interested to study carbamazepine’s effect on growth in human featus and performed an epidemiological study on the effect of antiepileptic drugs (AEDs) during pregnancy. This revealed that the most commonly used AEDs, among them carbamazepine, caused reduced head circumference and/or reduced the pregnancy duration. Interestingly the use of AEDs during pregnancy is increasing in Sweden. In summary, our aim was to investigate how the channelopathy in the megencephaly mouse regulates neural growth. This thesis discusses brain growth, neurogenesis, apoptosis and the effect of antiepileptic drugs on these.
1.1 NEURAL GROWTH AND THE NEURON

The mammalian embryo has three germ layers that develop into organ, bone, muscle or neural tissue; mesoderm, endoderm and ectoderm. Development of the human brain starts approximately three weeks after conception when signals to the ectoderm tissue initiate formation of the neural plate which folds to the neural tube. The upper part of the neural tube thickens and shapes the immature forebrain, midbrain and hindbrain. Neurons are initially produced in the neural tube and migrate to their final destination in the brain to form the different brain structures. Only half of the amount of neurons generated during development survives into adulthood, the rest is “cleared” away by apoptosis – programmed cell death. The mammalian brain consists of neurons and glia cells, which both have several subtypes. The neuron is specialized to transmit information to other nerve cells and does so by electric impulses and release of neurotransmitters (Fig. 1). The glia cells (astrocytes, microglia and oligodendrocytes) are mainly for support and maintenance. Most of the neurons are made prenatally and in humans the maturation of some brain structures continues during the first months in life. New neurons are generated throughout life in the mammalian brain from neural stem cells (NSC), which are pluripotent and can differentiate into astrocytes and oligodendrocytes (Altman and Das, 1965; Cameron et al., 1993; Gage, 2000; Kaplan and Hinds, 1977; Kuhn et al., 1996). In normal development, neuron generation is regulated so that the correct number of cells is produced in the proper place at the appropriate time. Dysfunction of this mechanism can cause several pathologies; where loss of neurons is found in neurodegenerative diseases like Parkinson’s and Alzheimer disease. Too many neurons or glia cells can cause overgrowth syndromes like cancer and megalencephaly (described later and see Appendix I, review by Almgren et. al.). (Volpe 2001)
Figure 1. A neuron and its primary function; transmitting signals along its axon, via the synapse to other neurons. Adapted from Brain Facts, Society for Neuroscience.

1.2 HIPPOCAMPUS

Structure
The hippocampus is the primal area of investigation in this thesis due to the fact that it is one of two areas in the brain where new neurons are generated throughout life and it is also a major site for epileptogenic activity. The hippocampus is located inside the temporal lobe and is part of the limbic system. It is involved in short term memory formation, learning and spatial navigation. The name "hippocampus" comes from the Greek word for seahorse (hippocampus) and is derived from its curved shape in coronal sections of the brain. The hippocampus consists of subfields of distinguishable anatomy; the dentate gyrus (DG), hilus (considered part of the dentate gyrus), and CA1-CA3. The CA1, CA2 and CA3 fields make up the hippocampus proper, also called Ammon’s horn (Lowry et al., 1954; O’Keefe and Nadel, 1978) (See Fig. 2).
Neural growth – with special emphasis on adult neurogenesis and the effect of antiepileptic drugs

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consists mainly of granule neurons, whereas the CA1-3 consists mainly of pyramidal neurons. The pyramidal cell layer is subdivided regarding to the morphology of neurons; CA1 contains small pyramidal neurons and CA2/CA3 large pyramidal neurons. These regions are also distinguished by their unique patterns of integration to the circuit. CA2 represents only a very small portion of the hippocampus and its presence is often ignored regarding hippocampal function. In our studies CA2 has been studied together with CA3. Interestingly, this small region seems extraordinarily resistant to situations that normally cause cellular damage, such as epilepsy (Nakamura et al., 1992). This is in contrast to CA1, which is extra sensitive to damage like hypoxia and epilepsy (Kawasaki et al., 1990).

Fig. 2. Hippocampus subregions and connectivity. Perforant pathway (pp) (red) connects entorhinal cortex (EC) and neurons in dentate gyrus (DG) and CA1. DG neurons projects to CA3 via the mossy fibre pathway (mfp) (blue). CA3 projects to CA1 via the Schaeffer collateral pathway (Scp) (green). Or, stratum oriens; Py, pyramidal cell layer; Rad, stratum radiatum; Lmol, stratum lacunosum moleculare; Mol, stratum moleculare; GrDG, granular layer of dentate gyrus; PoDG, polymorph layer of dentate gyrus; CA1, field CA1 of hippocampus; CA2, field CA2 of hippocampus; CA3, field CA3 ; rf, rhinal fissure

Connectivity

Information from the visual, auditory, and somatic associative cortices arrives first at the parahippocampal region of the cortex, and then passes through the entorhinal cortex and on to the hippocampus proper. The perforant pathway, which brings information primarily from entorhinal cortex, is generally considered the main source of input to the hippocampus (Naber et al., 2001; Witter, 2007). The DG receives input from neurons in layer II of the entorhinal cortex, and CA1 from layer III (Fig. 2). Within the hippocampus, DG neurons project mainly to CA3, the mossy fiber pathway (blue), whereas CA3 neurons project to CA1 (Schaeffer collaterals, green). CA1 efferents
(axons) in turn project back to the entorhinal cortex, forming a loop (yellow). The subiculum is the most inferior component of the hippocampal formation and is the main output of the hippocampus. It is located between the entorhinal cortex and the CA1. It receives input from CA1 and entorhinal cortical layer III pyramidal neurons. (Fig.2)

Each of the subregions in hippocampus has a unique functional role in the information processing of the hippocampus, but to date the specific contribution of each region is poorly understood. Even so is the specific niche in dentate gyrus, where adult neurogenesis takes place. Significant molecular expression patterns have been found distinguishing the different fields, but more research is required to elucidate their function as well as regulation and involvement in memory formation and learning (Zhao et al., 2001).

1.3 ADULT NEUROGENESIS

Neurogenesis is the formation of a new neuron. It was first believed that no neurons were generated in the brain after birth, and that the ones lost were never replenished. This dramatic view has changed gradually over the years initiated 1965 by the first evidence of adult neurogenesis in mammals (Altman and Das, 1965). During the last decade the field of neurogenesis has expanded fast and in 1998 a Swedish group proved generation of new neurons in the adult human brain (Eriksson et al., 1998). Sites of neurogenesis in the adult mammalian brain includes sub granular zone (SGZ) of the dentate gyrus in the hippocampus (Fig. 3) (Altman and Das, 1965; Hinds, 1968; Kaplan and Hinds, 1977) and the subventricular zone (SVZ) of the lateral ventricles. From the latter area, newly generated neurons migrate via the rostral migratory system to the olfactory bulb (Lois and Alvarez-Buylla, 1993). Olfactory learning, like odor discrimination, seems to be the key regulator in SVZ neurogenesis (Alonso et al 2006). In the hippocampal region the neural stem cells proliferate in the SGZ of the DG, at the border between the granular cell layer (GCL) and hilus (Cameron et al., 1993). The detailed mechanisms of SGZ and SVZ neurogenesis seem to differ a great deal, although they share some common regulators, reviewed by (Zhao et al., 2008). In this thesis the focus will be on hippocampal neurogenesis. In the SGZ a pool of progenitor cells is located, consisting of two types of neural progenitors, type 1 and 2. Distinct morphology and expression of molecular markers identifies them, type 1 has a radial process that spans the granular cell layer, reaching the molecular layer. Further they express nestin, glial fibrillary acidic protein (GFAP) and Sox2 (Fukuda et al., 2003; Garcia et al., 2004; Suh et al., 2007). Important to note is that the expression of GFAP, initially an astrocyte marker, does not make them similar to mature astrocytes. They divide consecutively, although with age at a slower rate, and give rise to mostly neurons but also to astrocytes and oligodendrocytes (Gage, 2000; Palmer et al., 2000). Type 2 progenitor cells have short processes and express nestin and Sox2. It is
debated whether type1 can give rise to type 2 cells. Type 2 cells have been reported
to be able to self renew and also to give rise to both neurons and astrocytes (Suh
2007). A third type of neural progenitor cell has been proposed, but in this thesis the
progenitors will be grouped and denoted neural stem cells (NSC). Neurogenesis
involves a series of consecutive steps; proliferation of precursor cells, migration,
differentiation into specific cell types, survival and integration in to the circuit (Fig 3).
Exactly how the progenitor cells are activated to proliferate is unclear, but influence of
the microenvironment is important. Extrinsic molecules expressed by neighboring
neurons or astrocytes like neurotransmitters, neurotrophins or growth factors seem
to regulate proliferation. For example, both vascular endothelial growth factor (VEGF)
and insulin-like growth factor-1 (IGF-1) are positive regulators of neurogenesis (Trejo
2001 from Parent). The Wnt-signaling pathway also induce proliferation of NSC (Lie et
al., 2005). γ-aminobutyric acid (GABA) and glutamate expression has on the other
hand been reported to reduce proliferation, but they induce differentiation,
promoting Neuro-D expression which is required for direction into neuronal and not
glial phenotype. Further GABA promotes migration of the immature neuron and
synaptic integration (Deisseroth and Malenka, 2005; Ge et al., 2006; Tozuka et al.,
2005). GABA is normally an inhibitory neurotransmitter, but NCS has elevated levels of
NKCC1, a chloride importer, which results in elevated levels of intracellular Cl⁻ this
makes GABA excitatory. This results in a reversed potential for GABA-induced currents
that is higher than the resting membrane potential during the first 2–3 weeks after the
birth of the new neuron. Thus, GABA initially depolarizes newborn immature neurons
in the adult dentate gyrus (Ge et al., 2006). GABA inhibitory and glutamate excitatory
inputs starts approximately after 3 weeks (Laplagne et al., 2006; Laplagne et al., 2007).
During differentiation the NSC gradually expresses overlapping markers of the neural
lineage, starting with doublecortin (DCX) and polysialylated neural adhesion molecule
(PAS-NCAM), followed by Calretinin, Calbindin and NeuN. The latter two continue to
get constitutive expressed in the mature neuron. Formation of dendritic spines and
axon starts already one week after birth and in the second week the immature
neurons are reported to form synapses with adult hilar and CA3 neurons. Although,
maturate of synapses continues for several weeks to achieve full complexity (Zhao
et al., 2008). The first three weeks of a new neuron is very critical, since the
integration and survival are determined during this phase. BDNF enhances survival of
immature neurons and sensory input is also an important factor (Rossi et al., 2006;
Tashiro et al., 2006). Approximately half of the newborn neurons die within two weeks
(Dayer et al., 2003). The time it takes for a cell to complete all steps of neurogenesis is
approximately seven weeks. Running and enriched environment induce hippocampal
neurogenesis in rodents, stress on the other hand has negative implications
(Kronenberg et al., 2003). The exact function of the new neurons in hippocampus is
not clear, but they appear to be important to manage a changing external
environment and to produce new temporary memories (Doetsch and Hen, 2005).
Fig. 3. SGZ neurogenesis is a process which can be divided into four steps, (1) Proliferation of NSC (turquoise), (2) differentiation into neuronal lineage, (3) migration into granular cell layer and (4) functional integration into the hippocampal network.

1.3.1 Neurogenesis vs. apoptosis
A balanced regulation of apoptosis and neurogenesis is seen in the adult rodent brain. The number of cells increases during the first year in life, giving rise to increased volume and density in the dentate gyrus (Bayer, 1982; Bayer et al., 1982). Although, no over-growth is seen, since the system eliminates excessive neurons and the ones failing to integrate to the circuitry (Buss et al., 2006; Oppenheim, 1991). The elimination of excess adult-generated neurons may be required for the maintenance of synapses and neuronal circuits to ensure efficient neural information processing. Over half of all new neurons in the DG die within 30 days, before maturation, double labeling with the apoptosis marker TUNEL and DCX reveals the immature phase (Biebl et al., 2000; Kempermann et al., 1997; Kuhn et al., 2005). Neurotrophic support is essential for the survival of immature neurons and BDNF seems to have an important role (Rossi et al., 2006; Scharfman et al., 2005). Signaling and synaptic activity are two other key factors for survival; signaling through the NMDA receptor plays an important role in neuronal survival during the time of the formation of dendritic spines and functional synaptic activity (Gould et al., 1994; Tashiro et al., 2006). Interestingly, enriched environment and special learning has increased survival of new DG neurons (Kee et al., 2007; Tashiro et al., 2007).

1.3.2 Neurogenesis and disease
Injury of the brain is known to increase neurogenesis in the affected area, as seen in stroke, brain trauma and seizures. Accumulations of new neurons at the site of injury can also be due to migration from the SVZ or SGZ as shown in several animal models, although evidence in humans are less (Jin et al., 2006; Nakatomi et al., 2002; Parent et al., 2006; Thored et al., 2007). Whether these newly generated neurons have a rescuing mechanism for self-repair or if they contribute to pathology is debated. In depression a lower neurogenesis and a reduced size of hippocampus is seen, in rodents and nonhuman primates (Kempermann et al., 2008; Perera et al., 2007; Reif
et al., 2006). In Parkinson’s patients a reduced cell proliferation is reported but in Alzheimer’s disease the findings are contradictory (Verret et al., 2007; Winner et al., 2007)

**Epilepsy**
In epilepsy groups of nerves signal abnormally causing seizures and/or sometimes convulsions and loss of consciousness. Over 50 billion people in the world are estimated to have epilepsy, which can be caused by brain injuries (trauma, infection, tumors or stroke), abnormal brain development or genetic predisposition. Research has identified over 30 genes causing epilepsy and some of them are involved in regulating the migration of new nerve cells. Mutations in these types of genes cause abnormal migration pattern in the developing brain with potential subsequent malformation resulting in epileptic foci. Other risk genes for epilepsy are those encoding ion channels, which regulate the firing pattern of the nerve cell. Mutations therein can give abnormal electric activity resulting in seizures. Many of the antiepileptic drugs downregulate or block ion channels to avoid over activity, see 1.7.

Seizures are divided in two types; generalized and partial. Generalized seizures arise when the excessive electric activity spreads over both brain hemispheres. One type of generalized seizure is the tonic-clonic seizure, involving convulsions and unconsciousness. A prolonged or multiple seizure activity without regaining consciousness is called status epilepticus and is associated with morbidity and mortality. In partial seizures the electrical disturbance is limited to a specific area of one cerebral hemisphere. Partial seizures can be classified as simple or complex. In simple partial seizures the patient experiences sensory and motor disturbances, but no change in consciousness. Complex partial seizures, which are the most common type, affect a larger area of the brain than simple partial seizures and are characterized by impairment of consciousness; patient may not respond to commands and exhibit unwanted movements. Partial seizures can diffuse and evolve in to a generalized seizure.

**Seizures and neurogenesis**
Mesial temporal lobe epilepsy (MTLE) is the most common type of epilepsy and is characterized by recurrent complex partial seizures and often associated with hippocampal sclerosis. The sclerosis is due to neuronal loss of pyramidal cells in CA1 and DG granule cells, primarily in hilus, through necrosis and apoptosis. This is followed by upregulation of growth factors and neuropeptides (Bengzon et al., 1997; Blumcke et al., 1999; Lee et al., 2002; Shetty et al., 2003; Wasterlain et al., 1993), enhanced cell proliferation and neurogenesis, and consequently a thickening of the hippocampal granule cell layer (Jessberger et al., 2005; Parent et al., 1997). Seizure-induced delayed cell death through apoptosis balances the neuronal population
Further structural abnormalities are astrogliosis, mossy fiber sprouting, and extension of hilar basal dendrites and ectopic migration of immature neurons into hilus (Blumke et al., 1999; Parent et al., 1997; Ribak et al., 2000). The mechanisms by which seizure activity drives neural stem cell proliferation are not well understood. BrdU labeling before induced seizures, in rodent models of epilepsy, has shown that a great majority of proliferating cells are mitotically active prior to seizure (Parent et al., 1999). Severity and duration of the seizure does not seem to reflect the level of proliferation, since even a single seizure induces proliferation (Bengzon et al., 1997). Although, more intense seizures seem to affect survival of newly generated neurons negatively (Mohapel et al., 2004). One explanation for how seizures initiate proliferation of neural stem cells (NSCs) is that they are capable to sense electrical activity. The excitatory stimuli act directly on hippocampal NSCs to favor neuron production by inhibiting expression of the glial fate genes (Deisseroth et al., 2004). Moreover, GABA receptors are upregulated in response to seizures and GABA receptors act excitatory on NCS (Fritschy et al., 1999; Tozuka et al., 2005). It has been suggested that trophic factors upregulated by seizures, such as brain derived growth factor, BDNF, induce NSC proliferation (Isackson et al., 1991; Shetty et al., 2003). In line with this Lee at al proposed that BDNF is required for basal neurogenesis (Lee et al., 2002). In addition, epigenetic factors such as changes in histone acetylation, induced by seizures, may ease transcription of necessary genes regulating cell cycle events (Jessberger et al., 2007). Most likely a combination of mechanisms works in synergy to promote neurogenesis in the epileptic brain.

1.4 HUMAN BRAIN GROWTH SYNDROMES – MEGALENCEPHALY
(See review by Almgren et. al. Appendix I)
Megalencephaly, also called macrencephaly, is a condition in which the patient has an abnormally large and heavy brain. By definition, the brain weight is greater than average for the age and sex of the infant or child, over the 98th percentile. This condition is associated with a number of rare disorders where the majority has not been neuropathologically well-defined. Subclassification of the megalencephalies has been challenging due to the large number of diverse etiologies that are associated with megalencephaly. However, metabolic megalencephalies are those that result from abnormal accumulation of metabolic substances within the cells of the brain leading to its enlargement, and anatomic megalencephalies are those secondary to increases in the size or number of cells without associated storage of abnormal metabolic products (Gooskens et al., 1988). In normal development, proliferation is regulated so that the correct number of cells is formed in the proper place at the appropriate time. Possibly the most common anatomic megalencephalies are those with unknown ethiology (idiopathic) that runs in families, so called familial isolated megalencephaly. Familial isolated megalencephalies are mostly autosomal dominant
but recessive cases also occur and those are generally more severe. Symptoms include delayed development, mental retardation, motor deficits, convulsive disorders and seizures. The idiopathic megalencephalies with neurological deficit and the unilateral megalencephalies (affecting one hemisphere) are believed to be caused by disturbed proliferation, survival or migration of neurons (Volpe, 1981; Volpe, 2001).

1.5 THE MOUSE MODEL OF MEGALENCEPHALY AND EPILEPSY

The mouse model for megencephaly, BALB/cByJ-Kv1.1\(^{mceph/mceph}\) (mceph/mceph) presents a unique and novel route towards understanding mechanisms of CNS growth (Donahue et al., 1996). These mice are homozygous for a mutation truncating the Shaker-like voltage gated potassium channel, Kv1.1 (KCNA1) (Fig. 4). In human mutations of Kv1.1 is associated with episodic ataxia, whether the mceph/mceph mice displays this phenotype is not investigated. The truncated Kv1.1 (MCEPH) protein retains only the N-terminal domain, the first transmembrane domain S1 and the first extracellular loop, and therefore lacks the voltage sensor and ion pore domains. These mice display motoric disturbances and complex partial seizures developing into generalized seizures, with myoclonic, tonic and tonic-clonic seizures progressing from 4 weeks of age (Petersson et al., 2003).

![Diagram](image)

**Fig. 4.** The Kv1.1 wild type protein consists of six transmembrane segments S1-S6 (S1-S6), the voltage sensitive P-loop is located between S5 and S6. N and C-terminal are intracellular (i.c). The truncated MCEPH protein has only the S1 segment, the intracellular N-terminal and the glycosylation site (G) at the first extracellular (e.c) loop.

Their brain size is significantly larger than wild type from three weeks of age (Diez et al., 2003). Histological examination of the Kv1.1 mceph/mceph brain has revealed absence of major structural abnormalities, edema, lesions, excessive myelination,
leukodystrophy and hydrocephalus. Epilepsy is commonly shown to result in degeneration and shrinkage of neural tissue. In contrast, the mceph/mceph brains show no overt neural atrophy (Petersson et al., 2003). Similar to other epileptic models, mceph/mceph has dramatic disturbances in expression of several growth regulating hormones and trophic neuropeptides (see Fig. 5). The altered expressions were colocalized in neocortex, hippocampus, amygdala and piriform/entorhinal cortex, which are the brain structures found enlarged in the mceph/mceph mice from 3 or 8 weeks of age by means of Magnetic Resonance Imaging. Upregulation of trophic molecules was seen already at 2 weeks of age which is before a brain structure enlargement (Diez et al., 2003). BDNF levels were increased as well as altered expression of molecules in the insulin-like-growth factor (IGF) system, known to have trophic effects (Diez et al., 2003; Petersson et al., 1999). Changes in neuropeptides, with known effect on neural development, are also seen in the mceph/mceph brain. Upregulation of enkephalin, galanin and neuroepeptide Y mRNA has been shown, in opposite to cholecystokinin which was down regulated, all in line with epileptic models (Petersson et al., 2000). Electrophysiological studies of enlarged Kv1.1 expressing hilar interneurons within mceph/mceph hippocampal slices showed an increased frequency of stimulus-induced pulse trains, reflected in an upward shift of the frequency vs. stimulation curve. Computer simulations of a hippocampal model neuron showed that reducing the number of potassium channels shifts the frequency vs. stimulation curve in the direction observed in mceph/mceph. Such an increased firing tendency in neurons can induce epileptic activity in model networks. However, no systematic change in membrane resistance or action potential shape was observed in mceph/mceph compared to wild type (Petersson et al., 2003). The mceph/mceph mice display megencephaly and so do the Kv1.1 knock-out mice, but the behavioral phenotype is more severe in the mceph/mceph mice (Persson et al., 2007).
Age related size increase and altered mRNA expression in *mceph/mceph* mice

![Diagram showing size increase and altered mRNA expression in mceph/mceph mice.]

**Fig. 5.** Size increase and altered expression of neuropeptides, trophic factors and other molecules in the *mceph/mceph* brain. Image adapted from Diez et al. 2003.

### 1.6 VOLTAGE GATED POTASSIUM CHANNELS – KV1.1

Channels that are opened or closed due to a change in membrane potential are called voltage gated; most of these are allowing the passage of one ion type through the pore (Na\(^+\), Ca\(^{2+}\), K\(^+\) or Cl\(^-\)). The largest family of ion channels is voltage gated potassium channels, and they serve different functions; mainly setting the resting membrane potential and repolarising the cell during the falling phase of the action potential (Fig. 6) (Hille, 2001). Activity-dependent changes of K\(^+\) channel properties or distribution, in nerve terminals, can generate a plasticity of intrinsic excitability, believed to contribute to some forms of memory storage (Zhang and Linden, 2003). The voltage gated potassium channel consists of four polypeptides also known as subunits. Each subunit consists of six transmembrane segments (S1-S6) connected by intra- and extracellular loops and one intra-membrane poor loop making the cell more...
permeable to K⁺ (Fig 4). A functional channel is formed by four subunits and the tetramer can be assembled by different types of subunits, although from the same family (Li et al., 1992; MacKinnon, 1991; Shen and Pfaffinger, 1995). The assembly of the tetramers takes place in the endoplasmatic reticulum (ER) (Rosenberg and East, 1992). The tetramerization domain (T1), located in the N-terminal, and the first transmembrane segment (S1) are important for the oligomerization (Babila et al., 1994; Shen et al., 1993). Today there are over 40 known voltage gated potassium channels, divided in to 12 subfamilies Kv1-Kv12 (Yu and Catterall, 2004). There are nine Kv1 subunit types; Kv1.1-Kv1.9 also known as Kcna1-9. Kv1.1 channels are mainly expressed in the nervous system, but are also found in cardiocytes and pancreatic beta-cells, gastrointestinal muscle cells, renal tissue and urinary bladder (Dou Y, Bian Z, Almgren M, Khan F, Johansson B, Arner A, Lavebratt C, submitted manuscript)(Escobar et al., 2004; Hatton et al., 2001). In the nerve cell they are predominantly localized to nerve terminals and axons, where they are involved in neurotransmitter release and repolarization of the action potential (Fig. 6-7). The most common Kv1 subunits in the brain are Kv1.1, Kv1.2 and Kv1.4, which can assembly to heterotetramer to form a Kv1 channel. Kv1.1 contains a strong ER retention motif, whereas Kv1.4 has a strong ER export signal (Li et al., 2000; Manganese et al., 2001). It has been proposed that for a tetramer to reach the plasma membrane a balance of the different trafficking motifs are required (Misonou and Trimmer, 2004). Kv1.1 and Kv1.2 may be implicated in early stages of myelination, they have both been found to interact with Nogo-A and contactin-associated protein (caspr), two central proteins in this process (Nie et al., 2003). The Kv1.3 and Kv1.4 subunits are involved in the regulation of oligodendrocyte precursor cell proliferation (Herrero-Herranz et al., 2007). In microglia, expression of Kv1.5 and Kv1.3 promotes cell cycle arrest and Kv1.5 knock out mice display increased proliferation (Pannasch et al., 2006).

The function of Kv1.1 has been investigated in knock out mice, which displays an epileptic phenotype (Smart et al., 1998). Electrophysiological studies have shown hyperexcitability of CA3 pyramidal cells in hippocampus, of auditory neurons and of pyramidal neurons in the neocortex (Brew et al., 2003; Lopantsev et al., 2003; van Brederode et al., 2001). A mutation of the Kv1.1 gene, causing a non-functioning Kv1.1 subunit, results in epilepsy and brain enlargement as seen in the mceph/mceph mice, for details see 1.5 and APPENDIX I. The Kv1.2 knock out mice display a reduced life span and exhibit spontaneous generalized seizures (Brew et al., 2007).
**Fig. 6. The action potential.** (1) The initial step of the action potential; Na⁺ channels open allowing sodium ions into the cell. This makes the membrane potential to become positive. (2) When the positive membrane potential is high enough the K⁺ channels open allowing a flow of potassium ions out of the cell. (3) At high membrane potential the Na⁺ channels close, which stops influx of positive charge. The K⁺ channels are still open it allows the efflux of positive charge so that the membrane potential falls. (4) When the membrane potential begins reaching its resting state the K⁺ channels close.

### 1.6.1 Kv1.1 and human diseases

In humans, mutations of the *Kv1.1* gene (also known as *KCNA1 HUMAN*) are associated with the autosomal dominant disorder episodic ataxia 1 (EA-1), continuous myokymia (a form of involuntary muscular movement) and epilepsy (Browne et al., 1994; Zuberi et al., 1999). To date 20 different mutations of *Kv1.1* are found in EA-1. Nineteen lead to amino acid substitution (missense mutation), whereas one leads to truncation in the C-terminal (Jen et al., 2007). The mutations are distributed over all segments S1-S6 throughout the *Kv1.1* gene. Functional studies of the mutations *in vivo* predict increased neuronal excitability (Eunson et al., 2000; Rea et al., 2002). The patient with the truncating mutation displays an unusual treatment-resistant EA-1 (Eunson et al., 2000). There seems to be a correlation between degree of ion-channelopathy and the severity of the disease.
Fig. 7. K⁺ channels in neurons. Neuronal K⁺ channels play an important role in regulation of the neuronal excitability. Kv1.1 channels are located on axons and synaptic terminals, where they regulate neurotransmitter release. Other potassium channels are located on the dendritic spines directing responsiveness to synaptic input and thereby the spike frequency of the action potential. (Figure adapted from Wickenden et al. 2002)

1.6.2 Potassium – regulation of proliferation and apoptosis

In addition to involvement in nerve and cardiac action potential, potassium channels are involved in a number of physiological processes including volume regulation, apoptosis, proliferation and differentiation. Many potassium channels have been related to proliferation and cell-cycle progression in mammalian cell lines and certain potassium channels show altered expression in cancer cells and tumors. K⁺ channel activation is important for the early G1 phase of the cell cycle; this has been demonstrated in numerous studies of Kv1.3 channels in T lymphocytes, see review by (Wonderlin and Strobl, 1996). The mechanisms by which K⁺ channels regulate cell cycle progression is unclear, but it seems to depend on its regulation of membrane potential, since K⁺ channel blockers inhibit proliferation due to membrane depolarization (Ouadid-Ahidouch et al., 2001; Wonderlin et al., 1995). Further, growth factors activate K⁺ channels and enhanced K⁺ channel activity is observed in tumor cells (Wang et al., 1997). Several studies show that voltage gated Kv1 channels upregulate proliferation of various cancer types; neuroblastomas, breast carcinoma, small lung cell carcinomas, prostate cancer and colon cancer (Wang, 2004). Kv1.1 has been implicated in breast cancer proliferation (Ouadid-Ahidouch et al., 2000).

Intracellular potassium levels also appear to promote critical events early in the apoptosis program. Potassium ion level is higher inside than outside the cell whereas
in apoptosis the cytosolic potassium steadily gets reduced, which decreases the cell size; this shrinkage appears associated with the activity of enzymes initiating apoptosis (Bortner and Cidlowski, 2007). This feature seems to be independent of cell type or stimulus used to induce apoptosis, although the activation and regulation of which ionic transport mechanisms that result in this volume decrease appears to be case specific (Beauvais et al., 1995; Benson et al., 1996; Bortner and Cidlowski, 2007). As a result of the potassium efflux, the concentration decreases from the normal physiological concentration of approximately 140 mM, to 30-50 mM. The loss of the intracellular potassium during apoptosis is an early necessary feature, although how it is regulated on the apoptotic signal is unknown (Park and Kim, 2002). Several studies has shown rescue of apoptosis by introduction of potassium to the cell and an increased apoptosis upon removal of intracellular potassium. Kv1.1, Kv1.2, Kv1.3 and Kv1.5 as well as other types of voltage gated potassium channels are known to be involved in regulation of apoptosis and or survival (Bortner and Cidlowski, 2007; Ekhterae et al., 2001; Lee et al., 2003).

Cell proliferation and apoptosis are two counterparts that share the responsibility for maintaining normal tissue homeostasis, and strict regulation is thereby important. Potassium channel’s involvement in these two events needs further investigation.

1.7 ANTI EPILEPTIC DRUGS (AEDs)
Many processes in the brain are involved in the development of a seizure, including neurons, glia cells, ion channels, receptors and inhibitory and excitatory synapses. The AEDs are designed to adjust these processes to induce inhibition instead of excitation in order to stop or at least milder seizure activity. AEDs have been used since the 1910, starting with phenobarbital and primidone, followed by phenytoin (1940) and carbamazepine and valproat in the sixties. During the 1990s several new drugs were developed and called “the new generation” AEDs; vigabatrin, lamotrigine, gabapentin (GBP), felbamate, oxcarbazepine, topiramate, levetiracetam, pregabalin, zonisamide and frisium. Although, the number of new improved AEDs, carbamazepine and valproat are still among the most commonly used (Paper IV). AEDs are categorized after their main mechanism of action, but many of them have several actions and others may even have unknown mechanisms of action. The main groups include sodium channel blockers, calcium current inhibitors, gamma-aminobutyric acid (GABA) enhancers, glutamate blockers, carbonic anhydrase inhibitors, hormones. (Table 1)
Table 1. Targets of antiepileptic drugs - mechanism of antiepileptic action

<table>
<thead>
<tr>
<th>AED</th>
<th>Na(^+) channel</th>
<th>Ca(^{2+}) channel</th>
<th>GABA</th>
<th>Glutamate</th>
<th>Other/unknown</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenobarbital</td>
<td>x</td>
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<td>x</td>
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<tr>
<td>Phenytoin</td>
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<tr>
<td>Fosphenytoin</td>
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<td>Ethosuximide</td>
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<td>x</td>
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<tr>
<td>Benzodiazepines</td>
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<td>x</td>
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<tr>
<td>Carbamazepine</td>
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<tr>
<td>Oxcarbazepine</td>
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<tr>
<td>Valporic acid</td>
<td>x</td>
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<td>x</td>
<td>x</td>
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<tr>
<td>Vigabatrin</td>
<td>x</td>
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<td>Lamotrigine</td>
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<tr>
<td>Felbamate</td>
<td>x</td>
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<tr>
<td>Topiramate</td>
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<tr>
<td>Gabapentin</td>
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<tr>
<td>Levetiracetam</td>
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<tr>
<td>Zonisamide</td>
<td>x</td>
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<tr>
<td>Pregabalin</td>
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</table>

1.8 TERATOGENIC AND NEGATIVE EFFECTS OF AEDs

A teratogen is defined as an agent, such as a virus, a drug, or radiation that causes malformation of an embryo or fetus. Teratogenic or other negative effects in utero have been reported for several AEDs including, carbamazepine (CBZ), valproat (VPA), phenytoin and others. CBZ and VPA are still among the most commonly used and reports show that these accounts for a major part of the reported negative effects of the offspring; ranging from malformations, central nervous system dysfunction and altered physical growth (15% of newborns exposed in utero had growth retardation after VPA exposure)(Dean et al., 2002; Harden, 2007; Kozma, 2001; Wide et al., 2004). In spite of this, it is important to keep the pregnant women with epilepsy on a stable antiepileptic treatment to avoid seizures which can threat both mother and fetus. Approximately 0.3% of all pregnant women has epilepsy and are in need of a balanced AED treatment (Morrell, 2002). To date, attention has mainly been given to the teratogenicity of AEDs, much less is known regarding the association of AEDs and epilepsy to preterm delivery and intrauterine growth restriction, which are predictors of childhood mortality and morbidity (Hvas et al., 2000; Wilcox and Skjaerven, 1992). Newborns of women with drug treated epilepsy had lower birthweight, length, and head circumference but normal gestational age compared to newborns of women without epilepsy, whereas none of these negative effects was present in cases of epilepsy without treatment (Hvas et al., 2000). Other studies report decrease in body dimensions, especially a reduced head circumference, after AED exposure in utero,
where polytherapy often shows stronger association than monotherapy (Bertollini et al., 1987; Wide et al., 2000). The negative effects of AEDs, especially new generation’s, needs to be investigated further and in larger populations in order to optimize treatment regimes for epileptic women.

The mechanisms of teratogenicity and how AEDs effect growth in utero are poorly understood. One hypothesis of teratogenesis involves oxidative damage from free radicals formed as reactive intermediates of AED metabolism (Wells et al., 1997). Another is apoptosis due to reduce expression of neurotrophins and levels of protein kinases, induced by AEDs, which are important for neuronal growth and survival. Neuronal apoptosis is believed to cause neural degeneration and possibly behavioral deficits. Blockage of synaptic transmission could also cause neurodegeneration via apoptosis and hence reduce the brain size, possibly HC and influence behavior. Many AEDs block Sodium (Na+) channels, Calcium (Ca2+) channels, AMPA receptors or NMDA receptors. Some AEDs modify the metabolism or release of GABA. CBZ’s anti-epileptic effect is primary through blocking sodium channels, and it has been proposed to modify adenosine A1 and GABA A receptors as well as L-and P-type calcium channels (Gasser et al., 1988; Granger et al., 1995; Olpe et al., 1991; Schirrmacher et al., 1995; Willow et al., 1985; Yoshida et al., 2007). VPA influences GABA concentrations in the brain and further it interferes with glutamate-mediated excitation and limits sustained repetitive neuronal firing through blockade of sodium channels (Brodie and Dichter, 1996; Meldrum, 1996; Taylor and Meldrum, 1995).. Studies by Bittigau et.al., demonstrate that phenytoin, phenobarbital, diazepam, benzodiazepine, vigabatrin, and valproate cause apoptotic widespread neurodegeneration in the developing rodent brain at plasma concentrations relevant for seizure control in humans (Bittigau et al., 2002; Bittigau et al., 2003). Further both CBZ and VPA have also been shown to regulate apoptosis via histone modifications in mice (Beutler et al., 2005; Jessberger et al., 2007).
2 AIMS OF THE STUDY

The general aim has been to understand how a potassium ion channel defect can affect hippocampal growth and neurogenesis, and how this can be treated. Specifically, the aims were:

- To characterize the Kv1.1 and MCEPH protein expression and trafficking.
- To determine which regions that are enlarged in mceph/mceph hippocampus and to determine if the overgrowth is due to larger cells or more cells.
- To unravel whether the excessive number of neurons in hippocampus is due to altered neurogenesis and/or apoptosis in the mceph/mceph DG.
- To clarify if antiepileptic drug treatment can protect from excessive number of neurons and astrocytes in mceph/mceph hippocampus.
- To identify transcripts involved in the overgrowth of the mceph/mceph hippocampus and the protective effect of antiepileptic drug treatment.
- To determine the effect of AEDs in humans in utero – with focus on head size of the newborn.
3 MATERIALS AND METHODS

3.1 MICE (PAPER I-III)

All studies were performed in accordance with guidelines from the Swedish National Board for Laboratory Animals. The spontaneously mutated BALB/cByJ-Kv1.1mcepht/mcephp, BALB/cByJ-Kv1.1+/ mice (wild type) and C3HeB/FeJ-Kcnai1tm1Tem (Kv1.1 knock out) (all originally obtained from The Jackson Laboratory, Bar Harbor, ME) were kept in a barrier animal facility at 12 h light: 12 h darkness, a temperature of 21-22°C and a relative humidity of 40-50%. Rodent breeding diet R36 (Lactamin AB, Stockholm, Sweden) and water were provided ad libitum.

3.1.1 BrdU administration (paper II)

The 5-bromo-2'-deoxyuridine (BrdU) (Sigma, St. Louis, MO) is a thymidine analogue which incorporates into proliferating cells during the S-phase of the cell cycle. BrdU was dissolved in 0.9% NaCl to a concentration of 10 mg/ml. The animals received intraperitoneal injections of 50 µg/g body weight twice a day for three consecutive days. One group of mice received the first injection at 3 weeks of age and was euthanized approximately 2 h after last injection. The next group received the same scheme of BrdU injections but was kept alive for additionally six weeks to study survival of newly proliferated cells. The last group was injected at 9 weeks of age and was euthanized 2 weeks later.

3.1.2 Antiepileptic drug (AED) treatment (paper III)

Carbamazepine (CBZ, Sigma-Aldrich, St. Louis, MO) was incorporated in pellets (3.5 g CBZ / kg pellets, Lactamin) and given orally to mice starting postnatal week 6 until 12 weeks of age. The expected daily intake was approximately 0.5 g CBZ / kg body weight. No sign of toxicity (i.e. decreased locomotion, drowsiness, or loss of weight) was found. The LD50 in mice of orally administrated CBZ is approximately 1,000 mg/kg, or above (reviewed in Schmutz et al., 1985).

3.2 PROTEIN ANALYSIS (PAPER I)

Antibody preparation

A rabbit polyclonal antibody against the N-terminal of Kv1.1 was generated using a synthetic peptide (CMSGENADEASTAPGHPODGSYPRQ) corresponding to amino acids 4–27. This antibody recognizes the truncated MCEPH protein as well as the full length wild type Kv1.1. Kv1 subunits have a high degree of homology and the peptide for immunization was selected to have as low similarity as possible to other Kv1 subunits. The peptide was conjugated to KLH and injected into rabbits for production of
antiserum. The antiserum was affinity purified against the peptide using standard procedures to achieve the final antibody, called anti-MCEPH.

**Preparation of samples**
Mice were euthanized using CO₂ and the brain was quickly dissected out and rinsed in ice cold PBS. The brain tissue was homogenized in RIPA lysis buffer or glycosylation lysis buffer. The homogenate was spun down to pellet debris. Transfected cells were harvested by trypsination followed by several washes in PBS. The cells were lysed in RIPA buffer and spun down to pellets.

**Western blot**
Brain or cell lysate was diluted with 2x Laemmli loading buffer with β-mercaptoethanol and applied on a 10% SDS-PAGE with a 4% stacking gel for size separation. Using wet transfer at 100 mV, proteins were transferred to nitrocellulose membranes (Hybond-ECL, Amersham Biosciences, Little Chalfon, UK). The membranes were blocked in 5% milk for 2 h and incubated over night with the primary antibody, at 4°C. The anti-MCEPH IgG was used at a 1:500 dilution; monoclonal anti-Kv1.1 IgG and anti-Kv1.2 IgG (Upstate Biotechnologies Inc, Waltham, MA, USA) were used at dilutions of 1:200 and 1:1000, respectively. This was followed by incubation for 2 h at room temperature with horseradish peroxidase coupled secondary antibodies; swine anti-rabbit IgG (DAKO, Glostrup, Denmark 1:2000) and goat anti-mouse IgG (Upstate Biotechnologies Inc. 1:5000). Finally, ECL Western blotting reagent (Amersham Biosciences) was used for detection according to the manufacturer’s protocol.

**Immunoprecipitation**
In immunoprecipitation a gentle lysis is used which does not disrupt protein-protein bindings; this allows interacting proteins to co-precipitate. Thereafter, an antibody was added to the tissue homogenate and allowed to bind its target. The antibody-antigen complexes were then immobilized on protein A coated Sepharose beads. The rest of the homogenate was removed and the beads were washed several times in PBS. The beads were then resuspended in a denaturing buffer containing SDS and β-mercaptoethanol, which releases the complexes from the beads and disrupts the protein-protein interactions. To detect the interacting proteins in the complexes, Western blot was performed as described above.

**Glycosylation analysis**
N-linked glycosylation can be used to study trafficking of glycoproteins. The glycosylation process starts in the ER where a high-mannose glycan is added to the protein, this is called core glycosylation. Next, the protein is transported to the Golgi complex were the glycan is modified. By using endoglycosidases such as EndoH and
PNGaseF, trafficking of a protein can be studied. EndoH cleaves off only core glycans, and PNGaseF cleaves off all N-linked glycans. Hence, when proteins are correctly processed through the ER and Golgi, they become resistant to EndoH. Sensitivity to EndoH indicates that the protein has not been processed beyond the ER, and hence unlikely reached the Golgi.

For glycosylation analysis brain lysate was denatured with 0.1% SDS and 1% β-mercaptoethanol at 100°C for 5 min. Samples were incubated with EndoH or PNGaseF according to the manufacturer’s protocol (New England Biolabs, Beverly, MA, USA) at 37° for 2 h. Western blot analysis was performed to detect changes in size of the studied proteins.

### 3.3 PLASMIDS (PAPER I)

The Kv1.1 gene consists of a single coding exon. This allows PCR amplification of the entire coding sequence using genomic DNA as a template. For oocyte expression studies (3.5) the complete Kv1.1 mRNA sequence [NCBI: NM_010595] was PCR amplified from genomic DNA of mceph/mceph and wild type mice using High Fidelity Taq polymerase (Roche Diagnostics, Basel, Switzerland). The PCR products were TA-cloned into pCR2.1 plasmids (Invitrogen, Carlsbad, CA, USA). The coding sequence was then subcloned into the oocyte expression vector pGEM-HE (Protinac GmbH, Hamburg, Germany) using the HindIII restriction sites at position 823 and 2712. The correct orientation of the insert was confirmed by restriction analysis and sequencing. Rat cDNA for Kv1.2 and Kv1.3 in the pGEM-HE vector was obtained from Protinac.

For expression in mammalian cells (3.4) the coding sequence of Kv1.1 was PCR amplified from genomic DNA of mceph/mceph and wild type mice using two different reverse primers that removed the stop codon and introduced a restriction enzyme site. The PCR products were first TA-cloned into pCR2.1 and then subcloned into the vectors pDsRed2-N1 and pZsGreen-N1 (BD Biosciences Clontech Palo Alto, CA, USA) to construct fluorescent fusion proteins.

### 3.4 CELL CULTURE AND TRANSFECTION (PAPER I)

Human embryonal kidney cells, HEK293A, were incubated in Dulbecco’s modified Eagle’s media (DMEM) supplemented with L-glutamine, 10% fetal bovine serum (Gibco, Rockville, MD, USA) and 100 μg/ml penicillin/streptomycin (Gibco) at 37°C and 5% CO2 humidified air. For transfection the cells were plated on 100 mm dishes. Cells were transfected at 80% confluency using Lipofectamine and Plus reagent (Invitrogen) according to the manufacturers protocol and analyzed 48 h after transfection.
3.5 ELECTROPHYSIOLOGY (PAPER I)

Voltage clamp measurements were performed in oocytes from *Xenopus laevis*, which were injected with *mcep*, *Kv1.2* and *Kv1.3* mRNA. Plasmids containing the selected genes were linearized and transcribed with the Message Machine Kit (Ambion, Austin, TX, USA). The oocytes were surgically removed from the frog under anaesthesia, and treated with Liberase (0.25mg/ml) for 3 h. The rinsed oocytes were incubated overnight in modified Barth’s solution. Thereafter they were injected with respective cRNA (50 nl/cell) using a Nanoject injector (Drummond Scientific, Broomall, PA, USA) and incubated at room temperature (20-22°C) for 16-24 h before starting the electrophysiology experiments (day 3 to 4 after injection).

The electrophysiological experiments were carried out with a two-electrode voltage-clamp (CA-1 amplifier, Dagan, Minneapolis, MN). The microelectrodes were filled with 3 M KCl solution, resulting in a resistance between 0.5 to 1.0 MΩ. All measurements were performed at room temperature (20-22 °C). The holding potential was set to -80 mV and the interval between the test steps was 2 s (Kv1.2) and 30 s (Kv1.3). The recorded current was filtered by a low-pass Bessel filter (5 kHz) and sampled with intervals of 2 ms (Kv1.2) and 4 ms (Kv1.3). In order to avoid different channel density levels, due to trafficking during the electrophysiological experiments, the measurements were restricted to a four-hour period. The conductance (G) was calculated from the current (I) and the associated voltage step (V) by:

$$ G = \frac{I}{V + 80 \text{ mV}} $$  \hspace{1cm} (1)

The conductance normalized to the maximal conductance under control conditions (G_{\text{max CTRL}}) were fitted to the Boltzmann equation:

$$ \frac{G}{G_{\text{max CTRL}}} = 1 / \left[ 1 + \exp \left( \frac{(V - V_{1/2})}{s} \right) \right] $$  \hspace{1cm} (2)

Where $V_{1/2}$ is the potential at half-maximal conductance, i.e. midpoint potential, and $s$ is the slope. Significance of differences was tested using the t-test.

3.6 IMMUNOHISTOCHEMISTRY (PAPER I-II)

*Kv1.1* and MCEPH (paper I)

For immunohistochemistry, mice were anesthetized with 2% isoflurane and perfused transcardially with a saline buffer followed by 4% paraformaldehyde in phosphate buffer with a flow rate of 5-10 ml/min for approximately 10 min. The volume of saline buffer was 20 ml at 37°C, followed by 20 ml of 37 °C paraformaldehyde and 35 ml ice-cold paraformaldehyde. Brains were dissected out, immersed in ice-cold fixative for 3 h and then rinsed in 0.1 M phosphate buffer (pH 7.4) containing 10% sucrose, 0.01%
sodium azide and 0.02% bacitracin (Sigma-Aldrich). Serial 14 µm sections were cut in a cryostat and mounted on gelatin/chrome-alum coated slides.

All slides were blocked in PBS with 5% goat serum and 0.23% Triton-X, followed by an overnight incubation at 4°C with the primary antibody diluted 1:400 in blocking solution. Slides were then incubated at 2 h in room temperature with a FITC labeled goat anti-rabbit IgG secondary antibody (Sigma-Aldrich, St. Louis, MO, USA 1:300). The slides were mounted with Vectashield antifade mounting media (Vector laboratories, Burlingame, CA, USA) and examined under an Axioskop 2 microscope (Zeiss, Oberkochen, Germany).

**NeuN and GFAP**

After fixation procedures like above these brains were dehydrated in a gradient of alcohol and xylene, followed by paraffin embedding. Forty µm thick sections were cut coronally using a microtome (Microm HM355, Microm Int., Walldorf, Germany). These sections were prepared for stereological analysis of cell number and divided in two subgroups by systematic uniform random sampling; choosing every tenth section with a random start per brain. The two subgroups were stained for neuronal nuclei (NeuN, Chemicon, Temecula, CA) and for glial fibrillary acidic protein (GFAP, Sigma-Aldrich, St. Lois, MO), respectively. The sections were sampled 400 µm apart covering the complete hippocampus, resulting in 5-7 slides per animal and stain. Deparaffinized sections were treated with 3% H₂O₂ for 10 min to block endogenous peroxidase activity. For both NeuN and GFAP staining the mouse on mouse (M.O.M) kit for immunodetection (Vector, Burlingame, CA) was used according to manufacturer’s instructions. Primary antibodies were diluted in M.O.M Diluent (NeuN 1:100, GFAP 1:500) and incubated at 4°C for 24 h. Biotinylated secondary antibody (anti-mouse IgG, M.O.M kit) was diluted in M.O.M Diluent (1:250) and incubated at 4°C for 24 h. ABC Vectastain Elite reagent (Vector) was applied for 2 h in RT to enhance signals. Detection was achieved with 3’3-diaminobenzidine substrate (DAB, Vector) for 5-15 min in RT. All slides were counterstained with hematoxylin (Histolab, Gothenburg, Sweden) for 2 min followed by dehydration and mounting with Pertex (Histolab).

**BrdU detection.**

Fixation was performed as above, whereafter brains were cut in 60 µm thick free-floating sections. To be able to detect the incorporated BrdU in the sections, DNA denaturation was necessary. This was done by treating the free-floating sections with sodium citrate buffer (0.03 mol/L NaCl, 0.3 mol/L sodium citrate, pH 8.5) containing 50% formamide, at 65°C for 2 h. After that the sections were rinsed in sodium citrate buffer for 10 min, incubated in 2 M HCl for 30 min at 37°C followed by a rinse in 0.1 M borate buffer (pH 8.5) and finally several rinses in TBS-T (TBS supplemented with 1% Triton-X 100). Immunoperoxidase staining was used to detect BrdU. Sections were
incubated in blocking solution (10% fetal calf serum in TBS-T) and then incubated in primary antibody (Rat monoclonal anti-BrdU IgG, 1:100, Abcam, Cambridge, UK) in blocking solution at 4°C over night. BrdU was detected with a biotinylated anti-rat IgG (1:200, Amersham Biosciences, UK) for 1 h at RT and washed three times in TBS-T. Next, ABC reagent (Vectastain Elite, Vector) was applied for 1 h. Detection was done with DAB-Ni (Vector) as chromogen, which gives a gray-black color.

**Ki67**

Ki67 is a nuclear antigen expressed in all proliferating cells during late G1, S, M and G2 phases of the cell cycle. Samples were prepared as in the BrdU protocol above. PBS containing 3% H2O2 was applied for 10 min to block endogenous peroxidase activity followed by washing in PBS and blocking for 1h at room temperature (5% horse serum in 1% TBS-T). Primary antibody rat anti-Ki67 IgG (1:200, Novocastra, Newcastle, UK) was incubated for 1 h at room temperature. Further detection steps were identical to those for immunoperoxidase staining above.

**Terminal dUTP Nick End Labeling (TUNEL).**

This assay enzymatically labels the free 3’OH ends of DNA in apoptotic cells. Samples were fixed and prepared as in section NeuN and GFAP. Deparaffinized sections were treated with 2% H2O2 in MeOH for 30 min in room temperature to block endogenous peroxidase activity. Permeabilization solution (0.1% sodium citrate, 0.1% TritonX-100) was applied for 30 min at room temperature. TUNEL reaction mixture (In Situ Cell Death Detection Kit, Roche Diagnostics, Germany) was applied to the slides and incubated at 37°C for 1 h. Tissues were washed in PBS and mounted with Vectashield.

**3.7 STEREOMETRY (PAPER II & III)**

To estimate the number of neurons and GFAP-positive astrocytes (from here on denoted astrocytes) within the hippocampus of wild type and *mceph/mceph* mice, 40 µm thick coronal sections were stained with NeuN and GFAP and analyzed with the optical fractionator with varying sampling fractions (Gundersen et al., 1988; West, 1993). Briefly, counting 100-150 cells in a defined fraction of each tissue section and through a known depth of the tissue section (h, i.e. 10 µm in this study), in a known fraction of tissue, allows for a mathematical estimate of the total population of cells. The optical fractionator sampling procedure results in estimates of cell number that are independent of regional or cellular volume, and independent of any tissue shrinkage or expansion during tissue processing. Sectioning the whole mouse brain yielded approximately 200 sections, out of which hippocampus stretched over approximately 60. Every tenth section (the section sampling fraction, ssf) was selected to systematically sample the hippocampus, yielding 5-7 sections per brain. This selection was done twice to allow for separate staining of GFAP and NeuN. Further sampling was conducted using a light microscope (Olympus BH-2) modified for
stereology with a 60X oil immersion objective (NA = 1.4), a computer-driven motorized Märzhäuser stage (Märzhäuser Wetzlar GmbH & Co. KG, Wetzlar-Steindorf, Germany), a microcator (Heidenhain, Traunreut, Germany) and a CCD camera interfaced to a PC with the software CAST (Visiopharm, Hørsholm, Denmark). The counting frames were randomly distributed over the defined hippocampal region with a fixed step length in the x- (dx) and y- directions (dy). The area sampling fraction (asf) equals the ratio between the area of the counting frame and the area of the sampling grid (Fig. 8). The counting frame was focused down through the optical disector height (h) and a cell was counted only if it fell within the inclusion lines of the counting frame and did not touch the exclusion lines. The height sampling fraction (hsf) is the ratio between the constant disector height (h) and the Q-weighted mean section thickness \( t_{Q} \), which is the measured section thickness weighted with the number of cells counted). Based on the above parameters and cell counts the total number of neurons and astrocytes were estimated:

\[
N = \sum Q \cdot x \frac{(1)}{hsf} \cdot x \frac{(1)}{asf} \cdot x \frac{(1)}{ssf}
\]

In which \( \sum Q \) is the total number of cells counted in the region of interest. In order to check for penetration of stain, section compression and lost caps, a z-axis distribution was performed. This is done by measuring the distance at all fields of view from the section top and bottom to all neurons and astrocytes. The z-axis distribution showed a slightly decreased cell density at the top and bottom of sections and relatively constant cell density in the middle of the sections. Subsequently, the counting frame was placed in the middle of the sections (Dorph-Petersen et al., 2001). All stereological analyses were performed in one hemisphere of the hippocampus.

A stereological analysis was also done to estimate the number of cells labeled with BrdU, Ki67 and TUNEL stain.

### 3.7.1 Quantification of BrdU and Ki67 labeled cells

BrdU-labeled cells in the granular cell layer (GCL) and the subgranular zone (SGZ) of the DG were counted with a modified fractionator method using the whole thickness of the section. The SGZ was defined as the approximately 20 µm band of GCL adjacent to the hilar surface. The sections were 60 µm thick and digital images of the SGZ and GCL on every sixth section (6-8 sections per animal) were captured with an Axioskop 2 microscope, using a 20X objective (Zeiss, Oberkochen, Germany). The migration pattern of the 6 weeks old proliferated cells were studied in GCL and hilus. This was done by counting all BrdU labeled cells in the outer half of GCL and in hilus in same manner as described above. As a complementary experiment to BrdU-staining the antibody Ki67 was used to measure proliferation in nine week old mice. Estimation of number of these cells was conducted in the same way as for the BrdU labeled cells.
The estimated numbers for proliferation, BrdU and Ki67, is good for relative comparison in this study since the whole section thickness was used (a height sampling section of one).

3.7.2 Estimation of Apoptosis

The number of apoptotic cells was quantified with a modified fractionator methodology. The number of TUNEL positive cells was estimated in every sixth section spanning the whole hippocampus, with an Axioskop 2 microscope, using a 20X objective (Zeiss, Oberkochen, Germany). The estimated numbers for apoptosis is good for relative comparison in this study since the whole section thickness was used (a height sampling section of one).

![Stereology](image)

Fig. 8. Stereology, partly adapted form Dorph-Petersen et al., J Comp Neurol, 2004

3.7.3 The Cavalieri method

**Structure volume estimate and shrinkage**

According to the principle of Cavalieri an unbiased estimate of the volume (V) of an object can be derived from the sum of areas (ai) of the individual profiles of the object. A set of n systematically positioned parallel sections, which are separated by a known constant distance t, are selected through-out the object. The areas of each parallel section were obtained with the 2D nucleator method (Gundersen, 1988). The estimated volume (V) is calculated using: \( V = t \times \Sigma ai \) (Gundersen & Jensen, 1987). To account for the total shrinkage and volume loss after histological processing, fixed tissue cubes of 2 mm³ were cut from mcep/mcep (n=3) and wild type (n=3) brains. Clean and dry pieces of tissue were weighed carefully before processing and converted to volume (V):

\[
V_{\text{Before}}(\text{tissue}) = W(\text{g}) \times 1.04(\text{cm}^3/\text{g})
\]
Processed tissue, 6-8 sections for Cavalieri principle, was converted to volume (V) according to:

\[ V_{\text{After(tissue)}} = t \times \Sigma a_i \]

Shrinkage was estimated according to following equation:

\[ \text{Volume shrinkage} = 1 - \frac{V_{\text{After(tissue)}}}{V_{\text{Before(tissue)}}} \]

### 3.7.4 Statistical analysis - stereology and Cavalieri

For cell number and volume, significance of difference in variance between groups was determined by the F-test, and difference in mean between groups was tested using unpaired two-tailed t-test for unequal variances.

### 3.8 EXPRESSION ANALYSIS (PAPER III)

#### 3.8.1 Microarray

Total RNA was isolated from mouse hippocampi with RNeasy kit (QIAGEN, Valencia, CA, USA). Double stranded cDNA was synthesized by reverse transcription of total RNA using random hexamers (SuperScript™ III First-Strand Synthesis System for RT-PCR, Invitrogen, Carlsbad, CA, USA). Sample labeling and hybridization was done individually with Pronto! Plus direct system, according to manufacturer’s manual (Promega, Madison, WI, USA). All samples were compared to universal mouse reference RNA from Stratagene (La Jolla, CA, USA). Reference RNA was prepared in parallel with all samples and subsequently hybridized onto all microarray slides. The microarray slides used for this study were the Mouse 70-mer oligo array 37k chip from Swegene DNA Microarray Resource Center (Department of Oncology, Lund University, Sweden). After sample labeling and hybridization, slides were scanned twice at 10 μm resolution with decreasing pmt (photomultiplier tubes) voltages using a Scanarray Express HT scanner. Image analysis was performed using SpotReader (Niles Scientific). Data files were processed using R (http://www.r-project.org) and packages from Bioconductor (www.bioconductor.org). Local background was not subtracted from the raw fluorescence intensities and data from each array was normalized using a printtip-stratified loess fit of log ratios (response) on average intensities (predictor) (Yang et al., 2001). Data points from spots near saturation were excluded from the loess fit. Following normalization, data from high pmt voltage scans were combined with data from lower pmt voltage scans by replacing data of probes near saturation in any of the high pmt voltage scans with the corresponding data from the lower pmt voltage data sets. Contrasts between samples of interest and spread measures were calculated using Limma (http://bioinf.wehi.edu.au/limma/) and probes were ordered by the false
discovery rate (Benjamini and Hochberg, 1995) reported by Limma when using method ‘fdr’ as correction for multiple testing (Smyth, 2004). In the weighted Limma calculations, spots classified as good by Spotreader were assigned weight one and the rest weight zero. Gene lists were analyzed for ontology annotations with The Ontology Annotation Treebrowser OAT (Bresell et al., 2006).

3.8.2 Real time PCR

Real time PCR was used to confirm mRNA expression patterns shown in the microarray study. The same cDNA samples as for the microarray analysis was used for the real-time quantitative PCR. cDNA was mixed with primers, probes and ABI TaqMan® Universal Master Mix No AmpErase® UNG (Applied Biosystems, Foster City, CA, USA). The assay was run on an ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems). Gene amplifications were performed in duplicates and data were obtained as threshold cycle (Ct) values. Relative gene expression was calculated according to a modified delta-Ct method, which allows normalization of gene expression with multiple endogenous control genes (Vandesompele et al., 2002). Target gene quantity was normalized against beta-actin (Actb) and glyceraldehyde-3-phosphate dehydrogenase (Gapdh) expression. The following genes were selected for verification; Adam22, AnxA3, Atpd, Csdc2, Fjx1, Lrrk1, Mic1, Nptx1, Sstr4 and Rgs2. Mean values of duplicates were used to calculate gene expression relative to that of Actb and Gapdh for each animal.

For verification of array results using real time PCR, differences between mouse groups were determined using unpaired one-tailed t-test, with significance set to $P = 0.05$.

3.8.3 In situ hybridization

In situ hybridization (ISH) was used to further confirm mRNA expression patterns shown in the microarray study. Sections from fresh frozen brains were cut at a thickness of 14 μm. High stringency in situ hybridization using oligonucleotide DNA probes was used according to publishers procedures (Schalling et al., 1988). The following probes were designed and synthesized (Thermohybaid); **Aatk** 5'-CAAGCATGAGCTATTATCGGCCTCAACCTACCTCTCGAGCTGGGAGA-3' and 5'-AATCCTGCCAGCTTTGTTTGCACCTCTCGAGCTGGGAGA-3'; **Bhlhb5** 5'-AGCGACGCTATCTCTGCTTTGTTTGCACCTCTCGAGCTGGGAGA-3' and 5'-CCATGCCCCAGCTATCTCTGCTTTGTTTGCACCTCTCGAGCTGGGAGA-3'; **Gprin1** 5'- GCTCTGGGATGCTTTGTTTGCACCTCTCGAGCTGGGAGA-3' and 5'-CCATGCCCCAGCTATCTCTGCTTTGTTTGCACCTCTCGAGCTGGGAGA-3'; **Ndn** 5'- CCGCCGACTACTCTCTGCTTTGTTTGCACCTCTCGAGCTGGGAGA-3' and 5'-CCATGCCCCAGCTATCTCTGCTTTGTTTGCACCTCTCGAGCTGGGAGA-3'; **S100b** 5'- CCGCCGACTACTCTCTGCTTTGTTTGCACCTCTCGAGCTGGGAGA-3' and 5'-CCATGCCCCAGCTATCTCTGCTTTGTTTGCACCTCTCGAGCTGGGAGA-3'; **Wasf3** 5'- GCACACTCTGAGCACCTCTCTCTGCTTTGTTTGCACCTCTCGAGCTGGGAGA-3' and 5'-CCATGCCCCAGCTATCTCTGCTTTGTTTGCACCTCTCGAGCTGGGAGA-3';
TGTCATACGCCATCA-3’, *Vgf* 5’-GGCCACCTAGACTTGTACGCCTTGATAAGGGTG
CAGAGTCTCAC-3’ and 5’-TGGGACTTATCTCGAATTCTGAGGCAAGGATGCT
AGCGCCTC-3’. The probes were labeled at the 3’ end with $^{33}$P-dATP using
deyoxynucleotidyltransferase. Sections was exposed to a β-absorbing film; Hyperfilm
MP (GE), for 7-14 days. A $^{14}$C step standard (Amershams Biosciences, St Louis, MO,
USA) was included to calibrate optical density readings and to convert measured
values into nCi/g. Expression on films, optical density, was measured with the image
analysis program, ImageJ (v.1.39j, http://rsb.info.nih.gov/ij/). Measurements were
performed on two sections in close proximity, on both hippocampal hemispheres.
A mean expression level of each brain relative to that of wild type brain was calculated,
first on each slide (1-2 sections per mouse group/slide) to avoid bias from interslide
variation, and then combining the duplicates.

For verification of array results using ISH, differences between mouse groups were
determined using unpaired one-tailed t-test, with significance set to $P = 0.05$.

### 3.9 EPIDEMIOLOGY – POPULATION BASED RISK ANALYSIS OF AEDs

We analyzed data from the Swedish Medical Birth Registry (SMBR) in which all
newborns in Sweden are registered (National Board of Health, 2003). Information on
maternal drug use was collected from a structured interview made by a midwife at the
first antenatal visit (usually around 10-12 gestational weeks). Thereafter, prescriptions
from the antenatal care system given after the first visit were recorded. In this study, a
record any time during pregnancy counted as use of antiepileptic drug. All infants born
1995-2005 where the mother reported use of AEDs were selected (n= 2,718). From
this group the following number of newborns was excluded: six with reported CNS
malformation and 64 who were twins or triplets, and 222 infants without known birth
weight or head circumference (HC). The remaining group consisted of 2,426
newborns, that is, 0.27% of all singleton infants with known birth weight and HC,
without known CNS malformations and with a record from the first antenatal care visit
(n=900,739 comprising the population group of comparison). No mother was younger
than 13 or older than 44 years. The range of infant HC was 20 – 40 cm. Population
control group comprised all singleton newborns born 1995-2005 without CNS
malformation, with $20 \leq HC \leq 40$ cm, $300 \leq$ birth weight $< 6,700$ g, where there was a
record from the first antenatal visit to the midwife (n = 900,739). The population
infants (controls) were grouped by birth weight in 500 g intervals. For each birth
weight group the mean and SD of HC were calculated for all newborns for each
stratum according to year of birth, maternal age (2 classes: < 35 years, $\geq$ 35 years),
parity (2 classes: 1, >1), maternal smoking (3 classes: 0 or no answer, < 10, $\geq$ 10
cigarettes per day), maternal body mass index (3 classes: < 19.8, 19.8 $\leq$ BMI $< 26$, $\geq$ 26
kg/m$^2$).
Quantitative analysis
For each infant exposed to AED in utero, its HC deviation from the mean birth weight specific HC, expected from the population data according to year of birth, maternal age, parity, smoking, and BMI was determined and expressed as the number of SDs in that stratum, a standard deviation score (SDS). The mean of these SDS was compared with zero in t-tests, using two-tailed tests, and the standard error of the mean (SEM) for the SDS. The effect of exposure to AED on HC was determined for all infants exposed to AED and also for some specific drugs individually, used in monotherapy or polytherapy. Differences in effects between the three periods 1995-1997, 1998-2001 and 2002-2005 were studied using analysis of variance.

Similar analyses were done for testing the hypothesis of reduced gestational age, ie pregnancy duration, as a result of AED treatment, without correction for birth weight. The study group again consisted of the 2,648 singletons to mothers reported on AED treatment during pregnancy for which there was no report on CNS malformation. One case lacked gestational age data giving 2,647 newborns. All had a gestational age above 23 weeks and below 44 weeks. The population control was similarly selected, including a gestational age of 24-43 weeks, among those mothers for whom there were records from the first antenatal visit.

Dichotomous analysis
We also estimated the impact of drug exposure on the occurrence of small HC (HC smaller than 2 SD below the expected mean) with a Mantel-Haenszel analysis, adjusting for the same confounders as above and using Miettinen’s method to estimate approximative 95% CI.

It must be called re-search because you have to re-do it over an over again!
4 RESULTS AND DISCUSSION

EXPRESSION AND FUNCTION OF THE MCEPH PROTEIN (paper I)

A truncated Kv1.1 protein in the brain of the megencephaly mouse: expression and interaction (paper I)

This study aimed to investigate the expression and trafficking of the truncated Kv1.1, MCEPH protein. Most truncated proteins are not expressed, with the exception of genes with one exon. Since Kv1.1 contains a single exon and its mRNA expression was increased in the mceph/mceph brain we hypothesized protein expression. To investigate this, a polyclonal anti-Kv1.1 N-terminal antibody was generated, that recognized both truncated and wild type Kv1.1 protein. Thorough testing on Kv1.1 knock-out mice showed that this antibody was cross reactive in western blotting, likely recognizing the homologous Kv1.2. However, the cross reactivity was significantly reduced when the antibody was used for immunohistochemistry on formaldehyde fixed tissue.

Protein expression
Western blot of brain lysate from mceph/mceph mice showed a unique band at approximately 30kDa. This corresponded well to the calculated weight of MCEPH which was 27kDa, proving that the peptide was expressed, although the amount of protein appeared to be very low. Immunohistochemistry on formaldehyde fixed brain sections showed MCEPH-like immunoreactivity (LI) primarily in hippocampus and ventral cortex, which are the structures enlarged in the mceph/mceph brain. In other brain regions with wild type Kv1.1 expression such as the cortex, only a few cells were labeled. MCEPH-LI was detected around the cell nuclei, possibly in ER, in contrast to the widespread Kv1.1 staining in fibers in wild type (Fig. 9).
Neural growth – with special emphasis on adult neurogenesis and the effect of antiepileptic drugs

Malin Almgren

Fig. 9. Immunohistochemistry. Kv1.1 expression in wild type hippocampus (A) is more localized to fibers. MCEPH expression in mceph/mceph mice hippocampus (B) is localized around nucleus of neurons in dentate gyrus (DG) and CA3.

**Trafficking and assembly**

Kv1.1 has a single glycosylation site in the first extracellular loop, which is preserved in MCEPH. This site made it possible to investigate MCEPH trafficking by glycosylation analysis. MCEPH was found to be core glycosylated which suggested that it gets trapped in the ER and not entering the Golgi. This is in line with the MCEPH-LI around the nuclei. Kv1 tetramers are assembled in the ER. Since MCEPH has an intact tetramerization domain, located at the N-terminal and first transmembrane domain, we hypothesized that MCEPH could assemble with other Kv1 subunits and that these complexes would be retained in the ER. In hippocampal tissue, no interaction between MCEPH and Kv1.2 could be detected using immunoprecipitation. However, in cell culture, the MCEPH protein was shown to bind full length Kv1.1, and in *Xenopus* oocytes MCEPH had a dominant negative effect on Kv1.2 and Kv1.3 currents. This was in line with the disturbed expression patterns of Kv1.2 and Kv1.3 previously seen in the mceph/mceph hippocampus (Petersson et al 2003). A possible explanation for the lack of interaction in hippocampal tissue could be the low level of MCEPH relative to Kv1.2 due to activation of ER assisted degradation (ERAD). This quality control system protects the cells from defect proteins. This system retains incorrect proteins and transports them to the cytosol where they are degraded by proteasomes (Ellgaard & Helenius 2003). Two other truncated Kv1.1 variants have previously been investigated in cell culture and *Xenopus* oocytes (Babila et al 1994, Folco et al 1997, Manganas et al 2001). Both were trapped in the ER and had a dominant negative effect on currents when coinjected with other Kv1 subunits.

Taken together, the MCEPH protein is expressed in the mceph/mceph brain, which constitutes the first demonstration of truncated Kv1.1 protein expression in brain. MCEPH could interact with Kv1.1 and had dominant negative effect on Kv1.2 and Kv1.3 currents in vitro. However, in hippocampal tissue no persistent interaction
between MCEPH and Kv1.2 could be detected, probably due to low MCEPH levels. A function of the MCEPH protein is in line with a recent study showing that the epileptic phenotype is more severe in the mceph/mceph than in the Kv.1 knock-out mice (Persson et al., 2007).

MORE CELLS IN THE mceph/mceph HIPPOCAMPUS (paper II)

Lack of potassium channel induces proliferation and survival causing increased neurogenesis and 2-fold hippocampus enlargement.

To characterize the effect of the potassium ion channelopathy causing brain enlargement in mceph/mceph growth features of the hippocampus were studied.

Cell number
Stereology analysis showed that mceph/mceph has a dramatic increase in number of neurons and astrocytes in the hippocampus. Hence the hippocampal enlargement in mceph/mceph is due to hyperplasia and hypertrophy, the latter shown for CA3 pyramidal cells (Lavebratt et al., 2006). The neuronal cell number in adult mceph/mceph versus wild type hippocampus was higher in DG, CA2/3 and hilus, but not in CA1. The increase of neurons in mceph/mceph DG was 2.1-fold ($P = 0.002$), in CA2/3 1.4-fold ($P = 0.04$), and in hilus 3.1-fold ($P = 0.002$). The number of astrocytes showed a similar regional pattern in increase as the number of neurons did; although a tendency for more astrocytes within CA1 was found. The increase of astrocytes in mceph/mceph DG was 2.7-fold ($P = 0.01$), in CA2/3 2.1-fold ($P = 0.03$), and in hilus 2.6-fold ($P = 0.001$), compared to wild type. The CA1 region tended to have more astrocytes with a 1.7-fold increase, but due to the large inter-individual variation, particularly in mceph/mceph, the difference did not reach statistical significance ($P = 0.06$) Neuron and astrocyte cell numbers are presented in Table 2.

Volume
All estimated volumes of mceph/mceph hippocampus layers; DG, hilus and CA1-3, were enlarged compared to wild type mice at 12 weeks of age (Table 2). The structure volume corresponded well with the cell numbers, thus no statistically difference in cell density between mceph/mceph and wild type hippocampi. Hence, the increased numbers of neurons and astrocytes seem to account for a significant part of the volume increase of hippocampus in adult mceph/mceph mice. To estimate onset of volume change, mceph/mceph and wild type hippocampus volume was analyzed at postnatal day 1 and 14. At day 1, the hippocampus of mceph/mceph mice was found to be smaller than wild type. At day 14, a small volume increase was seen in mceph/mceph DG. This data proposes that the excessive growth of mceph/mceph
hippocampus starts between birth and the second week of life. This is in line with the study by Diez et al. showing abnormal expression patterns of growth factors and neuropeptides in the mceph/mceph brain from two weeks of age (Diez et al., 2003). Furthermore, Kv1.1 is increased to adult levels from postnatal day 6 in wild type mice (Hallows and Tempel, 1998). This is in agreement with seizure onset from one week of age (Lavebratt unpublished data).

In all, the findings suggest that the overgrowth in mceph/mceph hippocampus is in part due to increased postnatal generation of neurons and astrocytes, in both young and adult mice.

Table 2. Neuron and astrocyte cell numbers and regional hippocampal volumes

<table>
<thead>
<tr>
<th></th>
<th>mceph/mceph</th>
<th></th>
<th></th>
<th></th>
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<th>wild type</th>
<th></th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>DG</td>
<td>CA1</td>
<td>CA3</td>
<td>hilus</td>
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<td>DG</td>
<td>CA1</td>
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<td></td>
<td>DG</td>
<td>CA1</td>
<td>CA3</td>
<td>hilus</td>
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<tr>
<td>Neurons</td>
<td></td>
<td>6,13E+05</td>
<td>2,85E+05</td>
<td>2,39E+05</td>
<td>1,86E+04</td>
<td></td>
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<td>1,70E+05</td>
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<td>2,82E+05</td>
<td>1,70E+05</td>
<td>6,10E+03</td>
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<tr>
<td>Astrocytes</td>
<td></td>
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<td>3,37E+04</td>
<td>2,23E+04</td>
<td>1,42E+04</td>
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<td>1,95E+04</td>
<td>1,05E+04</td>
<td>5,42E+03</td>
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<td>1,08E+09</td>
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Proliferation and survival

We hypothesized that the neuronal hyperplasia in mceph/mceph hippocampus would be the result of a progressive increase in adult neurogenesis as a consequence of the progressive seizure activity. Generally, seizures involving the hippocampus are
followed by a period of rapid neural loss, where after an increased neurogenesis is seen in the hippocampus (Parent et al., 1997). As expected, we found markedly elevated proliferation, the initial step of neurogenesis, in the hippocampal subgranular zone (SGZ) of mceph/mceph. Increased proliferation was detected at 3 weeks and 9 weeks of age (the ages studied), which was before onset of severe epileptic symptoms. The absolute level of proliferation was significantly higher in three week old compare to nine week old mice, whereas at both ages the mceph/mceph mice displayed approximately 3.5-fold higher levels than wild type. The difference in absolute level is in agreement with normal mice that have a high proliferation in SGZ during the first 3 postnatal weeks with a subsequent decline in proliferation rate (Kempermann et al., 2003; Lie et al., 2004). At 3 weeks of age the mceph/mceph mice show much milder seizures symptoms than at 9 weeks of age. The similar relative increase in proliferation rate despite more severe seizure behavior at 9 than 3 weeks is in line with Bengzon et al, who showed that severity and duration of the seizure does not seem to reflect the level of proliferation, given that even a single seizure induces proliferation (Bengzon et al., 1997). In contrast, the intensity of seizures has been suggested to influence the proliferation of neural progenitors. Parent et al show that cell proliferation only increased in animals that experienced nine or more class IV/V kindled seizures over three days (Parent et al., 1998; Parent and Lowenstein, 2002). Moreover, the severity of seizures seems to correlate negatively to survival of newly generated neurons (Mohapel et al., 2004). At 9-12 weeks of age the mceph/mceph mice experience class IV seizures or higher 36% of their time (Persson et al., 2007). Taken together, this indicates that the hyperplasia in mceph/mceph is a consequence not only of seizure-induced neurogenesis, but also of seizure-independent mechanisms, possibly enhanced survival. Generally, apoptosis is upregulated after seizures. Seizure-induced delayed cell death through apoptosis are proposed to balance the neuronal population (Humphrey et al., 2002). Hence, one would expect a lower survival in the epileptic mceph/mceph hippocampus compared to wild type. Survival of newly proliferated cells was measured by BrdU-labelling at 3 weeks of age and analyzed for presence of labeled cells 6 weeks later. The rate of survival appeared similar in wild type and mceph/mceph mice, but the latter displayed 3.5-fold more labeled cells. The survivors displayed neuronal morphology along with migration into the GCL in both genotypes. In agreement, previous reports show that most of the newly seizure-induced cells differentiate and mature into granular cells although many die through apoptosis between first and second week after they are born (Kempermann et al., 2003; Lie et al., 2004).

**Apoptosis**

Since we found hyperplasia in mceph/mceph and similar survival rate in the mceph/mceph and wild type mice, we hypothesized that apoptosis would not be upregulated in mceph/mceph enough to balance the excessive proliferation. Indeed,
the number of TUNEL positive cells was lower in the mceph/mceph than in the wild type hippocampus. The reduced apoptosis rate might be a consequence of reduced intracellular potassium level as this appears to promote critical events early in the apoptosis program (Sun and Guo, 2005). Accumulating evidence suggests that voltage-dependent potassium (Kv) channels have important roles in the generation and development of neuronal and non-neuronal cells. They have been implicated in processes such as proliferation, cell adhesion, migration, neurite outgrowth, and axon guidance (Ouadid-Ahidouche et al., 2000). The mceph/mceph mice lack functional Kv1.1, which contributes to regulate potassium ion levels in the cell. Hence, the ability for mceph/mceph neurons to reduce the $K^+$ concentration in order to induce apoptosis might be impaired. In fact, Kv1.1, Kv1.3 and Kv1.5 are known to be involved in regulation of apoptosis and survival (Bortner and Cidlowski, 2007; Ekhterae et al., 2001; Lee et al., 2003). Cellular suicide programs can be activated by neurotrophin deprivation in vertebrate neurons (Yuan and Yankner, 2000). Thus, upregulation of neurotrophins, such as BDNF, might reduce apoptosis and lead to enhance survival. Signaling components of BDNF and other trophic factors are dramatically upregulated in the mceph/mceph hippocampus (Lavebratt et al., 2006; Petersson et al., 1999). Equilibrium between neurogenesis and cell death is needed to maintain homeostasis. The enlargement of CA3 cannot be explained by adult neurogenesis since no significant upregulation of proliferation was detected here, however some of the mice analysed displayed massive proliferation in all regions of the hippocampus (Fig. 10, Almgren et al., unpublished results). Suggesting that modest proliferation is in fact present in CA3. Another possible explanation to the increased cell count in CA3 could be migration from either of the neurogenetic zones; both SVZ and SGZ are in close enough proximity. Neural progenitors in caudal SVZ can migrate to the hippocampus after ischemic injury (stroke) and contribute to CA1 cell regeneration, and migration from SVZ to hippocampus after status epilepticus has also been reported (Nakatomi et al., 2002; Parent et al., 2006). Finally, a reduced apoptosis level compared to wild type CA3 could explain the increased cell number in this area of mceph/mceph. Mice over expressing the anti-apoptotic protein Bcl-2 displays increased number of neurons in DG, but there is no increase of proliferation indicating that this is due to reduced apoptosis (Kuhn et al., 2005).
Fig. 10. Massive proliferation in mceph/mceph hippocampus labeled by BrdU at 9 and analyzed at 11 weeks of age.

POSSIBLE GENE CANDIDATES BEHIND THE HYPERPLASIA IN mceph/mceph HIPPOCAMPUS (paper III)

Carbamazepine protects against neuronal hyperplasia and abnormal gene expression in the megencephaly mouse.

As an attempt to find genes downstream the Kv1.1 involved in the hippocampal overgrowth of mceph/mceph we ran a microarray analysis to identify transcripts differently expressed between mceph/mceph and wild type hippocampus. In the comparison between mceph/mceph and wild type mice only six genes were found to be significantly differently expressed; Serpin, NpY, Fjx1, Penk1, Lrrk1, Vgf. Two of these genes, Neuropeptide Y and preproenkefalin, represented an immediate confirmation of the microarray results since our group previously showed that they are upregulated in mceph/mceph hippocampus with in situ hybridization and immunohistochemistry (Petersson et al., 2000). These two neuropeptides are commonly upregulated in epileptic models and NPY has been shown to increase proliferation in hippocampal cells (Hong et al., 1980; Howell et al., 2005; Howell et al., 2007). Fjx1 and Vgf were studied with qPCR and in situ hybridization, respectively, and found to be significantly upregulated in mceph/mceph.

Fjx1, encodes a transmembrane type II glycoprotein, a downstream target of Notch (Rock et al., 2005), known to regulate embryogenesis, tissue regeneration, and
carcinogenesis. *Fjx1* was recently identified as a new inhibitor of dendrite extension (Probst et al., 2007). We found *Fjx2* to be downregulated in the microarray analysis, but upregulated with qPCR. This difference could be due to variation in splicing, since the oligo on the chip is placed in the 3' UTR, whereas the qPCR primer is placed in the coding region of the only known exon.

**Vgf**, was recently shown to enhance neurogenesis in hippocampus (Thakker-Varia et al., 2007). *Vgf* mRNA synthesis seems to appear in neurons as they complete migration and begin to differentiate. Hippocampal *Vgf* mRNA is present in the CA1-CA3 region and at somewhat lower in the DG (Snyder et al., 1998). In *mceph/mceph* mice *Vgf* mRNA is upregulated primarily in the DG and CA3 (Fig 11). *Vgf* has a CREB binding site, which is necessary for induction by neurotrophins, and expression of VGF has been found to be induced by *Bdnf* (Alder et al., 2003). Furthermore, seizures induced by kainic acid have been found to increase *Vgf* mRNA levels in the dentate gyrus (Snyder et al., 1998a). The *mceph/mceph* mice, and other epileptic models, show marked upregulation of *Bdnf* mRNA and other neurotrophins and this could contribute to the high levels of *Vgf* found in these mice (Diez et al., 2003). Hence, VGF could be one of the regulators driving the hippocampal overgrowth in *mceph/mceph* mice.

![Fig. 11](image)  
**Fig. 11.** *Vgf* RNA expression detected by *in situ* hybridization in wild type, *mceph/mceph* and carbamazepine treated *mceph/mceph* mice.

Previously, we have shown that *Bdnf* is dramatically upregulated in *mceph/mceph* hippocampus (primers placed in coding exon VI). The failure to detect an increase in *Bdnf* upregulation in the microarray analysis could be explained by its complicated gene structure. In mice, this gene contains eight short 5' noncoding exons. Each of these exons can be alternatively spliced to the common coding exon VI. Each of these mRNA transcripts codes for an identical BDNF protein. However, the transcripts contain different 5' sequences. Each noncoding exon contains a unique promoter region with distinct chromatin architecture, which could modulate the expression of one splice variant compared to another (Aid et al., 2007; Tsankova et al., 2006). The oligo on the microarray slide is placed in the 3'UTR downstream the coding exon. Although no alteration yet is known in the 3'UTR, there may be such or other
upstream variations which could explain the failure to find expression similarity between primers placed in coding exon VI and the 3’UTR.

**CARBAMAZEPINE NORMALIZES THE NUMBER OF CELLS IN THE mceph/mceph HIPPOCAMPUS (paper III)**

**Protection against elevated cell numbers**
In an attempt to normalize the hippocampal overgrowth and abnormal cell number in *mceph/mceph* we investigated the effect of the antiepileptic drug carbamazepine (CBZ). In a previous study by our group CBZ was found to normalize hippocampus volume, despite remaining seizures (Lavebratt et al., 2006). Here we showed by stereological analysis of DG, hilus and CA1-CA3 that CBZ treatment completely protected from increased both neuron and astrocyte cell number. In CA1 of CBZ treated *mceph/mceph* mice both the number of neurons and astrocytes were slightly lower than in wild type. For exact cell number see Table 2. CA1 is known to be more sensitive to damage than the rest of hippocampus; hypoxia/ischemia and seizures induces cell death of the CA1 neurons (Kawasaki et al., 1990). Volume estimates, calculated using the Cavalieri principle, demonstrated a normalized size of the hippocampus by CBZ-treatment and thereby no significant difference in cell density between treated and non-treated *mceph/mceph*. Whether this normalization was due to a reduced proliferation or increased apoptosis or both remains to be studied.

**POSSIBLE GENE CANDIDATES BEHIND THE NORMALIZATION OF HYPERPLASIA IN mceph/mceph HIPPOCAMPUS (paper III)**

**CBZ altered expression – overgrowth protective genes?**
To find transcripts possibly regulating the normalization seen after CBZ treatment we ran a microarray analysis. Gene expression profiling comparing transcript levels in *mceph/mceph* enlarged hippocampus with those in normal-sized CBZ treated *mceph/mceph* and wild type hippocampus revealed many transcripts with differential level. In order to find genes which could potentially regulate the cell number, genes associated with proliferation or apoptosis were selected using an Ontology database. The following 7 genes were verified with ISH or qPCR and are described below; *Bhlhb5, Ndn, Mlc1, Sstr4, ApoD, Gprin1, Aatk,* and *Rgs2.*

*Bhlhb5,* is a member of a large group of transcription factors (basic helix-loop-helix), which are crucial regulators of neuronal cell generation and cell fate (Liu et al., 2000; Olson et al., 2001). *Bhlhb5* has been suggested to play an important role in glutamatergic neuron differentiation (Mattar et al., 2004), but further studies of
Bhlhb5 are needed to elucidate its function. mceph/mceph hippocampi showed lower levels of Bhlhb5 than wild type.

**Ndn**, is expressed predominantly in terminally differentiated postmitotic neurons and may be involved in permanent mitotic arrest (Yoshikawa, 2000). Necdin was reported to mediate intracellular processes essential for neurite outgrowth (Lee et al., 2005) and was suggested to induce cell cycle arrest and control neuronal apoptosis through interactions with the transcription factor E2F1 and the p75 neurotrophin receptor (Kuwako et al., 2004). In mceph/mceph Ndn was down regulated compared to CBZ-treated mceph/mceph. Hence, Ndn may contribute to the rescuing effect by CBZ, abrogating proliferation by differentiation.

Mutations in the **Mlc1** gene, which often result in lower expression of the protein, cause the progressive neurological disease megalencephalic leukoencephalopathy with subcortical cysts. The gene is expressed in astrocytes, ependymal and neuronal axons (Ilja Boor et al., 2006) (Teijido et al., 2004). The lower expression of Mlc1 seen in the megalencephalic mceph/mceph mice compared to wild type, correlates with the downregulation seen in megalencephalic leukoencephalopathy, both disorders also display seizures. CBZ increased the level of Mlc1 in mceph/mceph.

**Sstr4** encodes one of five G-coupled receptors through which somatostatin acts and is found on neuronal dendrites (Schreff et al., 2000). Four of the receptors (SSTR1, 2, 4, and 5) induce cell cycle arrest via PTP-dependent modulation of MAPK, associated with induction of the retinoblastoma tumor suppressor protein and p21(Patel, 1999). The increase of Sstr4 expression in mceph/mceph by CBZ treatment could hence contribute to mediate the protective effect of CBZ against excessive generation of cells.

**ApoD**, transports sterols, steroids and arachidonic acid for tissue repair and is highly expressed in brain (Seguin et al., 1995). Further, ApoD secretion is inversely correlated to cell proliferation and cell density and high ApoD levels is associated with cell cycle arrest (Sugimoto et al., 1994). The upregulation of this gene in treated mceph/mceph could contribute to the protection against excessive neurogenesis and gliogenesis, by reducing proliferation. The antipsychotic drug Clozapine was found to upregulate ApoD and other genes involved in lipid metabolism and neurotransmission (Thomas et al., 2003). Clozapine is commonly used for treatment of schizophrenia and bipolar disorder, and so is CBZ. Moreover, potential common mechanisms are not known and how CBZ interacts with ApoD remains to be elucidated.

**Gprin1**, is localized in the neural growth cone and is known to regulate neurite outgrowth via activation of Cdc42 (Nakata and Kozasa, 2005). Gprin1 was upregulated
in \textit{mceph/mceph} mice compared to wild type. This is in line with that neurons, at least CA3 pyramidal cells and hilar interneurons in DG, have enlarged soma size, since neurite and soma size correlate positively (Studer et al., 1994).

\textbf{Aatk}. High potassium level-induced hypophosphorylated \textit{Aatk} is associated with cell survival whereas low potassium level-induced hyperphosphorylated \textit{Aatk} mediates apoptosis in cerebellar granule cells (Tomomura et al, 2005). \textit{mceph/mceph} neurons lacks functional outward rectifier \textit{Kv1.1} that is involved in repolarization, hence intracellular potassium level may be elevated in \textit{mceph/mceph} neurons. Under this condition \textit{Aatk} could drive cell survival. The difference in intergroup expression between ISH and microarray analyses could be due to variation in RNA processing such as splicing between mouse groups and the different location of probes. \textit{Aatk} contains 14 known exons, and the oligo on the chip is placed in the 3' UTR, whereas the primers designed for ISH are placed in exons 6 and 11.

\textbf{Rgs2}, is widely expressed in mammalian tissues (Kehrl and Sinnarajah, 2002). Lower density of neuronal spines was found in \textit{Rgs2} knock-out mouse brain and it is believed that RGS2 has a role in synaptic development and basal electrical activity of hippocampal CA1 neurons. Furthermore \textit{Rgs2} knock-out T lymphocytes were reported to proliferate less. Both chronic and acute electro convulsive seizures are found to upregulate \textit{rgs2} in mouse brain. This is in line with the upregulation seen in the epileptic \textit{mceph/mceph} mouse.

\textbf{GABA receptor A5}

Several antiepileptic drugs reduce or interrupt seizures by suppression of synaptic neurotransmission via blockage of glutamate receptors or activation of GABA type A receptors (Meldrum, 1996; White et al., 2007). We show GABA receptor A5 upregulation after CBZ treatment; this may contribute to the normalization of cell number since suppression of synaptic neurotransmission may trigger apoptotic neurodegeneration (Bittigau et al., 2002; Katz et al., 2007). The upregulation of \textit{Gabra5} shown by microarray analysis was not tested with qPCR or ISH since this is in line with other studies (Nusser et al., 1998; Stell et al., 2003).

The ontology analysis of the transcripts differentially expressed between enlarged \textit{mceph/mceph} hippocampus and normal-sized CBZ-treated and wild type hippocampi revealed a large number of ion channel genes. Approximately 20\% of the annotated genes that were down regulated in untreated \textit{mceph/mceph} had channel/pore function. The known implications of ion channels in processes like volume regulation, apoptosis, proliferation and differentiation, makes this functional category highly relevant in this study. However, since there is likely a complex feed-back regulation, of ion-channel genes, to the CBZ treatment, the significance for this list of genes in the
regulation of the neurogenesis and apoptosis in mceph/mceph is unsure. Several other transcripts which we did not investigate are uncharacterized ESTs or genes that have not previously been described in the nervous system or with unknown ontology.

Apoptosis analysis of the CBZ treated hippocampus is in progress to reveal if the normalized cell number and size is due to an increase in programmed cell death.

**VGF**, which was found upregulated in mceph/mceph, could be one of the candidates driving the hippocampal overgrowth. It is proposed to be upregulated by seizures and neurotrophic factors. We show that Vgf expression is normalized by CBZ despite ongoing severe seizures similarly to what was seen for Bdnf. The CBZ treated mceph/mceph displayed class IV motor seizures (hindleg tonus) 13% of the time (Lavebratt et al, 2006).

### AED USE IS INCREASING AND HAS NEGATIVE EFFECT ON BODY DIMENSIONS IN UTERO (paper IV)

*Population-based study of antiepileptic drug exposure in utero - influence on head circumference in newborns. (Paper IV)*

In this study we analyzed the effect on head circumference of exposure in utero to AEDs during 1995-2005 in a large Swedish material. The analyses were adjusted for year of birth, maternal age, parity, smoking, and body mass index.

**Head circumference**
The most commonly used monotherapies were CBZ, followed by VPA, lamotrigine, and phenytoin. A significant reduction of mean birth-weight-adjusted head circumference (HC) was seen after CBZ and VPA monotherapy. The effect of CBZ and VPA was moderate – corresponding to less than one cm. However, it was shown that the rate of small HC (< 2 SD below the mean, corresponding to 2.2 – 5.4 cm below mean) was significantly increased after maternal use of AED in polytherapy. However, this was not found specifically for CBZ or VPA. CBZ has previously been shown to reduce body dimensions of the newborn, primarily HC (Hiilesmaa et al., 1981). However, a decline in the effect until the mid-nineties was previously suggested in an earlier Swedish study (Wide et al., 2000). No decline in effect of CBZ on HC over time was observed in the present study spanning 1995-2005 with more than double the population size. VPA was in a previous small study (n = 53) shown to reduce HC stronger than CBZ (Battino et al., 1992), however other small studies (n = 62 and 30) showed no VPA effect on HC (Arulmozhi et al., 2006; Bertollini et al., 1987).
None of the other four AEDs studied; phenytoin, clonazepam, lamotrigine and gabapentin, showed a significant effect on mean HC. For phenytoin, a teratogenic effect is known since long (Monson et al., 1973), but no effect on HC has been shown (Bertollini et al., 1987). Benzodiazepines usually have a low teratogenic effect and small studies of clonazepam in monotherapy (n=43) had little information value (Eros et al., 2002; Lin et al., 2004). Regarding gabapentin, few studies on the fetal effect have been published. In an investigation of the safety of gabapentin exposure in human pregnancy no increased risk was found compared to the general population but also this study had a low power due to the small number of patients (n=39) (Montouris, 2003). However, a recent report showed various malformations of the brain in mouse fetuses after gabapentin exposure in utero (Prakash et al., 2008). A dose-dependent teratogenic effect of lamotrigine has been published but effects on body dimensions have not been specifically studied (Morrow et al., 2006). There seems to be no clear-cut correlation between the teratogenic effect of an AED and the effect on mean HC. The mechanisms of teratogenicity are poorly understood and still less the effect on fetal growth, including head circumference.

**Gestational age**

CBZ has previously been proposed to reduce pregnancy duration (gestational age) (Wide et al., 2000). Analysis (adjusted for year of birth, maternal age, parity, smoking, and BMI) in this material supported an effect of CBZ mono- and polytherapy on gestational age (p = 0.003 and 0.05, respectively). Also, there was a tendency for clonazepam (p = 0.09) and gabapentin (p = 0.06) monotherapy to reduce gestational age. However, the mean reduction corresponded to only 3-4 days and may be explained by confounding by indication.

**Effect and use of CBZ and VPA over time**

To analyze difference in effect over time we divided the material into three groups; 1995-1997, 1998-2001 and 2002-2005. The use during pregnancy of CBZ was found to increase with 25% and VPA with 140%, between the first and last period in spite of recent reports on negative effects on newborns of these AEDs. The effect of VPA on mean HC changed over time and was lowest in the middle period (1998-2001) while no variation with time was seen for CBZ or all AEDs in group.

**Later cognitive effects?**

According to Dean and coauthors., autism, autistic and behavior disorders have been observed significantly more frequently in children exposed to VPA and CBZ monotherapy or polytherapy (Dean et al., 2002). Several studies indicate that a child’s IQ is negatively correlated with CBZ and VPA exposure in utero (Adab et al., 2001; Dean et al., 2002; Matalon et al., 2002; Ornoy and Cohen, 1996). Could a reduced head circumference be an indication of cognitive impairment later in life? A follow-up
study after 5.5 years of children with reduced HC at birth showed no significant remaining HC reduction (Gaily et al., 1990). However, no cognitive decline was measured, so this needs to be studied further.

AEDs are not only used to treat epilepsy: VPA, gabapentine, lamotrigine and CBZ are currently used as mood stabilizers in bipolar affective disorder in addition to other non-epilepsy diagnoses. We had no data on the indications for the prescription of the AEDs. Nevertheless, the negative effects of CBZ, VPA and gabapentin are most likely similar regardless of indication since the range of therapeutic dose is similar.
5 CONCLUDING REMARKS

Neurogenesis is a complex process and the details of its regulation are still not clear. Hippocampal neurogenesis is upregulated by exercise and in enriched environment and it is proposed to assist in learning and memory formation. Furthermore it is increased in diseases like stroke and epilepsy; however it is debated whether this process is due to self-repair or part of the pathology. In the present thesis a mouse model of epilepsy and excessive brain growth has been used to investigate how dysfunction of a potassium ion channel subunit, Kv1.1, can increase hippocampal neurogenesis, and how this hyperplasia can be treated.

This study demonstrates expression of a truncated Kv1.1 protein in brain. This MCEPH protein is trapped within the ER and has the ability to interact with Kv1 subunits resulting in a dominant negative effect on current. mceph/mceph mice constitute a tool to study Kv1.1 trafficking relevant for understanding epilepsy, ataxia and pathologic brain overgrowth. Furthermore, the rare phenotype seen in the mceph/mceph mouse i.e. epilepsy and brain enlargement is likely due to lack of Kv1.1 in the brain since Kv1.1 knock-out mice display seizures and similar brain enlargement. However, the truncated MCEPH protein likely adds a function in the mouse brain since the epilepsy phenotype is more severe in the mceph/mceph than in the knock-out mice.

Adult neuronal hyperplasia leading to a markedly enlarged hippocampus has previously not been reported. We have investigated steps in the chain of neurogenesis; proliferation, migration and survival, and drawn the conclusion that adult neurogenesis is enhanced in the mceph/mceph mice. These findings are in agreement with the 2-fold more neurons found in these mice. Thus, the hippocampus enlargement is at least in part due to an increased neurogenesis in the dentate gyrus in combination with an enhanced cell survival/reduced apoptosis. The reduced ability to regulate intracellular potassium levels in these mice may have implications on apoptosis and thereby favor survival of the new cells. The enlarged CA3 is in part due to increased cell number, neurogenesis has never been demonstrated in this region, and hence migration and/or reduced apoptosis are likely explanations. The hyperplasia and enlargement are due to lack of Kv1.1, and possibly reduced levels of other Kv1 subunits. In conclusion, the mceph/mceph mouse is a unique model of excessive adult neurogenesis, in an epileptic state, resulting in regional hippocampal enlargement.

CBZ was demonstrated to protect against an excessive increase in number of neurons and astrocytes in the hippocampus of the adult mceph/mceph mouse, despite ongoing
severe seizures. Genes which may mediate, or transcript levels that correlate to, this protective effect of CBZ includes NPY, Penk, Vgf, Mlc1, Sstr4, ApoD, Ndn, Aatk, Rgs2 and Gabar5. These genes have influence on proliferation, neurogenesis or apoptosis but more studies are required to unravel details on their function in hippocampus. A novel regulator of neurogenesis, Vgf, may be of particular interest for involvement in the rare brain growth and adult neurogenesis driven by the potassium channelopathy in the mceph/mceph mice. The anti-seizure mechanism of CBZ are mainly through blockage of Na⁺ channels, our results also support a CBZ effect on synaptic transmission through GABA A receptors. Previous studies have suggested that apoptotic neurodegeneration caused by blockage of synaptic transmission might explain some teratogenic effects of other AEDs. This mechanism needs to be studied in detail regarding CBZ treatment. The normalization of hyperplasia implies that CBZ may be used to treat certain types of megalencephaly.

We have used a large population-based cohort and shown that there is an apparent difference between AED drugs taken during pregnancy with respect to their effect on HC at birth. The strongest effects were seen for CBZ and VPA. The effects were of moderate size, but were statistically significant and remained from 1995 to 2005. The outcome of a reduced HC on cognitive development of the child is not known and needs further investigation. These findings should be considered in light of the fact that CBZ or VPA was given to 71% of the cases, a prescription rate that increased with 25% for CBZ and 140% for VPA over the last decade. Phenytoin, clonazepam, lamotrigine and gabapentin had no observable effect on HC. CBZ mono- and polytherapy significantly reduced gestational age and there was a tendency for clonazepam and gabapentin monotherapy to do the same. The relevance of the reduced gestational age is uncertain but these findings make further studies warranted in order to optimize treatment regimes for pregnant women. For example, more detailed studies on new generation AEDs are required to safely substitute CBZ and VPA in order to avoid negative effects in utero.
6 FUTURE PERSPECTIVES

There are several questions remaining regarding regulation and function of the increased number of neurons in the mceph/mceph mouse. Concerning the effect and mechanism of AEDs in utero further research is required. Below is a list of ongoing research projects and a number of experiments to be conducted in the future.

- The pool of neural progenitors in the dentate gyrus of mceph/mceph and wild type mice are being studied with MRS. This is done in collaboration with Dr. Mirjana Maletić-Savatić at Cold Spring Harbor Laboratory.

- Apoptosis in CBZ treated mceph/mceph hippocampus, which is currently under investigation.

- Screening patients with megalencephaly and epilepsy for mutations in Kv1 genes. Ongoing in collaboration with several European laboratories.

- Investigate the importance of the microenvironment on mceph/mceph neurogenesis in vitro and in vivo by transplantation of granule cells from mceph/mceph DG to wild type.

- Functional studies of gene candidates behind hyperplasia and its normalization by CBZ.

- It would be of interest to study the effect of additional AEDs on the hyperplasia in mceph/mceph brain; this may reveal mechanisms of normalization and regulation of neurogenesis and or apoptosis.
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APPENDIX I