SELMA KYLLIKKI KAASINEN

Putrescine Accumulation in Mouse Central Nervous System

Neuroprotection at the Expense of Learning Deficiency

Doctoral dissertation

To be presented by permission of the Faculty of Medicine of the University of Kuopio for public examination in Auditorium, Tietoteknia building, University of Kuopio, on Saturday 20th March 2004, at 12 noon

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ABSTRACT

Naturally occurring polyamines are positively charged molecules, which are ubiquitously present in the central nervous system. Polyamine metabolism consists of both synthesis and catabolism, processes which are strictly regulated by the enzymes ornithine decarboxylase (ODC), S-adenosylmethionine decarboxylase and spermidine/spermine N₁-acetyl-transferase (SSAT). Polyamines, spermidine and spermine, as well as the diamine putrescine bind to all anionic structures in neuronal and non-neuronal cells of the brain, and therefore they can influence many intracellular processes. In the brain, spermidine and spermine have been the most studied polyamines, so far, while putrescine has received less attention. Putrescine is produced in two ways: it is a product of activated polyamine biosynthesis and of the polyamine catabolic cycle. It is recognized that accelerated polyamine metabolism can lead accumulation of high amounts of putrescine. Therefore, in the literature putrescine accumulation is often considered to be a sign of neurotoxicity. The delayed increase in the putrescine levels which occurs after brain injury or neurotoxic insult is suggested to be a cause of delayed neuronal death. On the other hand, putrescine accumulation is linked to the repair functions and it has been proposed to have a neurotherapeutic role in neuronal damage.

We wanted to study the role and effects of polyamines, especially putrescine, in the brains of mice after excitotoxic stimuli. However, putrescine is normally undetectable, reaching measurable concentrations only after extracellular stress and hence, it is a difficult molecule to study. We exploited an earlier produced transgenic mouse strain with extra copies of SSAT in its genome. This results in accelerated polyamine catabolism, which in turn produces a large amount of putrescine in the tissues. This animal model allowed us to study the effects of SSAT overexpression and high putrescine concentration both under normal circumstances and after excitotoxic treatments. In addition we wanted to undertake a comprehensive behavioural profile of SSAT mice.

We showed that the brains of SSAT mice had a lifelong alteration in polyamine metabolism, accumulation of putrescine, the appearance of N₁-acetylspermidine and a decreased level of spermidine. The change in polyamine metabolism is the same as seen in wildtype mice and rats after stressful stimuli. SSAT mice had reduced mortality and they were able to tolerate excitotoxic stimuli induced by kainic acid and pentylenetetrazol (PTZ) showing less damage to pyramidal cells in hippocampus. The reduced PTZ induced seizure activity in SSAT mice, was reversed in syngenic mice by ifenprodil, a known NMDA receptor antagonist binding to a common binding site with polyamines. Further studies made in behavioural profiling revealed hypoactivity and reduced aggression of SSAT mice in addition to a sex dependent learning deficiency. Interestingly, also levels of several hormones were altered in transgenic mice. Adrenocorticotropic and corticosterones, known to be involved in the hypothalamic-pituitary-adrenal (HPA) axis, were significantly increased in SSAT mice, which may contribute to the long-term hyperactivation of the HPA system. On the other hand, concentrations of testosterone, thyroid stimulating hormone and thyroxine declined in SSAT mice. Both hyperactivity of HPA and reduced thyroid hormones may be involved in mediating the learning disabilities.

It seems likely that the accumulation of putrescine has a neuroprotective role in SSAT mice, but altered hormone levels, possibly as a result of the disturbed polyamine metabolism, are responsible for the behavioural and learning alterations in SSAT mice.
"Äiti - mulla on ikävä sua.
Yöllä yhdenlainen,
päivällä toinen.
Nuku hyvin äiskä.
T: Jenna-mussu"
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Kuopio, February 2004

Selma Kaasinen
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>AdoMet</td>
<td>$S$-adenosylmethionine</td>
</tr>
<tr>
<td>AdoMetDC</td>
<td>$S$-adenosylmethionine decarboxylase</td>
</tr>
<tr>
<td>AMPA</td>
<td>$\alpha$-amino-3-hydroxy-5-methyl-4-isoxazole-proprionic acid</td>
</tr>
<tr>
<td>Amyg</td>
<td>amygdaloid complex</td>
</tr>
<tr>
<td>CA1-CA3</td>
<td>subfields 1-3 of cornu Ammonis</td>
</tr>
<tr>
<td>cc</td>
<td>corpus callosum</td>
</tr>
<tr>
<td>Cerebral cortex</td>
<td>C.cx</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>cSAT</td>
<td>cytosolic spermidine/spermine $N^1$-acetyltransferase</td>
</tr>
<tr>
<td>DAB</td>
<td>3,3'-diaminobenzidine</td>
</tr>
<tr>
<td>dAdoMet</td>
<td>decarboxylated $S$-adenosylmethionine</td>
</tr>
<tr>
<td>DAH</td>
<td>diaminoheptane</td>
</tr>
<tr>
<td>DG</td>
<td>dentate gyrus</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>EPSP</td>
<td>excitatory post-synaptic potential</td>
</tr>
<tr>
<td>GABA</td>
<td>$\gamma$-aminobutyric acid</td>
</tr>
<tr>
<td>GFAP</td>
<td>glial fibrillary acidic protein</td>
</tr>
<tr>
<td>GrDG</td>
<td>granular layer dentate gyrus</td>
</tr>
<tr>
<td>Hil</td>
<td>hilus dentate gyrus</td>
</tr>
<tr>
<td>HPLC</td>
<td>high pressure liquid chromatography</td>
</tr>
<tr>
<td>i.p.</td>
<td>intraperitoneal</td>
</tr>
<tr>
<td>IPSP</td>
<td>inhibitory post-synaptic potential</td>
</tr>
<tr>
<td>i.v.</td>
<td>intravenous</td>
</tr>
<tr>
<td>KA</td>
<td>kainic acid, kainate</td>
</tr>
<tr>
<td>LTP</td>
<td>long-term potentiation</td>
</tr>
<tr>
<td>Mol</td>
<td>molecular layer dentate gyrus</td>
</tr>
<tr>
<td>MRI</td>
<td>magnetic resonance imaging</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>$N^1$-AC-SPD</td>
<td>$N^1$-acetyl-spermidine</td>
</tr>
<tr>
<td>NGS</td>
<td>normal goat serum</td>
</tr>
<tr>
<td>NMDA</td>
<td>$N$-methyl-D-aspartate</td>
</tr>
<tr>
<td>nSAT</td>
<td>nuclear spermidine/spermine $N^1$-acetyltransferase</td>
</tr>
<tr>
<td>ODC</td>
<td>ornithine decarboxylase</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>PAO</td>
<td>polyamine oxidase</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PFA</td>
<td>paraformaldehyde</td>
</tr>
<tr>
<td>PTX</td>
<td>picrotoxin</td>
</tr>
<tr>
<td>PTZ</td>
<td>pentylenetetrazol</td>
</tr>
<tr>
<td>PUT</td>
<td>putrescine</td>
</tr>
<tr>
<td>Pyr</td>
<td>pyramidal cell layer of hippocampus</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>s.c.</td>
<td>subcutaneous</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecylsulfate</td>
</tr>
<tr>
<td>sg</td>
<td>syngenic, a mouse without the mutated gene</td>
</tr>
<tr>
<td>SMO</td>
<td>spermine oxidase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>SPM</td>
<td>spermine</td>
</tr>
<tr>
<td>SPD</td>
<td>spermidine</td>
</tr>
<tr>
<td>SSAT</td>
<td>spermidine/spermine N(^1)-acetyltransferase</td>
</tr>
<tr>
<td>SSAT-OE</td>
<td>SSAT overexpressing mice</td>
</tr>
<tr>
<td>SSC</td>
<td>standard saline citrate</td>
</tr>
<tr>
<td>St Rad</td>
<td>stratum radiatum</td>
</tr>
<tr>
<td>St Or</td>
<td>stratum oriens</td>
</tr>
<tr>
<td>TBS</td>
<td>tris buffered saline</td>
</tr>
<tr>
<td>TdT</td>
<td>terminal deoxynucleotidyl transferase</td>
</tr>
<tr>
<td>tg</td>
<td>transgenic, a mouse with the mutated gene</td>
</tr>
</tbody>
</table>
Figure 1. SSAT overexpressing mouse with a littermate. The loss of hair and wrinkled skin are the most prominent features of the phenotype.
LIST OF ORIGINAL PUBLICATIONS

This thesis was based on the following publications referred to by their corresponding Roman numerals:


III Kaasinen S.K., Oksman M., Alhonen L., Tanila H. and Jänne J. (2004) Spermidine/spermine N<sup>1</sup>-acetyltransferase overexpression in mice induces hypoactivity and spatial learning impairment but no change in hippocampal long-term potentiation of NMDA toxicity. *Accepted to Pharmacology Biochemistry and Behaviour*

In addition, some unpublished data are presented.
# TABLE OF CONTENTS

ABSTRACT ................................................................. i  
ACKNOWLEDGEMENTS ............................................. ii  
ABBREVIATIONS ..................................................... iii  
LIST OF ORIGINAL PUBLICATIONS ............................... iv  

1 INTRODUCTION ....................................................................................................................... 17

2 REVIEW OF THE LITERATURE ........................................................................................................ 21

## 2.1. POLYAMINES IN THE CENTRAL NERVOUS SYSTEM (CNS) ...................................................... 21

### 2.1.1. POLYAMINE CONCENTRATIONS AND DISTRIBUTION IN THE BRAIN .............................. 21

### 2.1.2. MAINTENANCE OF INTRACELLULAR POLYAMINE HOMEOSTASIS ....................................... 24

#### 2.1.2.1. Polyamine biosynthesis ................................................................. 24

##### 2.1.2.1.1. Ornithine and ornithine decarboxylase (ODC) .......................................................... 24

##### 2.1.2.1.2. S-adenosylmethionine decarboxylase (AdoMetDC) ....................................................... 25

##### 2.1.2.1.3. Putrescine (PUT) .................................................................................. 27

##### 2.1.2.1.4. Spermidine synthase and spermidine (SPD) ............................................................... 28

##### 2.1.2.1.5. Spermine synthase and spermine (SPM) ................................................................. 28

#### 2.1.2.2. Polyamine catabolism ................................................................. 29

##### 2.1.2.2.1. Spermidine/spermine N1-acetyltransferase (SSAT) ....................................................... 29

##### 2.1.2.2.2. Acetylated polyamines .................................................................................. 30

##### 2.1.2.2.3. Polyamine oxidase (PAO) .................................................................................. 31

#### 2.1.2.3. Other amine oxidases .......................................................................................... 31

#### 2.1.2.4. Regulation of polyamine metabolism ........................................................................ 32

## 2.2. POLYAMINES IN NEUROPATHOLOGICAL STATES .................................................................... 33

## 2.3. THE HIPPOCAMPAL NEURONAL CIRCUITS AND THEIR ROLE IN .......................................... 37

### 2.3.1. THE ANATOMICAL STRUCTURE OF HIPPOCAMPUS .......................................................... 37

### 2.3.2. THE NEURONAL CIRCUITS OF HIPPOCAMPUS .................................................................. 38

### 2.3.3. HIPPOCAMPUS IN LEARNING AND MEMORY ................................................................. 40

### 2.3.4. GLUTAMATE RECEPTORS .......................................................................................... 41

#### 2.3.4.1. α-Amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA)/kainate receptors ................................................................. 41

#### 2.3.4.2. N-methyl-D-aspartate (NMDA) receptors ..................................................... 43

##### 2.3.4.2.1. Polyamine binding sites on the NMDA receptor .......................................................... 46

##### 2.3.4.2.2. Ifenprodil binding site on the NMDA receptor .......................................................... 47

### 2.3.5. GABA RECEPTORS .......................................................................................... 47

## 2.4. EXPERIMENTAL ANIMAL MODELS OF CHEMICAL BRAIN INJURY ....................................... 48

### 2.4.1. KAINIC ACID (KA) .......................................................................................... 49

### 2.4.2. PENTYLENETETRAZOL (PTZ) .................................................................................. 50

## 2.5. BEHAVIOURAL PHENOTYPING OF TRANSGENIC MICE ....................................................... 51

### 2.5.1. SHIRPA .......................................................................................... 51

### 2.5.2. RADIAL ARM MAZE .................................................................................. 52

## 2.6. TRANSGENIC MICE OVER-PRODUCING PUTRESCINE ........................................................... 53

### 2.6.1. ODC TRANSGENIC ANIMALS .................................................................................. 53

### 2.6.2. SSAT TRANSGENIC ANIMALS .................................................................................. 54
3 AIMS OF THE STUDY ............................................................................................................. 56

4 EXPERIMENTAL PROCEDURES ............................................................................................. 57
  4.1. ANIMALS (I-III) .................................................................................................................... 57
  4.2. BEHAVIOUR STUDY (III) .................................................................................................... 57
     4.2.1. SHIRPA PROTOCOL ........................................................................................................ 57
     4.2.2. EIGHT ARM RADIAL MAZE .......................................................................................... 58
  4.3. DRUG TREATMENTS (I-II) .................................................................................................. 59
     4.3.1. KAINIC ACID (I) .............................................................................................................. 59
     4.3.2. PENTYLENETETRAZOL (II) .......................................................................................... 60
     4.3.3. IFENPRODIL (II) .............................................................................................................. 60
  4.4. SAMPLE PREPARATION (I-III) ........................................................................................... 60
     4.4.1. PERFUSION AND SAMPLE COLLECTION (I-II) ............................................................. 60
     4.4.2. HISTOCHEMISTRY (I-II) ................................................................................................ 61
     4.4.3. IN SITU HYBRIDIZATION (I) .......................................................................................... 61
     4.4.4. NORTHERN BLOTTING (I) ............................................................................................. 61
     4.4.5. POLYAMINE ANALYSIS (I-II) ....................................................................................... 62
     4.4.6. MEASURING OF SSAT ENZYME ACTIVITY (II) .......................................................... 62
     4.4.7. HORMONE ANALYSIS FROM BLOOD SAMPLES AND TISSUE WEIGHT .... 62
     4.4.8. MRI ANALYSIS (II) ......................................................................................................... 63
  4.5. OTHER ANALYSES ............................................................................................................... 63
     4.5.1. SEIZURE SCORING (I-II) ................................................................................................ 63
     4.5.2. CELL COUNTS (I-II) ....................................................................................................... 63
     4.5.3. IMAGE ANALYSING METHOD (I) .................................................................................... 64
  4.6. STATISTICAL ANALYSES (I-III) .......................................................................................... 64

5 RESULTS .................................................................................................................................... 65
  5.1.1. POLYAMINE CONCENTRATIONS IN DIFFERENT BRAIN REGIONS .......... 65
  5.1.2. THE EFFECT OF KAINIC ACID ON POLYAMINE CONCENTRATIONS IN VARIOUS BRAIN REGIONS ............................................................................................................. 65
  5.1.3. THE EFFECT OF PENTYLENETETRAZOL ON POLYAMINE CONCENTRATIONS AND SSAT ACTIVITY IN VARIOUS BRAIN REGIONS .......... 67
  5.2. SSAT mRNA EXPRESSION IN SYNGENIC AND TRANSGENIC MICE (I) .......... 68
  5.3. KAINIC ACID (I) .................................................................................................................. 68
     5.3.1. SEIZURE ACTIVITY ......................................................................................................... 68
     5.3.2. LOSS OF NEURONS AND MORTALITY ............................................................................ 69
     5.3.3. GFAP EXPRESSION AFTER KA TREATMENT ................................................................. 69
  5.4. PENTYLENETETRAZOL (II) .................................................................................................. 70
     5.4.1. SEIZURE THRESHOLD .................................................................................................. 70
     5.4.2. NEURONAL DAMAGE .................................................................................................... 70
     5.4.3. PTZ COMBINED WITH IFENPRODIL ................................................................................ 70
  5.5. BRAIN METABOLITE ANALYSIS BY MRI (II) ................................................................. 71
  5.6. DIFFERENCES IN HORMONE CONCENTRATIONS BETWEEN SSAT SYNGENIC AND TRANSGENIC MICE ................................................................................................. 71
  5.7. BEHAVIOUR DATA (III) ........................................................................................................ 72
     5.7.1. COMPREHENSIVE BEHAVIOUR OF SSAT MICE ....................................................... 72
     5.7.2. LEARNING AND MEMORY ............................................................................................ 72
         5.7.2.1. Acquisition task .......................................................................................................... 72
         5.7.2.2. Retention ..................................................................................................................... 73
6 DISCUSSION .................................................................................................................................. 74

6.1. THE EFFECT OF NEUROTOXINS ON THE POLYAMINE CONCENTRATIONS IN SYNGENIC AND SSAT TRANSGENIC MICE ........................................................................ 74

6.2. NEURONAL DAMAGE AND SEIZURE ACTIVITY IN SSAT MICE ........................................ 77

6.2.1. NEUROPATHOLOGICAL CHANGES INDUCED BY NEUROTOXINS ............................... 77

6.2.2. PENTYLENETETRAZOL-INDUCED SEIZURE ACTIVITY ........................................... 79

6.3. THE INFLUENCE OF POLYAMINES ON BEHAVIOUR ......................................................... 80

6.3.1. BEHAVIOURAL EFFECTS OF POLYAMINES ............................................................... 80

6.3.2. LEARNING AND MEMORY IN SSAT TRANSGENIC MICE .......................................... 81

7 SUMMARY .................................................................................................................................... 83

8 REFERENCES .................................................................................................................................. 84

APPENDIX: ORIGINAL PUBLICATIONS I-III
1 INTRODUCTION

This study has examined the effect of accelerated polyamine catabolism and especially the influence of overproduced diamine putrescine (PUT) on brain polyamine homeostasis. In particular, we were interested in neuronal survival after a variety of excitotoxic insults. This study also evaluated how disturbed polyamine catabolism results in mouse behaviour.

In addition, this thesis is a survey of the vast literature published on polyamines describing how polyamines participate in the function of the brain under normal conditions and in neuropathological states. Polyamines, spermidine (SPD) and spermine (SPM) as well as PUT are small molecules that are positively charged at physiological pH. Due to their cationic nature polyamines can bind very tightly to negatively charged molecules: e.g. DNA, RNA, proteins and other negatively charged membrane constituents in the cell. However, still today there is no reliable measurement for the free intracellular polyamines. The binding efficiency of polyamines increases with the number of charges (PUT<SPD<SPM). Thus SPM possesses the highest binding affinity to cellular compartments. Their cationic nature makes polyamines difficult to study while the influence of polyamines on several intracellular functions has proved to be a challenge for scientists. For the last four decades, polyamines have both fascinated and frustrated scientists throughout the world.

The pioneers of polyamine research in the central nervous system are Dudley and Rosenheim (1927), Hämäläinen (1947), Rosenthal and Tabor (1956), Kewitz (1959) and Shimizu (1964), who determined for the first time the polyamine content in the brain. The work of Kremzner (1970), Shaskan (1973), Caldarera (1969) and Seiler with coworkers (1974) laid the foundation on for a better understanding of polyamines in relation to the development of the embryo, the different regions of the mature brain and the aging. It became evident that polyamines are distinctively present at a time of rapid growth and proliferation of nerve cells and non-neuronal cells within the central nervous system (Gilad and Gilad, 1992; Laitinen et al., 1982; Morrison et al., 1995; Slotkin and Bartolome, 1986) whereas in the mature brain the function of polyamines is probably not related to growth (Morrison et al., 1995; Slotkin et al., 2000).

Studies on neuronal damage models have shown that polyamine homeostasis is seriously disturbed as a result of extracellular insults leading to an increase of the polyamine synthesizing enzyme, ornithine decarboxylase (ODC), accumulation of PUT and also alterations in SPD and
SPM concentrations (de Vera et al., 1997; Gilad and Gilad, 1992; Lukkarinen et al., 1999; Paschen, 1992a; Reed and de Belleroche, 1990). Furthermore, it soon became evident that stressful stimuli to the cells also activate the backconversion of polyamines. The evidence for that is provided by the increased activity of the polyamine catabolic enzyme, spermidine/spermine N\textsubscript{1}-acetyltransferase (SSAT), and by the appearance of the acetylated form of polyamines, N\textsubscript{1}-acetylspermidine (Baudry and Najm, 1994; Ingi et al., 2001; Seiler and Bolkenius, 1985; Zoli et al., 1996b).

The accumulation of PUT following noxious stimuli is most probably the result of both enhanced biosynthesis and catabolism of polyamines. Both biosynthetic decarboxylases are involved in PUT accumulation: ODC activation produces PUT while on the other hand, the decrease seen in S-adenosylmethionine decarboxylase (AdoMetDC) activation after noxious stimuli appears to decelerate the turnover rate of PUT into SPD and leads to PUT accumulation. The reduction in AdoMetDC activity is also the reason for the decrease in the SPD pool after insults. In addition, activation of SSAT accelerates the reutilization of polyamines. Polyamine oxidase (PAO) is probably not a regulator of the polyamine interconversion cycle and hence, again putrescine is produced. The delay described in the increase of PUT after an insult is more likely due to its reutilization. However, the actual significance of the polyamine fluctuation at the time of neuronal trauma and shortly thereafter has lead to conflicting conclusions (de Vera et al., 1997; Gilad and Gilad, 1992; Kauppinen and Alhonen, 1995; Paschen, 1992a) and still today the role of polyamines in neuronal death is somewhat obscure.

A number of studies have shown indisputably that polyamines have binding sites at least on two types of glutamate receptor subtypes and on inwardly rectifying K\textsuperscript{+} channels (Ficker et al., 1994; Oliver et al., 2000). Ca\textsuperscript{2+} permeable \(\alpha\)-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) receptors are blocked by intra- and extracellular SPM and to lesser extent by SPD (DiScenna et al., 1994; Donevan and Rogawski, 1995; Isa et al., 1996; Washburn and Dingledine, 1996). On the other hand, in \(N\)-methyl-D-aspartate (NMDA) receptors, polyamines have several binding sites on the outer side of the membrane and probably one binding site on the open channel pore. Polyamines, mainly SPD and SPM, have dual properties at the NMDA receptor by both increasing and decreasing NMDA receptor activation. PUT is believed to have a weak antagonistic effect on NMDA receptors binding to the open channel pore (Kashiwagi et al., 1997; Romano et al., 1991; Williams, 1997a; Williams et al., 1990; Williams, 1989). In the light of diverse properties of polyamines at excitatory glutamate receptors, polyamines are thought to have an important role in synaptic plasticity and neuronal signalling.
In the following studies we used transgenic mice overexpressing the polyamine catabolic enzyme, SSAT, under the control of its own promotor (Pietila et al., 1997). Non-transgenic littermates having the same genetic background (BALBe/DBA 2 strain), here called syngenic mice were used as control animals in each study.

In order to investigate the role of polyamines in neuropathological states, we used two different excitotoxic drugs, kainic acid (KA) and pentylenetetrazol (PTZ), to mimic epileptic like seizure-induced neuronal damage. Two separate models complement each other in terms of the effect measured and emphasize the generability of any results. KA activates NMDA receptors through AMPA/KA receptors and induces an inflow of Ca$^{2+}$ ions, which in turn is toxic to neurons. Conversely, PTZ inhibits the activation of GABA receptors which then promotes the enhanced excitement of glutamate receptors. We compared KA induced neuronal cell loss and mortality, and further, PTZ induced kindling in a combination with ifenprodil between syngenic and SSAT transgenic mice. In addition, we subjected the animals to a behavioural test battery to study possible abnormal behaviour of the animals due to their disturbed polyamine homeostasis. Finally, we also performed blood sampling to analyse the hormone concentrations of mice.

We found out that overexpression of SSAT enzyme contributes to the disturbed polyamine metabolism in every brain area of SSAT mice. The main changes were an overproduction of PUT, the appearance of $N^\text{i}$-acetylsperrmidine and a decrease in SPD concentrations. Although PUT is a known precursor of $\gamma$-aminobutyric acid (GABA), the accumulation of PUT did not influence GABA levels in CNS. SSAT mice were distinctively protected from the neuronal death induced by KA or PTZ and they had reduced overall mortality. Further, the threshold to PTZ induced seizure activity was increased in SSAT mice. This difference between transgenic and syngenic mice disappeared, when PTZ infusion was combined with ifenprodil. The results obtained from two different excitotoxic models indisputably favour the neuroprotective response of accelerated polyamine catabolism and indeed, the overaccumulation of PUT. Instead, the comprehensive behaviour analysis battery (SHIRPA) revealed the hypoactivity of SSAT mice. The animals were less aggressive and had reduced muscle tone when compared to the syngenic mice. Moreover, especially female SSAT mice were shown to have a learning deficiency. Sex dependent impairment in learning may imply an interaction between polyamines, estrogen and NMDA receptors, which also warrants further studies.
Figure 2. The structural formula of polyamines and acetylated polyamines. In addition, the structural formula of the inhibitor of polyamine biosynthesis (D,L)-2-(difluoromethyl)ornithine (DFMO), the competitive antagonist of N-methyl-D-aspartate (NMDA) receptors, ifenprodil and the terminal catabolite of putrescine, γ-aminobutyric acid (GABA).
2 REVIEW OF THE LITERATURE

2.1. POLYAMINES IN THE CENTRAL NERVOUS SYSTEM (CNS)

Natural polyamines are low molecular weight aliphatic polycations, which contain two to four primary amine groups at each carbon chain (Figure 2). SPM was the first polyamine found in 1678 by A. Leeuwenhoek, who observed SPM phosphate crystals in human semen, but the discovery was not understood until 1791 when L.N. Vauquelin rediscovered SPM. Later, in 1889, von Udránszky and Baumann described that the source of putrefying odours of corpses is a diamine, named as PUT. It was not until the 20th century when the brain polyamines were discovered. In 1925 Dudley and Rosenheim found SPM, at that time called neuridine, in brain tissue. By 1930's, the polyamines had been characterized to comprise of five compounds: PUT, cadaverine, 1,3-diaminopropane, SPD and SPM (Cohen, 1998; Seiler, 1994), of which the last two have been most extensively studied.

We can consider that the history of brain polyamine research began in the 1950's when increasing interest in polyamines and improved analytical techniques allowed the study of brain polyamines among several species; in humans by Hämäläinen (1957), in rat by Rosenthal and Tabor (1956), in pig by Kewitz (1959), in rabbit by Shimizu et al. (1964) (referred to in Kremzner, 1970) and in mouse (Shimizu et al., 1965) as well as the comparison of polyamine distribution (Perry et al., 1967; Shaw and Pateman, 1973; Shimizu et al., 1964) in the central nervous system (CNS). However, the progress in the study of the involvement of polyamines in biological processes in CNS was quite slow until the 1970's.

2.1.1. POLYAMINE CONCENTRATIONS AND DISTRIBUTION IN THE BRAIN

It was first observed in rabbits (Shimizu et al., 1964 in (Kremzner, 1970)) that the concentration of SPM is highest in cortical grey matter, subsequently the same observation was made later in rats, sheep and humans (Kremzner, 1970). On the other hand, SPD and PUT occurs at the greatest concentrations in cortical white matter (Kremzner, 1970) and in mice PUT is found in both grey and white matter (Fischer et al., 1972). Polyamines are unevenly distributed in the
brain and the main regional differences in polyamine concentrations are seen in cerebral cortex, cerebellum, medulla and pons. In rats, the SPD concentration is lowest in cerebral cortex while medulla, pons and cerebellum have the highest concentration. The SPM concentration is highest in cerebellum and cerebral cortex and lowest in medulla and pons (Shaskan et al., 1973). Shaskan and Snyder (1973) proposed that the regional increases and declines in brain polyamine concentrations might be due to the transfer of polyamines between the areas.

Changes in polyamine concentrations during the development of CNS (Caldarera et al., 1969; Pearce and Schanberg, 1969; Shimizu et al., 1965) have likewise been under intense study. In rats, both SPD and SPM concentrations increase during the developmental phase in the foetus (Kremzner, 1970) being about 20-fold higher at the time of birth in comparison with those of mature brain. There is a remarkable drop in both polyamine concentrations at birth. SPD concentration increases rapidly during the subsequent weeks in the post-natal phase reaching the highest concentration in youth and then there is a new decrease until the concentration of adults is reached (Pearce and Schanberg, 1969). In mature brain, the concentration of SPM is fairly stable during aging (Pearce and Schanberg, 1969; Shaskan, 1977). The changes in the SPM concentration in mice are similar to those in rats (Shimizu et al., 1965) but the mature level is reached in a much shorter (10-12 days) time (Laitinen et al., 1982). In contrast, the SPD concentration of newborn rats decreases in cerebral cortex during the first three to four weeks (Kremzner, 1970; Shaskan, 1977) as it does in mice brain (Shimizu et al., 1965) although in a shorter period (Laitinen et al., 1982). Thereafter the amount of SPD increases for one week until it reaches the level of adults. In addition to SPD and SPM, the PUT concentration is also known (Laitinen et al., 1982) to be very high in foetus and it declines in a similar fashion as SPD during the post-natal development in mice. In chick embryos (Caldarera et al., 1969), SPD and SPM concentrations were opposite to the situation in rats and mice. In the brain of fish the concentrations of polyamines, mainly PUT and SPD, increase during the whole life-span of the fish while the SPM pool remains stable (Seiler and Lamberty, 1973). One exceptional observation is derived from human brain studies (McAnulty et al., 1977; Seiler and Lamberty, 1975) revealing that PUT has the highest concentration of polyamines in all studied brain regions during foetal and postnatal development.

At first sight, these multiple changes in polyamine concentrations during animal development and aging might appear confusing but they are often connected to the simultaneous changes occurring in DNA (Chiu and Oleinick, 1998; McAnulty et al., 1977; Shimizu et al., 1965), neurotransmitters (Gilad and Kopin, 1979), nerve growth factors (Dornay et al., 1986; Gilad and Gilad, 1989), polyribosomes (Caldarera et al., 1969) and RNA (Shaskan et al., 1973). Therefore, the question arises of whether the polyamines are important in growth and proliferation of cells in...
the CNS. The high molar ratio of spermidine to spermine coincides with rapidly proliferation in nerve cells and non-neuronal cells. A correlation between polyamines and nerve growth was found by Gilad (Dornay et al., 1986; Gilad et al., 1985; Gilad et al., 1986; Gilad et al., 1989; Gilad and Gilad, 1983; Gilad and Gilad, 1988). The increase of SPD concentration in rat brainstem correlates with the rapid proliferation of oligodendroglial cells and myelination during the post-natal phase (Kremzner, 1970). Further, in the brainstem of the human foetus, an enhanced PUT accumulation has been observed during myelination and in the forebrain during neuroblast multiplication (McAnulty et al., 1977). PUT accumulation is also observed in mouse nerve cells and in oligodendrocytes (Fischer et al., 1972) as well as in synaptosomes of cerebral cortex (Seiler and Deckardt, 1976). The increases of both PUT and SPD concentrations are involved in the extension of fibrous zones of growing fish brain whereas the SPM content, found to be present to mainly in grey matter, does not change (Seiler and Lamberty, 1973).

Several studies have indicated (Antrup and Seiler, 1980; Bernstein and Muller, 1999; Gilad et al., 1995; Gilad and Kopin, 1979; Laitinen et al., 1982; Ohkaya et al., 1997; Seiler and Sarhan, 1980; Shaskan et al., 1973; Shaw, 1979; Sparapani et al., 1998) that an activation of a polyamine biosynthetic enzyme, ODC correlates with changes of SPD and SPM concentrations during the development of brain as well as in mature brain. In foetal brain, ODC activity increases until birth, then declines rapidly at birth and increases again in the following seven days after birth. At 10-12 days, ODC activity reaches the activity level of the mature brain (Gilad and Kopin, 1979; Laitinen et al., 1982). In humans (Morrison et al., 1998), however, the ODC activity during aging correlates with the PUT concentration (Morrison et al., 1995). The enormous increase in post-natal ODC activity occurs simultaneously with the major phase of migration of cerebellar neurons (Gilad and Kopin, 1979) and also the time of Schwann cell proliferation (Ohkaya et al., 1997). In contrast to ODC, the other polyamine synthesizing enzyme, AdoMetDC displays a low activity in the prenatal phase but after birth the activity increases slowly with age (Antrup and Seiler, 1980; Laitinen et al., 1982; Morrison et al., 1993a; Morrison et al., 1993b; Shaskan et al., 1973; Shaw, 1979). However, minor changes in the activity of AdoMetDC are observed simultaneously with the migration phase and proliferation of neurons (Gilad and Kopin, 1979; Shaskan et al., 1973). The greatest AdoMetDC activities are found in the brain regions of cerebral cortex, cerebellum and medulla-pons regions (Morrison et al., 1993a; Shaskan et al., 1973).

There is no doubt that polyamines have a specific role in the development and proliferation of neurons and non-neuronal cells in brain. Their function may be directly related to synaptic reorganization and neuronal maturation (Dornay et al., 1986; Morrison et al., 1995; Morrison et al., 1993a; Morrison et al., 1998), or they may act indirectly by regulating specific proteins that
control synaptic responses or neurotrophic functions (Dornay et al., 1986; Gilad et al., 1989; Laitinen et al., 1982; Morrison et al., 1998; Slotkin and Bartolome, 1986; Slotkin et al., 2000). In mature brain, the function of polyamines is unrelated to growth (Morrison et al., 1995; Morrison et al., 1993a; Slotkin et al., 2000).

2.1.2. MAINTENANCE OF INTRACELLULAR POLYAMINE HOMEOSTASIS

2.1.2.1. Polyamine biosynthesis

It is generally accepted that in vertebrates the polyamine metabolism pathways: synthesis and catabolism are similar in the peripheral organs and in the central nervous system (CNS) (Seiler, 1994). All cells produce polyamines, except anuclear red blood cells that accumulate polyamines by uptake and binding (Seiler, 1990). Polyamine metabolism is a cyclic process, which is controlled by biosynthesis and backconversion of polyamines (Figure 3). The major precursor for polyamine synthesis is L-ornithine. In the CNS, arginine is the source of L-ornithine since it is catabolized by the enzyme arginase. Thereafter, L-ornithine initiates polyamine biosynthesis by being decarboxylated to PUT by ornithine decarboxylase (ODC). The activation of ODC is the rate-limiting step for the entire biosynthesis chain. Another key enzyme for polyamine synthesis is S-adenosylmethionine decarboxylase (AdoMetDC), which catalyzes the decarboxylation of S-adenosylmethionine (AdoMet) to decarboxylated S-adenosylmethionine (dAdoMet) that is the source of the aminopropyl residue in SPD and SPM. AdoMet is derived from the reaction of adenosine triphosphate (ATP) and L-methionine, which is thus the other amino acid required for the synthesis of polyamines. Combination of PUT and the aminopropyl residue yields SPD in a reaction catalyzed by spermidine synthase. Similarly, spermine synthase transfers another aminopropyl residue to SPD yielding SPM. Both spermidine and spermine synthases produce also 5'-methylthioadenosine, which is reused for the formation of ATP (Cohen, 1998; Jänne et al., 1991b; Morgan, 1999; Pegg, 1986; Seiler, 1990; Seiler, 1994; Shaw, 1979; Tabor and Tabor, 1984).

2.1.2.1.1. Ornithine and ornithine decarboxylase (ODC)

The source of the amino acid L-ornithine (Figure 2 and 3), is not entirely clear. Part of the ornithine, which is present in the blood plasma of animals, may originate from the diet (Morgan, 1999). One pool of ornithine is derived from arginine by arginase catalyzed hydrolytic cleavage,
which is the final step in the urea cycle (Cohen, 1998; Seiler, 1994; Yu et al., 2001). The fact that all the enzymes involved in the urea cycle are not present in every cell and further, that arginase is still present in those cells makes the brain polyamine metabolism even more interesting. The existence of two arginase subtypes (A1 and A2) was found some time ago (Spector et al., 1985). Interestingly, the subtype A1 is expressed more strongly in the mouse brain (Yu et al., 2001) and there it does seem that the role of arginase in the brain is to produce ornithine (Morgan, 1999; Yu et al., 2001).

Ornithine decarboxylase (Figure 3) is one of the best studied enzymes in mammals. Mammalian ODC was discovered in 1968 simultaneously in three different laboratories, one of them being in Finland (see review (Jänne et al., 1991b)). ODC is a dimer with a subunit weight of 53 000 and its $K_m$ value for ornithine at physiological pH is 75 µM (Pegg, 1986; Seiler, 1994). It is very unstable having a half-life of between 10 and 60 minutes. The human ODC gene is located on the short arm of chromosome 2 (Jänne et al., 1991b; Winqvist et al., 1986) and sequencing of ODC has revealed (Hickok et al., 1987; Hölttä et al., 1989; Jänne et al., 1991b; Kahana and Nathans, 1984; Kontula et al., 1984; McConlogue et al., 1984) high conservation among different species. The ODC enzyme is present both in the cytoplasm and nucleus (Cintra et al., 1987) at very low levels (Jänne et al., 1991b; Morgan, 1999; Seiler, 1994) showing its highest activity just prior to cell proliferation (Shaw, 1979). It is also found in the membrane fraction (Gilad et al., 1996a).

2.1.2.1.2. S-adenosylmethionine decarboxylase (AdoMetDC)

S-adenosylmethionine decarboxylase is another polyamine synthesizing enzyme whose activation is needed for the synthesis of higher polyamines (Figure 3). It was discovered by G. Cantoni in 1953 in E.coli (Cohen, 1998) and later from the cytosol fraction of several animal tissues including brain (Tabor and Tabor, 1984). The nucleotide sequence coding for the human (Maric et al., 1995) and mouse (Nishimura et al., 2002) AdoMetDC enzyme is known. The human gene is mapped on chromosome 6 and its mouse counterpart is located on chromosome 10 (Maric et al., 1995; Nishimura et al., 2002). Although AdoMetDC is less inducible than ODC, it often accompanies the induction of ODC and its half-life is also less than one hour (30-60 minutes) (Jänne et al., 1991b). AdoMetDC is markedly activated by PUT (Seiler and Dezeure, 1990). On the other hand, inhibition of AdoMetDC leads to an enhanced accumulation of PUT in vivo (Jänne et al., 1991b). We can therefore view it as another rate-controlling enzyme of polyamine
Figure 3. The metabolism of polyamines. The biosynthetic pathway is presented with light arrows and the catabolic pathway is shown with dark arrows. The enzymes of polyamine biosynthesis are in light grey boxes, catabolic enzymes are in dark grey boxes and the recently found SMO is presented in the light box.
biosynthesis in conjunction with ODC. PUT induces the formation of mature AdoMetDC from an inactive proenzyme (Pajunen et al., 1988; Pegg et al., 1988; Seiler and Dezeure, 1990). The $K_m$ value for AdoMet at physiological pH is 65 $\mu$M. Mammalian AdoMetDC is comprised of four subunits of which the $\alpha_2$ subunit is the largest ($M_w$ 31 000) having a covalently bound pyruvate group at the amino terminus with the $\beta_2$ subunit being the smallest ($M_w$ 7 000). The pyruvate group serves as the coenzyme needed for the catalytically active enzyme (Cohen, 1998; Jänne et al., 1991b; Morgan, 1999; Pegg, 1986; Seiler, 1994).

### 2.1.2.1.3. Putrescine (PUT)

Putrescine (Figure 2) has two special features among polyamines. Unlike other polyamines, PUT was first isolated from plants in 1907 but it took over fifty years until it was isolated from the brain by Kewitz (Cohen, 1998; Kewitz, 1959). Secondly, the name "polyamines" is somewhat misleading when PUT is concerned since it has only two amino groups in the carbon chain being in fact a diamine, 1,4-diaminobutane (Cohen, 1998; Seiler, 1994; Seiler and Al-Therib, 1974; Shaw, 1979). However, PUT is usually included with the polyamines. It is called PUT because of the putrefying odours liberated in its synthesis. Since the binding energy of polyamines increases with their number of charges, PUT which has only two amino groups has the lowest binding affinity of all polyamines (Jänne et al., 1991a; Seiler, 1990). In addition, PUT is present in normal brain tissue only at very low levels (Seiler, 1994; Seiler and Bolkenius, 1985; Shaskan and Snyder, 1973; Shaw, 1994; Shaw and Pateman, 1973). These facts together make PUT a difficult molecule to study.

In mammals, there is only one synthetic pathway (Figure 3) for the formation of PUT and hence it is a major precursor for the other polyamines and their acetylated derivatives. It seems that in the prenatal phase, PUT found in the brain tissue is derived from ODC activity whereas in the mature brain PUT is mainly the product of an active catabolic cycle. This assumption is based on the observations whereby ODC activity decreases upon normal aging (Gilad and Kopin, 1979; Laitinen et al., 1982) whereas AdoMetDC (Laitinen et al., 1982; Shaskan and Snyder, 1973) and polyamine oxidase (PAO) activities, both of which display only modest activity in the developing foetus, increase during aging (Bolkenius and Seiler, 1986; Seiler, 1994).

In addition to being a precursor of the higher polyamines, PUT is also one of the sources for GABA, which is an inhibitory neurotransmitter in mammalian brain. Seiler and coworkers discovered the synthesis of GABA from PUT (Laschet et al., 1992; Seiler et al., 1980a; Seiler et
al., 1979) (Figure 2). Although, only a minor portion of GABA is formed from PUT, several possible routes have been demonstrated for the synthesis of GABA in the brain with one additional route in peripheral tissue (Cohen, 1998; Seiler, 1994).

2.1.2.1.4. Spermidine synthase and spermidine (SPD)

Spermidine (Figure 2) was found by Dudley in 1927 from a tissue extract when purifying SPM phosphate and later from brain by Kewitz (Cohen, 1998) whereas spermidine synthase, the enzyme responsible for SPD synthesis, was found many decades later in bovine brain by Raina et al. 1984 (Morgan, 1999; Raina et al., 1984). The active human spermidine synthase (M_w 35 000) is comprised of two subunits (Kajander et al., 1989) and the gene coding for human spermidine synthase which is located on chromosome 1, is well characterized (Myöhänen et al., 1991; Wahlfors et al., 1990). The enzyme activity is mainly regulated by the availability of its substrate, dAdoMet (Jänne et al., 1991b; Pegg, 1986; Tabor and Tabor, 1984). The enzyme is inhibited by SPD and 5'-methylthioadenosine (Morgan, 1999). Active spermidine synthase does not require cofactors for its aminopropyltransferase reactions (Figure 3) (Pegg, 1986; Seiler, 1990). The precise catalytic mechanism of SPD synthesis (Seiler, 1994) and the subcellular localization of spermidine synthase (Shaw, 1994) are still uncertain but the enzyme is a stable protein expressed constitutively and has a half-life of several days (Morgan, 1999; Seiler, 1990).

2.1.2.1.5. Spermine synthase and spermine (SPM)

In 1927 Dudley and O. Rosenheim identified a substance called neuridine which is now known to be same as SPM (Cohen, 1998). The polyamine, SPM (Figure 2), is formed from SPD and dAdoMet in a reaction catalysed by spermine synthase (Figure 3). The reaction is analogous to the one catalysed by spermidine synthase and it produces one molecule each of 5'-methylthioadenosine and SPM. Spermine synthase consists of two subunits of equal size (M_w 45 000) (Kajander et al., 1989; Pajula et al., 1979) and the genomic sequence coding for the human spermine synthase has been determined (Korhonen et al., 1995). As with spermidine synthase, spermine synthase is regulated by the substrate, dAdoMet and its needs no cofactors for its activity (Jänne et al., 1991b; Morgan, 1999; Seiler, 1990; Tabor and Tabor, 1984). The enzyme is inhibited by its products, SPM and 5'-methylthioadenosine (Morgan, 1999). Since both of the synthases use the same substrate, the activity of these enzymes is also regulated by competition (Pegg, 1986). Interestingly, spermine synthase appears to be present only in eukaryotic cells (Jänne et al., 1991b; Kuopio Univ. Publ. G. A.I. Virtanen Institute for Mol. Sci.
Tabor and Tabor, 1984) and its activity is higher in brain tissue and exocrine glands as compared with other tissues (Seiler, 1994). The underlying factors for this tissue distribution are not well understood (Jänne et al., 1991b; Seiler, 1994).

2.1.2.2. Polyamine catabolism

In principle, the polyamine biosynthetic reactions presented above are irreversible and therefore another set of reactions is needed to convert SPM back to PUT. This occurs during the catabolic cycle of polyamines. One of the key enzymes of polyamine metabolism, spermidine/spermine $N^1$-acetyltransferase (SSAT), is responsible for the degradation of SPM and SPD (Figure 3). Polyamine catabolism is also known as interconversion pathway, in the first phase SSAT acetylates the primary amino groups of SPM and SPD into $N^1$-spermine and $N^1$-spermidine with acetyl-CoA. Thereafter, polyamine oxidase (PAO) converts acetylated polyamines into SPD and PUT, respectively, and releases 1-acetylaminopropanal ($N$-acetyl-3-aminopropionaldehyde) and hydrogen peroxide (Morgan, 1999; Pegg, 1986; Seiler, 1987; Seiler, 1994; Seiler and Al-Therib, 1974; Seiler et al., 1980b; Shaw, 1979; Tabor and Tabor, 1984).

An alternative route for backconversion of SPM to SPD was proposed quite recently (Vujcic et al., 2002a; Wang et al., 2001). It was suggested that a flavin-containing enzyme, named spermine oxidase (SMO), could act by oxidizing SPM to SPD without any prior acetylation by SSAT.

2.1.2.2.1. Spermidine/spermine $N^1$-acetyltransferase (SSAT)

Spermidine/spermine $N^1$-acetyltransferase is the third rate-controlling enzyme, together with ODC and AdoMetDC, in polyamine metabolism and is responsible for the first step in the interconversion cycle of SPM and SPD into SPD and PUT, respectively (Figure 3) (Cohen, 1998; Morgan, 1999; Seiler, 1994). Seiler and Al-Therib described the existence of the polyamine catabolic cycle in 1974 in rat brain (Seiler and Al-Therib, 1974). In mammals, the molecular weight of one SSAT enzyme subunit is 18 000 - 20 000 and the native protein is 65 000 - 80 000 M₉ (Coleman et al., 1995; Libby et al., 1991). Therefore, the active SSAT enzyme is either a dimer or a tetramer (Casero and Pegg, 1993). Like the other two important enzymes, SSAT is also very unstable with a short half-life (15-60 minutes). SSAT has hardly detectable baseline activity in cells, but SSAT is very sensitive to changes in intracellular metabolism. The $K_m$ values for the physiological substrates of SSAT are as follows: SPM 34 µM, $N^1$-acetylspermidine 51 µM, SPD
130 (Seiler, 1994) and acetyl-CoA 1.5 μM (Casero and Pegg, 1993). The nucleotide sequence of SSAT is known for the human (Xiao et al., 1991), hamster (Pegg et al., 1992) and mouse (Fogel-Petrovic et al., 1993a) genes. The human SSAT gene is localized on the short arm of the X chromosome (Xiao et al., 1991). The amino acid sequence of SSAT is highly conserved showing 95% similarity between species. Such a high similarity of proteins is usually considered to indicate an important function for the protein in cellular metabolism. In the differentiated cells of mature brain as much as 70% of the total PUT is derived from the interconversion pathway while only 30% of the total PUT is newly synthesized by ODC (Seiler and Bolkenius, 1985). Therefore, we can assume that one of the main physiological roles of this complex system, in which SSAT plays a key role, is to protect cells from accumulating higher polyamines, SPD and SPM (Vargiu and Persson, 1994).

Most probably, there are two types of spermidine/spermine $N_8$-acetyltransferases: a nuclear SSAT (nSAT) and a cytosolic SSAT (cSAT) enzyme of which the latter is inducible and the former is a basal enzyme. The nuclear enzyme produces $N_8$-acytyspermidine, which is probably transported into the cytoplasm or excreted out of the cell, whereas the cytosolic SSAT produces both $N_1$-acetylspermine and $N_1$-acytyspermidine (Cohen, 1998; Pegg, 1986; Seiler, 1994). In addition, there seems to be another enzyme in the cytosol, which does not react with antibodies specific for the known inducible cSAT enzyme (Persson and Pegg, 1984) although it has the same catalytic properties as inducible cSAT. Apparently, the inducible cytosolic SSAT is capable of catalyzing only the $N_1$ amino groups of polyamines and further acetylation to the $N_8$ form is not observed (Della Ragione and Pegg, 1983; Morgan, 1999). The catabolic reaction where the primary aminopropyl residues of SPD and SPM are acetylated by SSAT, is irreversible and hence, another enzyme is needed to complete the interconversion cycle of higher polyamines.

2.1.2.2.2. Acetylated polyamines

Positively charged polyamines bind efficiently to the anionic compartments of cells. Acetylation of polyamines is a process where the positively charged aminopropyl residues are displaced from polyamines and hence, in an acetylated form, polyamines (Figure 2) are liberated from their anionic binding sites. Thereafter acetylpolyamines are either excreted into the extracellular space or are further processed by deacetylation or terminal degradation in the cell (Figure 3). Polyamine acetylation appears to occur independently both in the cytoplasm and in the nucleus of cells (Morgan, 1999; Ortiz et al., 1983; Seiler, 1994; Sessa et al., 1994). There is another polyamine acetylating enzyme, spermidine $N_8$-acyltransferase, which produces $N_8$-
acetylspermidine, this enzyme is located in the nucleus (Seiler, 1987). It is suggested that $N^8$-acetylspermidine is exported into cytosol where it is subsequently deacetylated by cytosolic acetylspermidine deacetylase (Morgan, 1999; Seiler, 1987; Seiler, 1994). The function of $N^8$-acetyltransferase is not entirely clear but it may inactivate SPD, which in its acetylated form, can penetrate through the nuclear membrane (Morgan, 1999; Seiler, 1987).

2.1.2.2.3. Polyamine oxidase (PAO)

Oxidization of acetylated polyamines is the final step in the polyamine interconversion pathway and is catalyzed by polyamine oxidase. PAO cleaves the secondary amino group from $N^1$-acetylspermine and $N^1$-acetylspermidine forming SPD and PUT, respectively (Figure 3) (Morgan, 1999; Seiler, 1994). Hölttä (1977) described the enzyme, which has a molecular mass of 60 000 and contains a tightly bound flavin adenine dinucleotide (FAD) molecule. The physiological pH optimum for PAO is pH 10 where the $K_m$ values for the natural substrates are 40 µM for SPM, 0.6 µM for $N^1$-acetylspermine, 5.0 µM for $N^1,N^{12}$-diacetylspermine and 14 µM for $N^1$-acetylspermidine (Morgan, 1999; Seiler, 1994; Seiler, 1995). Polyamine oxidase is selective for the 3-aminopropyl residues of SPM and the 3-acetamidopropyl residue of $N^1$-acetylated polyamines and hence, does not react with $N^8$-acetylated SPD (Seiler, 1995). Only recently, were the cDNAs encoding for human PAO and mouse PAO sequenced. The human gene is localized on chromosome 10 and the mouse gene on chromosome 7. Comparison of amino acid sequences showed 82 % similarity between species (Vujcic et al., 2002b).

The enzyme is localized in peroxisomes and cytosolic fractions in rat liver (Hölttä, 1977). The subcellular localization in brain is not known (Seiler, 1994) although peroxisomal structures exist in non-neuronal cells and therefore, the neuronal distribution is presumably similar to that in liver (Seiler, 2000). PAO is a stable protein and the half-life is counted in days. In the prenatal brain PAO activity is very low but increases rapidly after birth and the activity is retained at a comparatively high level in differentiated cells (Seiler, 1995). Therefore, we can assume that the reutilization of polyamines regulates the intracellular polyamine concentrations in differentiated cells and the rate of recycling is controlled by SSAT.

2.1.2.3. Other amine oxidases

In addition to PAO, polyamines can be oxidized by other amine oxidases. A polyamine degradation pathway is catalyzed by copper ($Cu^{2+}$) containing amine oxidases (Seiler, 2000). In
mammals, the well characterized enzyme, diamine oxidase (DAO), prefers diamines as substrates (Morgan, 1999; Seiler, 2000). DAO degrades polyamines into aminoaldehydes, ammonia and hydrogen peroxide (Figure 3). Aldehydes are not converted back into the polyamine pool.

2.1.2.4. Regulation of polyamine metabolism

In addition to growth factors and hormones, intracellular polyamine metabolism is mostly regulated by a feedback mechanism depending on polyamine concentrations. Cellular polyamine pools are affected by the rate of polyamine biosynthesis, catabolism, excretion, uptake and degradation. The main contributors to polyamine metabolism are the three key enzymes, ODC, AdoMetDC and SSAT. They all are present at very low levels in quiescent cells and yet, they are rapidly induced in response to almost any stimuli. However, the up-regulation is transient because of the short half-life of these enzymes (Seiler, 1990; Seiler, 2000).

The activity of polyamine biosynthetic enzymes, ODC and AdoMetDC, are controlled at different levels (Persson et al., 1999). The increase in ODC enzyme synthesis does not correlate with the amount of ODC mRNA, suggesting that regulation occurs at the translational level whereas the increase in AdoMetDC activity is found simultaneously with the increased level of AdoMetDC mRNA. Both enzymes are down-regulated by an excess of polyamines and a depletion of polyamines activates their synthesis (Persson et al., 1999; Seiler, 1994). The ODC enzyme has a unique feature among short-lived proteins having specific inhibitory protein, ODC antizyme (Canellakis et al., 1979), which inhibits the activity of ODC in brain (Kilpelainen et al., 2000; Sakata et al., 1997). The antizyme and ODC enzyme form a complex, which is rapidly degraded by the 26S proteasome (Kilpelainen et al., 2000).

The regulation of SSAT gene expression by polyamine concentrations represents a unique feature in the regulation of polyamine metabolism. Unlike the polyamine biosynthetic enzymes which are negatively controlled by polyamines, the enzyme responsible for the catabolic cycle of polyamines is positively regulated by an excess of polyamines SPD and SPM (Fogel-Petrovic et al., 1993b; Seiler, 1994). Moreover, SSAT expression is mostly controlled at the post-transcriptional level (Desiderio et al., 1993; Fogel-Petrovic et al., 1996a; Fogel-Petrovic et al., 1996b; Shappell et al., 1993).

Attempts have been made to localize the specific intracellular stores of polyamines (Sarhan and Seiler, 1989) but no such stores have yet been identified (Morgan, 1999). In cases when the intracellular need exceeds the cellular production of polyamines, the cells take up polyamines, mainly PUT and SPD, from the extracellular space (Khan et al., 1991). However, small amounts of
SPD, SPM and PUT are present in nuclei (Sarhan and Seiler, 1989), but their transport mechanism into the nuclei is not known although the excretion of polyamines especially SPD back to cytoplasm probably occurs through its acetylation by a specific enzyme, spermidine $N^8$-acetyltransferase (Seiler, 1994). PUT and SPM but not SPD are reported to have a specific uptake system by synaptosomes (Gilad and Gilad, 1991a; Masuko et al., 2003) and polyamines may have a modulatory role in synaptic transmission by regulating the uptake of neurotransmitters (Law et al., 1984). SPM appears to have a regulatory role in mitochondrial DNA synthesis (Tassani et al., 1995) and its uptake by peripheral mitochondria is quite well understood (Dalla Via et al., 1996; Tassani et al., 1996). Xie et al. (Xie et al., 1997) have suggested that the regulation of polyamine transport is more important than the regulation of ODC activity as a means of preventing the accumulation of toxic levels of polyamines in the cell.

The polyamine homeostasis is a good example of a precisely regulated metabolic pathway. Reutilization of polyamines minimizes their consumption whereas tightly regulated metabolism provides an adequate supply of polyamines for cell growth and prevents cellular toxicity by excess of polyamines.

### 2.2. POLYAMINES IN NEUROPATHOLOGICAL STATES

In addition to the fact that the natural polyamines PUT, SPD and SPM take an active part in cell proliferation and development of central nervous system, their cellular homeostasis is easily disturbed by a variety of extracellular or intracellular stresses or insults. The alteration of polyamine metabolism has been studied in diseases and neuropathological states such as Alzheimer's disease (Morrison et al., 1993b; Morrison et al., 1998; Morrison and Kish, 1995), brain tumors (Goldman et al., 1986; Rohn et al., 2001; Yamazaki et al., 1986), epilepsy (Laschet et al., 1999) and psychiatric disorders (Lees, 2000; Yamakura and Shimoji, 1999). In particular, polyamine metabolism has been also shown to be disturbed in numerous experimental models: in functional activation models (Baudry and Najm, 1994; de Vera et al., 2002; Halonen et al., 1993; Hayashi et al., 1993; Ingi et al., 2001; Shimosato et al., 1997), in head injury models (Dogan et al., 1999; Gilad et al., 1996b; Henley et al., 1996; Zoli et al., 1991), in several ischemic models (Baskaya et al., 1997a; Gilad and Gilad, 1991b; Johnson, 1998; Keinanen et al., 1997; Lukkarinen et al., 1998; Paschen, 1992b; Zoli et al., 1996b), after neurotoxic insults (Camon et al., 2001; Liu et al., 2001; Lombardi et al., 1993; Reed and de Bellerocche, 1990; Vivo et al., 2002) or in pharmacological treatments (Camon et al., 1994; De Sarro et al., 1993; Gimenez-Llort et al.,
Neuronal injuries result in biosynthetic and/or catabolic changes in polyamine metabolism. In experimental brain injury models, a rapid increase in ODC activity occurs by 4 hours, being the first change observed in polyamine metabolism (Dempsey et al., 1988; Henley et al., 1996; Paschen, 1992b; Paschen et al., 1991; Zoli et al., 1991). However, due to the short half-life of the enzyme, ODC activity returned to the basal level by 1-3 days (Henley et al., 1996; Keinanen et al., 1997; Paschen, 1992b). The activity of the other regulatory enzyme of polyamine biosynthesis, AdoMetDC, has been shown to decline after ischemia (Paschen, 1992b; Paschen, 1994). A delayed accumulation of PUT was also observed and high levels of PUT are maintained for several days (Dogan et al., 1999; Gilad et al., 1993b; Henley et al., 1996; Paschen et al., 1991; Rao et al., 2000). The cellular content of the higher polyamines, SPD and SPM, however, remain virtually unaltered. In fact, the SPD content tends to decrease after cranial impact while that of SPM seems to remain unchanged (Dogan et al., 1999; Gilad et al., 1993b; Henley et al., 1996; Paschen et al., 1991; Rao et al., 2000). Unchanged SPD and SPM levels might be explained by the enhanced SSAT activity, which primes the back-conversion pathway of polyamines after the insult (Babu et al., 2001; Ingi et al., 2001; Rao et al., 2000; Zoli et al., 1996b). Therefore, the normally undetectable N₁-acetylated spermidine appears in notable amounts in injured cells (Rao et al., 2000).

The trend observed in the changes of polyamine metabolism in pharmacological models of neuronal injury is similar to that seen in experimental brain injury models but some differences do exist. As with experimental models, pharmacological treatments induce ODC and expand the PUT pool. Excitatory amino acids and excitotoxins are able to induce severe disturbances in polyamine metabolism (Hayashi et al., 1993; Martinez et al., 1991; Paschen et al., 1993). Kainic acid, an agonist of AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid) and kainate receptors, have been shown to increase the PUT level by up to 20-fold (Baudry and Najm, 1994; de Vera et al., 1991; de Vera et al., 1997; Martinez et al., 1991). It seems that the dramatic accumulation of PUT leads to a marked decrease in SPD and SPM concentrations and the changes in polyamine concentrations are suggested to correlate with the brain damage (de Vera et al., 2002; Vivo et al., 2002). Studies supporting this view indicate that the PUT concentration increases in the most vulnerable regions such as in the ischemic core (Henley et al., 1996; Paschen, 1994) or at the site of injection (Baudry and Najm, 1994; de Vera et al., 2002). PUT is also reported to accumulate in the surrounding areas to the injury (Baskaya et al., 1997a). In addition, studies following the extracellular flow of polyamines have revealed an increase in extracellular PUT.
(Gimenez-Llort et al., 1996b; Vivo et al., 2002) and SPD concentrations (Vivo et al., 2002), yet contrary findings have also been reported (Gilad et al., 1993a; Gilad et al., 1993b). Therefore, some authors (Camon et al., 2001; Els et al., 2001; Rohn et al., 2001) have suggested that extracellular polyamines could be used as clinical markers of injury severity: the more severe the injury, the more polyamines are released to cerebrospinal fluid (Els et al., 2001). Although the above proposal is supposedly difficult to apply in clinical purposes, some attempts have been made for better adjustment of changes in polyamines in correlation to injured cells. Antibodies raised against ODC enzyme and polyamines have provided new perspectives the localization of polyamines in brain (Bernstein and Muller, 1999; Dempsey et al., 1988; Fujiwara and Masuyama, 1995; Gilad et al., 1999).

The accumulation of PUT near or at the site of insult in the brain is suggested to possess a neuroprotective role, a mechanism that is activated following brain injury (de Vera et al., 1997; Gilad and Gilad, 1991b; Gilad and Gilad, 1992; Kauppinen and Alhonen, 1995; Wengenack et al., 1997b). A treatment with a combination of polyamines PUT, SPD and SPM protected neurons in the hippocampal CA1 region after ischemia and a protective role of extracellular polyamines was suggested (Gilad and Gilad, 1991b). Similarly, a combined treatment with polyamines accelerated the recovery of neurons after facial nerve injury (Gilad et al., 1996b). Results obtained from animal kindling experiments (Hayashi et al., 1992) showed that the increase in PUT concentration inhibited the development of kindling. Some polyamine derivatives are found to be potent antagonists for AMPA receptors and considered to be neuroprotective in transient global ischemia (Yoneda et al., 2002). In addition, the use of the excitatory amino acid antagonist, ifenprodil (Williams, 2001), has shown a protective effect in the formation of ischemia-induced lesion size and in the damage to the blood-brain barrier (Baskaya et al., 1997b; Dempsey et al., 2000; Dogan et al., 1997).

The transport of polyamines has been the focus of drug development for treatment of the neurological diseases. The brain is a rather closed system, due to the presence of the blood-brain barrier which is disrupted only in pathological states. Any potential molecule would need to possess certain qualities such as lipid solubility or the presence of a specific carrier protein. Despite being water-soluble, cationic like molecules, polyamines are able to pass through the barrier (Khan et al., 1991; Shin et al., 1985). The system that is responsible for polyamine transport through blood-brain barrier has not yet been elucidated, but it has been shown that certain normally impervious molecules can penetrate the blood-brain barrier when polyamines, especially PUT, are present without causing detrimental effects to the BBB (Bondy and Walker, 1986; Wengenack et al., 1997a; Wengenack et al., 1997c). In addition to facilitating the transport
Selma Kyllikki Kaasinen: Putrescine accumulation in mouse CNS – neuroprotection at the expense of learning deficiency

through BBB, PUT modified nerve growth factor (NGF) has been demonstrated to be relatively stable in the brain and was able to elicit a bioresponse as well (Poduslo et al., 1998). Furthermore, the N-methyl-D-aspartate receptor antagonist, ifenprodil, known to bind to the polyamine binding, has been reported to decrease ischemic injury both in the brain and blood-brain barrier (Baskaya et al., 1997b; Dempsey et al., 2000).

Despite all the studies made so far on polyamines and their contribution to neuronal injury, there is no agreement on whether polyamines can protect from detrimental sequelae or whether polyamines promote the development of neuropathological outcomes. There seems to be a controversy between the modes of treatments: when polyamines are administered by peripheral injection they show a protective effect on neuronal survival in a variety of injury models whereas intracranially administered polyamines cause motor activity like behaviour, seizures and neuronal injury. Lombardi et al. (Lombardi et al., 1993) suggested that the release of polyamines into the extracellular space from injured neurons or glial cells could potentiate the excitotoxic action of NMDA receptors while elevated levels of intracellular polyamines have a neuroprotective effect (de Vera et al., 1997). PUT accumulation seen in the injured area after experimental head injury or after neurotoxic insults is thought to indicate the actual involvement of polyamines in the development of seizures (De Sarro et al., 1993; de Vera et al., 2002; Gimenez-Llort et al., 1997) leading to neuronal death (Henley et al., 1996; Kindy et al., 1994; Liu et al., 2001; Paschen, 1994). Since the regulation of polyamine metabolism is so tight, it is highly likely that an excess of polyamines is toxic to the cell. For instance, SPM is the most stable polyamine and its cellular content changes only in response to dramatic insults. Therefore, an accidental fluctuation in the SPM content would appear to be disastrous to the cell. This view is supported by studies dealing with the toxicity of SPM accumulation after pharmacological manipulation (Doyle and Shaw, 1996; Segal and Skolnick, 2000) and ischemia (Graham et al., 1996). The use of pharmacological compounds as inhibitors or activators of the enzymes of polyamine metabolism have brought new insights into brain polyamine research. α-Difluoromethylornithine (DFMO) is the best-known compound affecting polyamine synthesis by inhibiting ODC and depleting PUT and SPD pools. Kindy et al. (Kindy et al., 1994) and Rao et al. (Rao et al., 1995) reported that DFMO prevented delayed neuronal death in the hippocampal CA1 region after ischemia. Polyamines are positive modulators of the N-methyl-D-aspartate (NMDA) receptor and activation of NMDA receptors results in increased Ca^{2+} ion influx into the neurons. Therefore, an increase of polyamine pools would maintain the activation of NMDA receptors, evoking a toxic influx of Ca^{2+} ions into the neurons, leading to cell death while an inhibition of polyamine synthesis by DFMO would deactivate NMDA receptors.

The polyamines, SPM and SPD, have a higher affinity for the polyamine binding site of NMDA receptors (Ransom and Stec, 1988) than PUT (Seiler, 1994). In addition, PUT is present at low levels in quiescent cells even after various stimuli as compared with the concentrations of SPD and SPM. Therefore PUT, which has been proposed be harmful to neurons (Paschen, 1994), is actually the least effective polyamine at the polyamine site of NMDA receptor. Furthermore, the dual effect of polyamines on NMDA receptors is attributable to two different polyamine binding sites on the outer membrane of the NMDA receptor. At lower doses, SPM and SPD have agonistic effect on NMDA receptor activity and higher doses of polyamines inhibit the activity of NMDA receptor (Williams et al., 1992). Therefore, any increase in extracellular polyamines should rather inactivate NMDA receptors and hence block the inflow of Ca\(^{2+}\) ions into the cells, not to cause a detrimental effect. According to Gilad and coworkers (Gilad et al., 1993a; Gilad et al., 1993b), the extracellular concentration of PUT does not increase after ischemia.

### 2.3. THE HIPPOCAMPAL NEURONAL CIRCUITS AND THEIR ROLE IN LEARNING AND MEMORY

#### 2.3.1. THE ANATOMICAL STRUCTURE OF HIPPOCAMPUS

Hippocampus (Latin word for "seahorse") is involved in memory storage, and is a part of the limbic system, which mediates emotions and aspects of learning and memory. The two hippocampi lie medially to the lateral ventricles beneath the cerebral cortex and corpus callosum in both hemispheres of the brain (Kelly and Dodd, 1991; Martin and Jessell, 1991b). The two hippocampi with their associated structures form the three-dimensional structure called the hippocampal formation (Figure 4). It extends rostro-caudally over and behind the diencephalons starting from the basal forebrain and ending in the temporal lobes. Much of the information concerning the hippocampal formation originates from the classical studies of Ramón and Cajal in 1911 and Lorente de Nó in 1933, 1934. The hippocampal formation consists of six cytoarchitectonically different regions but here only the hippocampus is considered (Amaral and Witter, 1995).

Hippocampus is a folded structure of neurons comprising of two separate sheets; dentate gyrus and Ammon's horn (in Latin cornu Ammonis) of which the dentate gyrus consists of three layers: granular cell layer, molecular cell layer and polymorphic cell layer, also known as hilus. Ammon's horn is subdivided into CA1, CA2 and CA3 fields (Amaral and Witter, 1995; Bear et al.,
Selma Kyllikki Kaasinen: Putrescine accumulation in mouse CNS – neuroprotection at the expense of learning deficiency

The principal cell layer of the hippocampal CA1, CA2 and CA3 fields is the pyramidal cell layer that is comprised of a 3 to 4 cell layers thick structure. The majority of neurons are pyramidal cells, whose size and connectional differences determines the hippocampal subfields. A various populations of basket cells are located in the pyramidal cell layer and their axons innervate the pyramidal cells. The hippocampal pyramidal cell layer is surrounded by the stratum oriens that contains the dendritic tree of the pyramidal cells (Amaral and Witter, 1995; Paxinos and Watson, 1986).

2.3.2. THE NEURONAL CIRCUITS OF HIPPOCAMPUS

The hippocampal formation possesses unique, one-way projections running from the subiculum to the CA1 region. There are three major excitatory pathways in the hippocampus: perforant pathway, mossy fiber pathway and Schaffer collaterals (Figure 4). The cortical input arises from the perirhinal cortex and terminates in the entorhinal cortex. The perforant pathway originates from the entorhinal cortex and enters into the dentate gyrus and the stratum lacunosum moleculare that is adjacent to CA3. The second pathway is known as the mossy fiber pathway, because the axons originating from the granule cells of dentate gyrus are called mossy fibers and further, there are no other projections than mossy fibers from the dentate gyrus to the CA3 region (Amaral and Witter, 1995; Bear et al., 2001; Kandel et al., 1991; Witter and Groenewegen, 1992; Witter et al., 2000). The axons branch in the CA3 region. One branch runs out of the hippocampus into the fornix that is a major output pathway of hippocampus (Bear et al., 2001). The other branch, known as Schaffer collaterals, is the last excitatory pathway of hippocampus. The neurons of CA3 region send their axons to the stratum radiatum where the collaterals synapse with the dendrites originating from the neurons of CA1 region (Amaral and Witter, 1995; Bear et al., 2001; Kandel et al., 1991; Witter and Groenewegen, 1992). Interestingly, CA2 region does not receive inputs from the CA3 region, but the neurons of CA2 region send projections to the CA1 region. From the CA1 region, the neurons project back to the entorhinal cortex as well as into the other cortical and subcortical regions (Amaral and Witter, 1995).

Glutamate and other EAAs can activate long-term potentiation (LTP) at any synapse of the known hippocampal pathways. LTP is a long-lasting enhancement of synaptic transmission (Bear et al., 2001; Bliss and Collingridge, 1993). In the regions of CA1 (Schaffer collaterals) and dentate (perforant pathway) LTP is induced by NMDA receptors whereas in the CA3 region (mossy fiber pathway) the induction of LTP is the responsibility of the non-NMDA receptors (Chen and Tonegawa, 1997; Kakegawa et al., 2002). In both cases, the activation of EPSP contributes to the
2 Review of the literature
Figure 4. The structure of hippocampus and the pathways of hippocampal circuits. The uppermost pictures present the sagittal view of mouse and human brain. Hippocampus is indicated as grid area and amygdaloid complex as crossbar. The picture in the middle is a coronal section of a mouse brain indicating hippocampal pyramidal subfields, cortical and subcortical areas. The lowest picture presents the cellular layers of hippocampus. The hippocampal circuits are shown with arrows: 1. Mossy fiber pathway, 2. Schaffer collaterals. The perforant pathway, running from the entorhinal cortex to DG, is not indicated. Abbreviations are as follows: alv, alveus hippocampus; Amyg, amygdaloid complex; CA1-CA3, sub fields 1-3 of cornu Ammonis; cc, corpus callosum; DG, dentate gyrus; GrDG, granular layer dentate gyrus; Hil, hilus dentate gyrus; Mol, molecular layer dentate gyrus; Pyr, pyramidal cell layer of hippocampus; St Rad, stratum radiatum; St Or, stratum oriens

release of $\text{Ca}^{2+}$ ions into the postsynaptic site, which play a role in the induction of LTP. During activation, new AMPA receptors are inserted into the synaptic membrane, the postsynaptic membrane becomes more sensitive to EAAs and the potential differential becomes greater (Bettler and Mulle, 1995; Feldmeyer et al., 1999; Kauer et al., 1988; Lees, 2000). In the CA1 region, $\text{Ca}^{2+}$ entry via receptor-gated ion channels activates $\text{Ca}^{2+}$ dependent protein kinases (PKC), $\text{Ca}^{2+}$/calmodulin -dependent kinase II (CamKII) and tyrosine kinases that are involved in phosphorylation cascades in cells. Especially CamKII is thought to act as a molecular form of memory (Bliss and Collingridge, 1993; Chen and Tonegawa, 1997). In conclusion, LTP evokes long-lasting, sometimes permanent changes of synapse modification and cell function, known as synaptic plasticity, which in turn, is necessary for learning and information storage.

2.3.3. HIPPOCAMPUS IN LEARNING AND MEMORY

Apparently, the hippocampus is not a storage site for very long-term information such as childhood memories or habits. Rather the hippocampus is necessary for more conscious efforts. In humans, lesions in the hippocampal formation lead to learning impairment although these individuals are able to remember passed events (Rolls, 2000). Animals with damage in the hippocampus or in areas close to it have difficulties in solving space cued and acquisition tasks like the Morris water maze and the radial arm maze (Eichenbaum, 2001; Jarrard, 1993; Letty et al., 1995). In other words, performances that require solving problems by using working memory or remembering locations by using spatial memory, also known as declarative memory, which is an information storage for facts and events (see (Bear et al., 2001; Eichenbaum et al., 1996; Rolls, 2000) are directed by hippocampus.
2.3.4. GLUTAMATE RECEPTORS

The chemical synaptic neurotransmission in the CNS is both excitatory and inhibitory (see below). The major excitatory transmitter in the brain is the amino acid, glutamate. The other endogenous excitatory amino acid (EAAs) acting on the glutamate binding site is L-aspartate (Farooqui, 1991; Loscher, 1998). The release of EAAs from the presynaptic site contributes to the depolarization of the postsynaptic membrane potential, known as the excitatory postsynaptic potential (EPSP) (Bear et al., 2001). Postsynaptic receptors activated by glutamate are subdivided into four types of receptors. Three of them, AMPA receptors, kainate receptors and NMDA receptor are named according to their different chemical agonists, α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA), kainic acid (KA) and N-methyl-D-aspartate (NMDA), respectively (Figure 5). They are all ionotropic receptors because they are coupled with ionophores. In contrast, the fourth type of glutamate receptors is a G-protein-linked receptor with several receptor subtypes; this type is called the metabotropic receptor (Bear et al., 2001; Kandel and Schwartz, 1991). However, an ionotropic kainate receptor coupled with G-proteins does exist (Cunha et al., 1999). All the receptors activated by neurotransmitters are membrane-spanning proteins having both the binding site for the specific neurotransmitter on the region exposed to the extracellular site and an ion channel through the membrane. The approximate molecular masses of the proteins for AMPA, kainate and NMDA are 52 kDa, 76 kDa and 209 kDa, respectively (Farooqui, 1991). The membrane-spanning proteins are composed of four or five subunits, each of which contains four or five membrane-spanning α-helical regions. Together the subunits form a pore, an ion channel through the membrane and the binding of the neurotransmitter to the receptor is needed to open that channel (Bear et al., 2001; Kandel and Schwartz, 1991).

2.3.4.1. α-Amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA)/kainate receptors

Ionotropic glutamate receptors, AMPA and kainate, are distinguished by their ligand binding sites and by their subunits (Figure 5). However, AMPA and kainate receptors have several common features and therefore the receptors are often referred as non-NMDA receptors (Bettler and Mulle, 1995; Kandel and Schwartz, 1991). Both receptors gate a low conductance cation channel that is permeable to Na\(^+\) and K\(^+\) although Ca\(^{2+}\) permeable AMPA receptors have also been found (Bettler and Mulle, 1995; Itazawa et al., 1997; Mahanty and Sah, 1998). Neither, AMPA nor kainate receptors have a binding site for NMDA, but they are affected by KA and quisqualate A.
The AMPA type of glutamate receptors represents the fastest form of excitatory transmission in the mammalian brain. The AMPA receptors are comprised of four subunits (GluR1, GluR2, GluR3, GluR4) of which the GluR2 subunit is impermeable to Ca\(^{2+}\) ions (Donevan and Rogawski, 1995). Kainate receptors are comprised of five subunits (GluR5, GluR6, GluR7, KA1, KA2) of which 7 out of 9 subunits are the result of different exon splicing and/or RNA editing (Bettler and Mulle, 1995; Lees, 2000).

AMPA and kainate receptors are widely distributed in the vertebrate nervous system, although there is some variation in composition of non-NMDA receptor subtypes between brain areas as well as between neurons and non-neuronal cells. The density of AMPA receptors is highest in hippocampus and cerebral cortex, being somewhat similar to that of NMDA receptors (below) (Bettler and Mulle, 1995; Farooqui, 1991; Washburn et al., 1997). Hippocampal pyramidal neurons mainly show an outwardly rectifying, Ca\(^{2+}\) impermeable response to kainate and AMPA while non-pyramidal neurons show inward rectifying currents in response to AMPA; these are known as Ca\(^{2+}\) permeable AMPA receptors (Donevan and Rogawski, 1995; Washburn and Dingledine, 1996).

Also kainate receptors coexist in the same brain areas with AMPA receptors. The highest density of kainate receptors is found in the mossy fiber system and in the inner and outer layers of cerebral cortex (Farooqui, 1991). Hippocampal CA3 pyramidal cells contain GluR6 receptor subunits where they participate in mossy fiber-CA3 synaptic transmission. Also kainate receptors modulating synaptic transmission at CA1 interneurons contain GluR6 subunits (Mott et al., 2003; Mulle et al., 2000; Rodriguez-Moreno et al., 2000). There are subtype differences in the receptors even between the dendrites, axons and soma of the same neuron. In addition, AMPA and kainate receptor subtypes can coexist in one cell but their subtypes do not coassemble (Bettler and Mulle, 1995; Lees, 2000; Rodriguez-Moreno et al., 2000; Vignes et al., 1997; Washburn et al., 1997; Wenthold et al., 1996).

The function of AMPA receptors is to mediate excitatory synaptic currents (Bettler and Mulle, 1995) and it has been shown that the activation of AMPA receptors can initiate and maintain seizures (Loscher, 1998). In addition, Ca\(^{2+}\) permeable AMPA receptors show faster desensitisation and higher single-channel conductance in some cortical and hippocampal interneurons when compared to AMPA receptors of other neurons (Bettler and Mulle, 1995; Greig et al., 2000; Itazawa et al., 1997) highlighting the importance of AMPA receptors in the excitatory action in these brain areas (Kyrozis et al., 1995).

Kainate receptors have been proposed to act as modulatory receptors at the synapse because kainate receptors are present also presynaptically (Bortolotto et al., 1999; Kamiya, 2002;
Lees, 2000; Reid and Bliss, 2000; Schmitz et al., 2001) where they regulate glutamatergic neurotransmission (Bettler and Mulle, 1995; Kandel and Schwartz, 1991; Lees, 2000; Reid and Bliss, 2000). However, kainate receptors are also involved directly in the excitatory action within AMPA receptors because the activation of kainate receptors leads to a down-regulation of GABA receptors (Lees, 2000; Reid and Bliss, 2000; Vignes and Collingridge, 1997). In particular, the GluR6 subunit seems to be important in sensitising kainate receptors because GluR6 deficient mice have an elevated seizure threshold to KA (Mulle et al., 1998).

Ca$^{2+}$ permeable AMPA receptors that lack the GluR2 subunit have most probably binding sites for polyamines. One site may be near the cytoplasmic side and the other at the extracellular site near the pore (Washburn and Dingledine, 1996). Both extracellular and intracellular polyamines act as open channel blockers at AMPA receptor (Bowie et al., 1998; Fletcher and Lodge, 1996; Isa et al., 1996; Koh et al., 1995). In addition, polyamines mediate the inward rectification of kainate receptors containing GluR6 subunits by blocking the channel pore (Mott et al., 2003; Washburn and Dingledine, 1996).

2.3.4.2. N-methyl-D-aspartate (NMDA) receptors

The agonist N-methyl-D-aspartate selectively activates the third ionotropic glutamate receptor (Figure 5). The NMDA receptor, as well as other glutamate receptors, is comprised of four cation selective subunits of which each subunit (NR1, NR2A, NR2B, NR2C, NR2D with the splice variants) has four transmembrane regions (M1, M2, M3, M4) with extracellular N-terminal domains. The N-terminus is thought to participate in the binding of glutamate (Kandel and Schwartz, 1991; Yamakura and Shimoji, 1999) and Zn$^{2+}$ ions (Paoletti et al., 1997; Traynelis et al., 1998; Zheng et al., 1994). In addition to glutamate, the NMDA receptor requires binding of the coagonist glycine for activation. The binding site for glycine is in the transmembrane region M1 of the NMDA receptor subunit NR1. The transmembrane region M2 lines the channel pore and the N terminus of the M2 segment contains the binding site for magnesium (Mg$^{2+}$) ion. The sensitivity to Mg$^{2+}$ ion varies between different NMDA receptor subtype compositions (Kandel and Schwartz, 1991; Loscher, 1998; Yamakura and Shimoji, 1999). Accordingly, M3 and M4 membrane spanning regions are associated with NMDA receptor channel blockers such as MK-801, phencyclidine and polyamines (Chao et al., 1997; Gallagher et al., 1997; Kashiwagi et al., 1996; Kashiwagi et al., 2002; Kashiwagi et al., 1997; Yamakura and Shimoji, 1999). Thus, one NMDA receptor subunit contains all of the regulatory features needed to create a functional channel: the transmitter binding site, the cation binding site, the agonist/antagonist binding sites and glycine.
AMPA/KAINATE RECEPTOR

NMDA RECEPTOR

GABA RECEPTOR

Figure 5. The structure of excitatory glutamate receptors, (A) AMPA/kainate and (B) NMDA receptors, and a structure of GABA receptor (C). Four out of five receptor subunits in the membrane are presented as squares, figures indicate the binding sites of amino acids and drugs, arrows indicate the direction of ions through the open channel pore. Mg$^{2+}$-ion has a binding site in a pore of NMDA receptor. Abbreviations used are AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid; BZD, benzodiazepine; Ca$^{2+}$, calcium ion; Cl$^-$, chloride ion; GABA, γ-amino butyric acid; Glu, glutamine; Gly, glycine; KA, kainic acid; Mg$^{2+}$, magnesium ion; Na$^+$, sodium ion; NMDA, N-methyl-D-aspartate; PA, polyamines; PTX, picrotoxin; PTZ, pentlenetetrazol; binding site (Farooqui, 1991; Kandel and Schwartz, 1991).

The NMDA receptors are ubiquitously present in the brain. However, the highest density of NMDA receptors is found in the hippocampus, striatum, thalamus and cerebral cortex (Farooqui, 1991). It appears that both subunits, NR1 and NR2, are needed for a functional NMDA receptor having a heteromeric structure (Petralia et al., 1994). Since both of the subunits have either splice variants (NR1) or subtypes (NR2), there are several alternatives for the functional NMDA receptor (Loftis and Janowsky, 2003; Nankai et al., 1998). The composition of the NMDA receptor subunits changes in the brain during foetal development (Cathala et al., 2000; Nankai et al., 1998; Okabe et al., 1998; Virgo et al., 2000; Williams et al., 1991a), during aging (Clayton et al., 2002), in diseases and injuries (Hynd et al., 2001; Virgo et al., 2000) as well as between the brain areas and neurons of the mature brain (Loftis and Janowsky, 2003; Mortensen et al., 1999; Nankai et al., 1998; Yamakura and Shimoji, 1999).

The NMDA-activated receptor-channel complex has some unique features. First, the channel is permeable to Ca$^{2+}$, Na$^+$ and K$^+$ ions. Second, at the resting membrane potential (-65 mV), the channel pore of the NMDA receptor is plugged by a Mg$^{2+}$ ion and hence, the ion transfer between the extra- and intracellular space through the NMDA receptor is prohibited. The NMDA receptors usually coexist with AMPA and kainate receptors at the same post-synaptic membrane and therefore, the release of glutamate from the presynaptic site activates each type of glutamate receptor. However, at normal resting membrane potential, the binding of glutamate to the NMDA receptor only opens the pore. The intracellular ionic current increases due to release of Na$^+$ ions into the cell through AMPA and kainate receptors. When adequate depolarisation (20-30 mV) is reached, the voltage-dependent Mg$^{2+}$ ion pops out of the pore of the NMDA receptor and Ca$^{2+}$ and Na$^+$ ions are able to flow freely into the cell through the NMDA receptor channel (Bear et al., 2001; Kandel and Schwartz, 1991; Loscher, 1998). Thus, both the binding of glutamate to the NMDA receptor and cell membrane depolarisation are necessary for the full activation of NMDA receptors. The non-NMDA receptors control EPSP, where the current flow through the NMDA receptor lasts only as long as AMPA and kainate receptors are activated (Bear et al., 2001; Kandel

and Schwartz, 1991). Thus AMPA and kainate receptors from the post-synaptic site, contribute to the phenomenon of the silent synapses where glutamate binding to the post-synaptic receptors does not change the resting membrane potential (Lees, 2000).

2.3.4.2.1. Polyamine binding sites at NMDA receptor

When the polyamine binding sites in the Ca\(^{2+}\) permeable AMPA receptors (Donevan and Rogawski, 1995; Isa et al., 1996; Washburn and Dingledine, 1996), K\(^+\) channels (Ficker et al., 1994; Oliver et al., 2000) and NMDA receptors were discovered (Ransom and Stec, 1988; Romano et al., 1992; Williams et al., 1990; Williams et al., 1991b), it offered a general understanding to the prolonged controversy of the role of polyamines. Polyamines can influence membrane potential via the K\(^+\) channels, AMPA and NMDA receptors both on the intracellular and extracellular sides of the cell membrane (Figure 5). SPM has attracted the main interest in binding studies because SPM with its four amino groups has the highest binding affinity of all polyamines. Conversely, PUT has received least attention. Intracellular SPM is responsible for the block of the inward rectifier K\(^+\) channels (Kir) (Williams, 1997a; Williams, 1997b) and AMPA/kainate receptors (Bowie and Mayer, 1995; Donevan and Rogawski, 1995; Isa et al., 1996; Kamboj et al., 1995) whereas the extracellular polyamines, mainly SPM and SPD, control the excitability of NMDA receptors (Araneda et al., 1999; Araneda et al., 1993; Singh et al., 1990; Subramaniam and McGonigle, 1994; Williams, 1997a; Williams, 1997b; Williams et al., 1991b). Polyamines are able to promote both inhibition and stimulation of NMDA receptors. The theory of polyamines modulating NMDA receptors was first proposed by Ransom and Stec (1988), who showed that both SPD and SPM increased the binding affinity of MK-801, the open-channel blocker of the NMDA receptors. The efficiency of SPM and SPD on binding to the ion channel blocker is dependent on the saturating condition of glutamate and glycine (Ransom, 1991; Romano et al., 1991; Sharma and Reynolds, 1999; Williams, 1997b; Williams et al., 1991b). This mechanism is known as glycine-independent stimulation (Reynolds and Miller, 1989; Sacaan and Johnson, 1990; Traynelis et al., 1995; Williams, 1989), which is controlled by the NMDA receptor subunits, NR1 and NR2 (Kashiwagi et al., 1996; Williams et al., 1995; Williams et al., 1994; Yamakura and Shimoji, 1999; Zhang et al., 1994). The second mechanism, glycine-dependent SPM stimulation is the result of a decreasing agonist concentration and dissociation of glycine from the NMDA binding site and it is observed in NR1/NR2A and NR1/NR2B receptors. Two inhibitory mechanisms of polyamines apply when there is a decrease in glutamate affinity or when extracellular SPM is strongly voltage-dependent (Williams, 1997a; Williams, 1997b; Williams et
al., 1994; Yamakura and Shimoji, 1999). However, there is a variation in the sensitivity of SPM binding at the NMDA receptor and this is probably due to developmental changes in the NMDA receptor conformation (Subramaniam and McGonigle, 1993; Subramaniam and McGonigle, 1994; Williams et al., 1991a) as well as due to the differences in the channel subunits of NMDA receptors between brain areas (Ientile et al., 1999; Mortensen et al., 1999).

2.3.4.2.2. Ifenprodil binding site on the NMDA receptor

A drug named ifenprodil (Figure 2) is a neuroprotective agent known to act as a noncompetitive antagonist of polyamines at or near the polyamine recognition site of NMDA receptors (Figure 5) (Legendre and Westbrook, 1991; Mott et al., 1998; Ransom, 1991; Reynolds and Miller, 1989; Williams, 2001). Ifenprodil attenuates the enhanced binding of MK-801 by polyamines, and the elevated concentrations of polyamines SPD and SPM, but not PUT, are able to inhibit the high-affinity binding of ifenprodil (Berger and Rebernik, 1999; Reynolds and Miller, 1989; Shaw, 1994). In CNS, the ifenprodil binding site distribution parallels that of NMDA receptors, having the highest density in the hippocampus, in the striatum and in the cerebral cortex (Dana et al., 1991). Moreover, like the polyamine sensitivity, also ifenprodil sensitivity to NMDA receptors changes during development and there are differences in ifenprodil sensitivity to NMDA receptors even within a single neuron (Kew et al., 1998). Ifenprodil has been shown to interact with the subunit NR2B of the NMDA receptor (Gallagher et al., 1996; Grimwood et al., 2000; Perin-Dureau et al., 2002; Zhang et al., 2000) although an interaction with other NMDA receptor subunits has also been postulated (Masuko et al., 1999; Pahk and Williams, 1997; Williams, 1993; Williams, 2001). Ifenprodil may have a state-dependent (concentration and voltage) made of action with NMDA receptors (Legendre and Westbrook, 1991; Reynolds and Miller, 1989; Williams, 1993; Zhang and Shi, 2001). Whether ifenprodil acts at the same sites as polyamines or has a different binding site at NMDA receptors is still not resolved.

2.3.5. GABA RECEPTORS

The amino acid, γ-aminobutyric acid (GABA) is the major inhibitory synaptic neurotransmitter in CNS, while glycine inhibits only a minority of neurons. GABA receptors are composed of two subtypes, GABA$_A$ and GABA$_B$ receptors, which consist of three subunits ($\alpha$, $\beta$ and $\gamma$) with splice variants. Five subunits are needed to form an ion permeable channel pore...
(Figure 5). The subunit $\alpha$ is needed for binding of transmitter, the subunits $\alpha$ and $\beta$ bind barbiturates and the subunit $\gamma$ is responsible for benzodiazepine binding at GABA receptors. Both barbiturates and benzodiazepines activate GABA$\mathrm{A}$ receptors although in different ways (Bear et al., 2001; Kandel and Schwartz, 1991; Poulter et al., 1999; Xin et al., 2001). Also antagonists, such as picrotoxin and PTZ, bind to the same site at GABA$\mathrm{A}$ receptors (Huang et al., 2001). GABA receptors are ubiquitously distributed in the brain, being located in many of the non-pyramidal neurons (Ghez, 1991; Martin and Jessell, 1991a; Mason and Kandel, 1991). The majority of the basket cells in the dentate gyrus are GABA-ergic and therefore act as inhibitory interneurons (Amaral and Witter, 1995). In contrast to the amino acids glutamate and glycine, GABA is synthesized only by the neurons that release it. GABA is synthesized from various metabolic sources by specific enzymes in the cytosol of axon terminals. As with the excitatory receptors, also inhibitory receptors are transmitter-gated ion channels. However, inhibitory channels are permeable to a natural anion: Cl$^-$, which hyperpolarizes the membrane raising the membrane potential away from the threshold to trigger an action potential. The action is called the inhibitory post-synaptic potential (IPSP) (Bear et al., 2001; Kandel and Schwartz, 1991; Rabow et al., 1995). Recent studies have revealed that GABAergic cells, such as hippocampal interneurons, can also be excitatory (Lamsa and Taira, 2003). GABA receptors have an important role in epilepsy and in various psychiatric disorders (Martin, 1991; Rabow et al., 1995).

### 2.4. EXPERIMENTAL ANIMAL MODELS OF CHEMICAL BRAIN INJURY

The release of the excitatory neurotransmitter, glutamate, activates NMDA and non-NMDA (AMPA and kainate) receptors on the postsynaptic site of axon terminal. Even a brief exposure to high glutamate concentrations will result in neuronal death. The activated NMDA and non-NMDA receptors allow Ca$^{2+}$ ions to pass through the opened receptor–gated ion channels. An excess inflow of Ca$^{2+}$ ions into the neurons and the increased Ca$^{2+}$ concentration in the neurons can activate Ca$^{2+}$ dependent proteases, second messenger cascades leading to apoptosis and may produce the generation of free radicals which are toxic to the neurons. The neurotoxic effect of glutamate and its derivatives is called excitotoxicity and it is thought to play a major role in various pathological diseases of CNS as well as occurring after traumatic insults to brain (Bear et al., 2001; Bettler and Mulle, 1995; Chen et al., 1997; Choi, 1992; Kandel and Schwartz, 1991).
2.4.1. KAINIC ACID (KA)

Originally kainic acid was isolated from the Japanese seaweed *Digenea simplex*. It is an analogue of glutamate which acts as an excitatory neurotransmitter at AMPA and kainate receptors. KA is a neurotoxic agent causing an intense excitation of neurons in CNS, which is seen as epileptic-like seizure activity (Johnston et al., 1974; Olney et al., 1974; Sperk et al., 1983) and the neuropathological outcome is similar to that seen in epileptic patients. Therefore, KA is widely used in drug-induced models of epilepsy (Ben-Ari et al., 1981; Lotham and Collins, 1981). The development of seizures is classified into different stages. In the early phase after KA treatment, the animals perform a staring behaviour and later a myoclonic twitching, restricted to the head and to the one side of the forelimbs. In the later stages, animals suffer a generalized clonic convulsion, so called "wet dog shakes" and develop severe seizures with rearing and falling. In the last stage, animals experience a tonic seizure with whole body extension, which can be fatal. The development of seizures takes place from 2 to 6 hours in mice and rats, thereafter the animals are exhausted and seem to recover from the treatment. In the following 24 hours, the animals present a motor hyperactive behaviour and some animals elicit occasional convulsions days after KA treatment. Both hyperactivity, spontaneous convulsions and even biting occur also in handling of the animals (Halonen et al., 1995; Milgram et al., 1988; Sperk, 1993; Sperk et al., 1983).

Neuronal damage develops as fast as 2 to 4 hours after KA treatment. KA activate neurons ubiquitously in the brain, but the most affected brain areas are the amygdaloid complex, cerebral cortex, hippocampal formation and thalamus. The first signs of histological changes are detected as condensed nuclei and vacuolisation of neurons and as swelling of astrocytes and edema. Several types of glial cells show enhanced proliferation. In the electron microscope, the axo-dendritic and axo-somatic synapses also appear dilated. In the hippocampal formation, the pyramidal neurons of CA1 and CA3 regions and the hilus of the dentate gyrus are the most vulnerable to KA, while neurons of hippocampus CA2 region are almost resistant. Pyramidal neurons show a shrunken profile with darkly stained cytoplasm and nuclei. Moreover, mossy fiber sprouting is a common feature after KA (Tauck and Nadler, 1985). Later the pathological changes become complex and all the tissue elements are involved in the process. The edema declines in 48 hours and injured neurons die by necrosis and apoptosis. Days after the treatment, the destroyed neurons are replaced by astrocytes (Choi, 1992; Nitecka et al., 1984; Sperk, 1993; Sperk et al., 1983). Finally, the distribution and severity of neuronal damage correlate with the intensity of the KA-induced seizure activity.
There may be several underlying mechanisms by which KA causes neuronal damage. The reason why certain areas, such as hippocampal pyramidal neurons, are vulnerable to KA is because these areas contain a high concentration of kainate receptors. The stimulation of excitatory pathways by KA leads to a strong excitation in the interneurons of DG and in the pyramidal neurons of CA3 and CA1, which in turn, causes seizures. The activation of kainate receptors activates also NMDA receptors that allow Ca\textsuperscript{2+} ions to flow into the cell. Massive Ca\textsuperscript{2+} influx is toxic to neurons and it causes direct, local neuronal lesions, so called axon-sparing lesions in these areas (Reid and Bliss, 2000; Sperk, 1993). KA is able to inhibit GABA receptor activation, and this amplifies the KA-excitatory effect (Ben-Ari and Cossart, 2000). In addition, prolonged stimulation of the excitatory pathway mediates the excitation to distant areas, such as cortical areas and amygdala, and evokes distant neuronal damage (Balchen et al., 1993; Sperk, 1993). Finally, KA induced neuronal damage may also be related to secondary seizure related neuropathological events such as hypoxia, hypoglycemia or edema (Sperk, 1993).

2.4.2. PENTYLENETETRAZOL (PTZ)

Pentylenetetrazol is a GABA\textsubscript{A} receptor antagonist binding to the picrotoxin site on GABA\textsubscript{A} receptors. PTZ inhibits the activation of GABA\textsubscript{A} receptors and hence enhances the action of excitatory receptors. Huang et al. (2001) have shown that PTZ inhibits the GABA-activated Cl\textsuperscript{−} current in a concentration-dependent but in a voltage-independent manner by decreasing the opening of the GABA receptor channel. PTZ is mainly used as a kindling model, where repeated electrical or chemical stimuli cause a long-lasting change in the properties of neurons and animals develop seizures. Kindling mimics the development of epileptogenesis in humans (Ekonomou et al., 2001; Huang et al., 2001; Martin, 1991). As with KA, also the seizure development after PTZ treatment is divided into different phases. The first signs of PTZ-induced behaviour are staring, twitching of vibrissae and facial clonus, which are followed by myoclonic jerks, forelimb clonus and sometime axial convulsive waves through the body. In the third phase, clonic convulsions with rearing and falling appear. In addition, violent running and bouncing behaviour is observed before the last phase of seizure development. More generalized seizures and tonic convulsions with loss of body control are evident in the PTZ-treated animals (Franke and Kittner, 2001; Liu et al., 1999; Loscher, 1998; Medina et al., 2002).

Histological and anatomical changes have been mainly studied after repeated exposures to PTZ and the changes are not so drastic than after KA administration. The first signs of altered neurological morphology occur within 24 hours in pyramidal cells of hippocampus CA1 and CA3.
regions and in granule cells of dentate gyrus (Franke and Kittner, 2001; Rauca et al., 2000). The most obvious change is the shrinkage of the cell body; triangular shape and dark-blue stain of neurons while the normal neurons are clear, slight by stained and round cells with visible Nissl-bodies. The delayed neuronal loss can be observed as long as 15 weeks post-kindling (Franke and Kittner, 2001).

2.5. BEHAVIOURAL PHENOTYPING OF TRANSGENIC MICE

Gene mutations produce genetically engineered mice can either overexpress an extra gene in the genome as is the case with our transgenic animals or lack a certain functional gene from their genome in the case of knock-out mice. In both scenarios, the gene mutation often causes a change in the phenotype of the animals. The phenotype is a display of the genotype at the anatomical, behavioural, biochemical, molecular, physical and physiological levels. Behavioural phenotyping is needed to evaluate behavioural alterations in genetically engineered mice (Crawley, 2000) taking into consideration also the behavioural profile of the background mouse strain (Rogers et al., 1999; Voikar et al., 2001). One of the aims of behavioural neuroscience is to design a standardized behavioural profiling battery that would be available to every laboratory working with mutant mice. The standardized protocol would include also the recommendations for breeding, housing and testing conditions. Hence, the behavioural phenotyping would become more reliable and allow comparison of the results obtained in different laboratories (Arndt and Surjo, 2001; Bolivar et al., 2000; Rogers et al., 1997; van der Staay and Steckler, 2001).

2.5.1. SHIRPA

SHIRPA protocol is designed for a routine comprehensive screening and testing of mouse behavioural and functional phenotype (Crawley, 1999; Rogers et al., 1997). The term SHIRPA is an abbreviation from the collaborative partners involved in designing of the protocol. SHIRPA protocol is divided into three stages of which the first is the behavioural observation profile assessment, the second stage includes the functional phenotype assessments and histopathological analysis. Finally, the third stage includes the assessments for the neuropsychiatric functions such as the light-dark box, the elevated plus maze and the Morris water maze (Crawley, 2000; Rogers et al., 1997; Rogers et al., 1999).
2.5.2. RADIAL ARM MAZE

Radial arm maze (RAM) is one of the most commonly used behavioural paradigms (T-maze, Barnes maze) in the assessment of learning and memory processes in rats and mice. Radial mazes require the animal to orientate according to known landmarks and to choose specific arm (Figure 6). In the end of each arm there is a reward, which can be food or water (Crawley, 2000). The eight arm maze (8-RAM) was first developed for rats by Olton and Samuelson in 1976 (Olton et al., 1979; Olton and Samuelson, 1976) and later modified for mice (Crusio et al., 1993; Laviola et al., 1992; Pick and Yanai, 1983). The 8-RAM performance describes both spatial memory, also known as working memory, and long-term memory of the animal. The spatial memory is employed in a daily acquisition task and the long-term memory is exploited in a memory retrieval task where mice perform an earlier learned task in the maze after a “holiday” period.

Figure 6. The radial eight arm maze surrounded by the landmarks that offer directional information to mouse during trial.
2.6. TRANSGENIC MICE OVER-PRODUCING PUTRESCINE

As already discussed, PUT with its low affinity for EAA receptors and almost undetectable intracellular levels has been difficult to study in terms of the influence of PUT on neuropathological states. In addition, the experiments based on different kinds of administration e.g. intracranial or systemic pharmacological treatments, have produced with conflicting results. In an attempt to study the role of PUT in normal and in neuropathological conditions we first produced two different mouse lines overaccumulating PUT. In one strain PUT is produced from ornithine since the mouse line carries extra copies of ODC. In the other strain, there is backconversion of polyamines i.e. there are extra copies of SSAT in the genome of this mouse strain.

2.6.1. ODC TRANSGENIC ANIMALS

The first PUT overaccumulating transgenic mouse line produced (Halmekytö et al., 1991a; Halmekytö et al., 1991b) carried the human ODC gene under its own promoter and overexpressed ODC in all tissues. Very high basal activity of ODC (80-fold compared to syngenic mice) resulted in an overaccumulation of newly synthesized PUT in tissues. The ODC overexpressing mice have been studied extensively to characterize their phenotype (Jänne et al., 1991a; Jänne et al., 1999; Kauppinen and Alhonen, 1995). A reduced free magnesium $\text{Mg}^{2+}$ concentration was observed in the brain of ODC transgenic mice (Kauppinen et al., 1992). Magnesium ions are known to block NMDA-activated ion channels and hence to inhibit the excitatory current through the channel. Therefore, it was proposed that ODC transgenic mice might be sensitive to glutamate-activated neurotransmission through NMDA receptors. However, electrophysiological \textit{in vitro} studies revealed that synaptic plasticity was not reduced in ODC transgenic mice (Pussinen et al., 1998). The subsequent \textit{in vivo} studies supported this view. These animals were protected from seizures produced by either electric stimulation or chemically induced kindling by PTZ (Halonen et al., 1993). In addition, ODC overexpressing mice showed impaired learning in the Morris water maze test (Halonen et al., 1993). When ODC transgenic mice were subjected to ischemia-induced neuronal injury (Lukkarainen et al., 1995) the increase in ODC activity and the massive accumulation of PUT apparently did not enhance neuronal death but rather seemed to represent an adaptive response to the insult. The influence of ODC overexpression on the outcome of brain ischemia was later studied also with transgenic rats carrying the human ODC gene (Lukkarinen et
al., 1997; Lukkarinen et al., 1999; Lukkarinen et al., 1998). These studies supported the results obtained with mice, suggesting that PUT is not causally involved in the maturation or spread of the stroke lesion in vivo. On the contrary, the activation of ODC is more likely to have a neuroprotective action after the insult (Lukkarinen et al., 1999; Lukkarinen et al., 1998). The protective effect of ODC is supported by the observation that neurotrophins, such as brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF) and neurotrophin-3 (NT-3), are significantly elevated in ODC transgenic mice (Reeben et al., 1996). Finally, a long-term survival study showed that a life-time constitutive activation of ODC and the accompanying PUT accumulation did not cause neuronal degeneration in the central nervous system (Alhonen et al., 1995).

2.6.2. SSAT TRANSGENIC ANIMALS

The other transgenic mouse line overproducing PUT in its tissues carries additional mouse SSAT gene copies in the genome of BALBc/DBA 2 strain of mice (Figure 1) (Pietila et al., 1997). Approximately 20 copies of the transgene result in increased SSAT mRNA and 1.5-fold increase in SSAT activity in the brain tissue. ODC activity is enhanced up to 10-fold as compared with the syngenic mice. Further, the PUT concentration is 20-fold above the normal level, N1-acetylated spermidine, which is not normally found, is also readily detectable to the brain tissue, and the concentration of SPD is decreased (Jänne et al., 1999; Pietila et al., 1997). The fact that SSAT activity was not related to the markedly enhanced SSAT mRNA expression may be due to the translational regulation of SSAT (Fogel-Petrovic et al., 1996b). A similar phenomenon is also evident in transgenic mice over-expressing the SSAT enzyme under the control of metallothionein I promoter (Suppola et al., 1999). In spite of the minor change in the SSAT activity, tissue polyamine pools revealed a dramatic distortion accompanied by bizarre phenotype changes, such as hairlessness and wrinkled skin (Pietila et al., 1997; Pietila et al., 2001). In addition, SSAT transgenic mice have been shown to be very sensitive to the polyamine analog, N1,N11-diethynorspermine (DENSPM) (Alhonen et al., 1999; Vujcic et al., 2000) and the female mice were found to be infertile (Min et al., 2002; Pietila et al., 1997).

While a possible neuroprotective role of ODC overexpression in the central nervous system is supported by several studies, the consequences of SSAT overexpression in the central nervous system of SSAT transgenic mice have gained little attention beyond the basic metabolic characterization. However, the accumulation of PUT in brain tissue is even more pronounced in the SSAT transgenic mice than in ODC transgenic animals. Therefore, the SSAT transgenic mouse

line could serve as a very interesting model when studying the effects of activated polyamine
catabolism, especially PUT overaccumulation in CNS: its role in neurobiological functions, in
behaviour and in neuropathological states.
3 AIMS OF THE STUDY

The experimental study was designed to elucidate the effects of accelerated polyamine catabolism in mouse CNS. We were interested in neuronal survival after excitotoxic insults in mice overexpressing SSAT. This study also shows how disturbed polyamine catabolism results in an alteration in mouse behaviour. The mouse line carries extra gene copies of the polyamine catabolic enzyme, SSAT, under the control of its own promoter. The overexpression of SSAT gene leads to a permanent distortion of polyamine metabolism, such as an increase in PUT and N\textsuperscript{1}-acetylspermidine and a decrease in SPD and SPM pools in all tissues. SSAT transgenic mice offered us an opportunity to study the role of polyamines, especially the overaccumulation of PUT in the brain. The role of PUT in CNS has received distinctly less attention than that of the higher polyamines.

In order to investigate the physiological consequences of accelerated polyamine catabolism in the brain; and in particular its putative role in excitotoxicity, we:

1. Studied the alterations in brain polyamine concentrations in SSAT overexpressing mice with two commonly used models of excitotoxicity, namely treatment with kainic acid and PTZ to examine the changes in polyamine concentrations after a neuropathological insult.

2. Analyzed the concentrations of other brain metabolites in SSAT mice.

3. Examined whether the SSAT overexpression and the consequent PUT accumulation after the neurotoxic insult have a neuroprotective role or whether they induce neuronal death.

4. Characterized the brains of SSAT mice at a histological and molecular level.

5. Undertook a comprehensive behavioural performance assessment in SSAT mice.
4 EXPERIMENTAL PROCEDURES

4.1. ANIMALS (I-III)

All animals used in this study were from the hybrid strain BALBc X DBA/2 mice. SSAT overexpressing mouse line UKU165b were produced by the microinjection technique (Pietila et al., 1997) in the A.I. Virtanen Institute for Molecular Sciences. After the experiments, mice were either CO₂ anaesthetized and decapitated or pentobarbital anaesthetized (40 mg/kg) before preparation of the samples as described in detail later. The experiments were approved by the Institutional Animal Care and Use Committee of the University of Kuopio and by the Provincial, Government of Eastern Finland.

4.2. BEHAVIOUR STUDY (III)

Altogether 69 adult (3-4 months) SSAT syngenic and transgenic mice were used in the behavioural study. They were housed one mouse per cage under controlled conditions (temperature +21°C, lights: 07.00 a.m. – 07.00 p.m.). All experiments were carried out between 08.00 a.m. and 04.00 p.m.

4.2.1. SHIRPA PROTOCOL

SSAT syngenic and transgenic mice were subjected to behavioural and functional analysis of mouse phenotype using SHIRPA protocol (Rogers et al., 1997) with some modifications. We set up a behavioural observation assessment according to the instructions introduced on the web page: www.mgu.har.mrc.ac.uk/mutabase/shirpa-summary.html. The secondary screen of SHIRPA protocol was utilized only to a limited extent. Mice were tested twice, before the eight-day and two-day radial maze periods without familiarization to the equipment made according to the instructions as recommended on web pages. We used the scoring system of the standardized primary screen where the behaviour of mice was recorded in a viewing jar and arena as well as their activity in arena. In addition, we recorded the general health and physiological condition of the mice, such as body weight, body length, temperature and skin colour. The entire behavioural
battery comprised of 34 recording parameters. To avoid incidental findings and to better manage such a large data base, we first tested which parameters were interrelated using Spearman rank correlations for the pooled data of all animals. This first screening yielded five groups of intercorrelated parameters (p<0.05) that were named as follows. 1. Activity (body position, spontaneous activity, locomotor activity); 2. Aggression (aggression, provoked biting, irritability); 3. Defense behavior (trunk curl, limb grasping); 4. Grip strength; 5. Muscle tone (touch escape, toe pitch, body tone, limb tone); 6. Rear elevation (pelvic elevation, tail elevation). The remaining parameters were ignored in this study. Scoring of each test parameter was normalised on a scale from 0 to 4 to allow parameter grouping.

4.2.2. EIGHT ARM RADIAL MAZE

The eight arm radial maze (8-RAM) is an acquisition task, which is based on food rewards. The 8 arms radiated out from an octagonal Plexiglas center, 22 cm in diameter. The Plexiglas arms were 25 cm long, 6 cm wide and 6 cm high. The mouse was expected to pick up the food reward from the end of every arm. While in the acquisition task mice should use their spatial memory by following the landmarks around the maze and also, remember which arms they have already visited. Repeated trials correlate inversely with the learning efficiency of the mice. This acquisition task was originally developed by Pick and Yanai (Pick and Yanai, 1983) and adapted for the mouse by Crusio et al. (Crusio et al., 1993). We used food fasting instead of water deprivation. Every arm ended with a perforated wall behind which there were cereal rewards. This system confirmed that the maze environment was filled with uniform scent and mice were not able to use their sense of smell while searching for the food reward. The food reward was placed behind a small barrier in front of the wall, which, in turn, prevented animals from seeing the food. At the beginning of each trial, a mouse was placed in the centre of the maze for 20 seconds. Thereafter the guillotine doors were lifted up and mouse was able to enter into a chosen arm. Between the visits to the arms, the mouse was kept in the centre area for 5 seconds by sliding the guillotine doors down. The purpose of this was to prevent animals from utilizing non-memory based strategies.

The SSAT transgenic and syngenic mice were accustomed to rice cereals (Kellogg's) beforehand and their food was restricted for two days before the first trial and the food restriction was maintained through the training period. The body weight was maintained on 80-85% of the normal body weight. They were introduced to the maze before the trial by allowing them to move and eat cereal rewards freely for 10 min on two successive days. The landmarks we used for the
mice were a black cross and a triangle on the white painted wall around the maze. Between male and female mice, the maze was cleaned with mild detergent to remove the odours of the opposite sex.

In the following nine days mice were trained once per day and after each training period they received a food reward in their own cages. The mice were removed from the 8-RAM after final retrieval of all rewards or they were allowed to make 16 entries or spent 20 min at the most in the 8-RAM, whichever came first. An entry was counted when all four paws had entered the arm. An entry into an arm from which it had already retrieved the food pellet was deemed an error. Because of the large inter-individual variability in the number of errors before finding the most difficult i.e. the 8th reward, the performance was evaluated on the basis of total number of errors made before the 7th correct choice. Therefore time and visits were recorded as follows: a) the number of correct entries before seven correct, b) the number of error entries before seven correct, c) time spent during the trial. Finally, after two weeks rest, the mice were allowed to perform the acquisition task for two more days to reveal their long-term memory.

4.3. DRUG TREATMENTS (I-II)

4.3.1. KAINIC ACID (I)

SSAT syngenic and transgenic mice were treated subcutaneously (s.c.) with KA, which was dissolved in 0.9 % sodium chloride. Saline-injected mice served as controls. To discover the appropriate level of neurotoxic effect in the hybrid strain BALBc X DBA/2 of mice, doses of KA ranged from 10 to 40 mg/kg in preliminary experiments. Thereafter, both syngenic and transgenic mice were divided into groups by age and they were treated with a single dose of KA (34 mg/kg) to determine if there was a correlation between the age of the mice and the effect of KA. The age distribution of KA treated mice was from 3 weeks to 8 months.

Time points used in KA experiment were dependent on whether we wanted to determine the total size of KA induced injury (7 days) or to analyze the expression level of SSAT and glial fibrillary acidic protein (GFAP) mRNAs by in situ hybridization (0 h, 6 h, 12 h, 1 day, 3 days and 7 days).
4.3.2. PENTYLENETETRAZOL (II)

Two separate experimental set ups were performed in the study to analyse the effects induced by PTZ. The administration of PTZ intravenously (i.v.) induced convulsions that were recorded for each animal. A single dose of PTZ (35 mg/kg) intraperitoneally (i.p.) was used to study the total neuronal damage induced by the neurotoxin in both SSAT syngenic and transgenic mice (see materials and methods in publication II). All animals in the PTZ infusion were decapitated at the end of the infusion whereas the mice treated with a single i.p. dose were decapitated at the 4h time point.

4.3.3. IFENPRODIL (II)

The N-methyl-D-aspartate receptor antagonist ifenprodil was used in combination drug experiments with PTZ. It was administered i.p. as a single dose before PTZ infusion (see materials and methods in publication II for details). All animals treated with ifenprodil-PTZ combination were decapitated at the end of the infusion.

4.4. SAMPLE PREPARATION (I-III)

4.4.1. PERFUSION AND SAMPLE COLLECTION (I-II)

The mice prepared for the histochemistry were perfusion-fixed intracardially 7 days after KA and 4h after PTZ injections. The animals were anaesthetized with pentobarbital (40mg/kg, i.p.) and treated with 100 U of heparin. Thereafter mice were perfused with saline and 4% PFA in 0.1M phosphate buffered saline (PBS) for 10 minutes. Brains were removed and post-fixed for 5 h in 10 volumes/weight of the same fixative. The fixed brains were either cryoprotected with 20% sucrose before freezing in isopentane-liquid nitrogen (I) or embedded in paraffin (Shandon Hypercenter XP, Life Sciences International Ltd., England) (II) and stored at +4°C.

The fresh frozen brain samples used in in situ hybridization were embedded into cork plates with saline and frozen on dry ice. Brain samples needed for quantitative analysis were dissected on ice, collected into 1.5 ml tubes and snap frozen in liquid nitrogen. All frozen samples were stored at -80°C.
4.4.2. HISTOCHEMISTRY (I-II)

The brain samples prepared for the histochemistry were cut rostro-caudally either with a cryotome (Leica JungCM 3000, frozen brains) or a microtome (Microm, Germany, paraffinized brains) into 8-10 µm thick sections and collected onto SuperFrost*/Plus (Menzel-Gläser, Germany) microscope slides. The slides were air-dried and stored in -20 and +4°C, respectively. The staining procedure was as described in the materials and methods of publications I and II.

The brain sections were stained in order to identify the cytoarchitecture and different anatomical regions of the brain. In addition, staining was needed for the determination of the distribution and severity of KA and PTZ induced neuronal damage.

4.4.3. IN SITU HYBRIDIZATION (I)

The in situ hybridization protocol was used to detect the expression of SSAT and GFAP mRNA in the brain tissue of the SSAT mice in normal conditions and after KA treatment. The levels of mRNA expression were followed at set time points (5 syngenic and 5 transgenic per group) after KA. The fresh-frozen brains were cut into 10-µm slices on a cryostat (Leica JungCM 3000) at -20°C and the sections were collected onto SuperFrost*/Plus microscope slides (Menzel-Gläser, Germany) which were stored at -20°C until used. The sections were hybridized as described (see materials and methods I) by using the following synthetic oligonucleotides: 5'-CCAACAATGCTATGTCCTTCAGGGGTCCAGTGCTC-3' (for SSAT) and 5'-CCATCCCGCATCTCCACCGTCTTTACCACGATGTTCTCTTG-3' (for GFAP). The autoradiographic XAR-5 films (Kodak, USA) were exposed to the hybridized slides for 14-21 days at +4°C.

4.4.4. NORTHERN BLOTTING (I)

The expression of GFAP mRNA at 24 hours after KA treatment (25 mg/kg) was analysed with Northern blot from both (four) syngenic and (four) transgenic mice. The fresh-frozen cerebral cortices were homogenized for the acidic guanidium thiocyanate/phenol/chloroform extraction. Purified RNA samples were processed for Northern blot system (see materials and methods I for details) and GFAP mRNA was detected from the exposed Biomax film (Kodak, USA).
4.4.5. POLYAMINE ANALYSIS (I-II)

The determination of polyamine concentrations in tissue samples was performed by using high-performance liquid chromatography (HPLC). The protocol for the analysis has been described by Hyvönen et al. (Hyvönen et al., 1992). Briefly, the fresh-frozen brain samples were homogenized with 2 volumes of homogenization buffer (25 mM Tris, pH 7.4, 0.1 mM EDTA, 1 mM DTT). The tissue homogenates (20 µl) were diluted in 1/10 with 5% sulphosalicylic acid and 10 µM diaminoheptane was used as an internal standard. The solutions were incubated overnight in a freezer to obtain a better precipitate before centrifugation and filtration.

4.4.6. MEASURING OF SSAT ENZYME ACTIVITY (II)

The same tissue homogenates prepared for the polyamine analysis were further prepared for the analysis of SSAT enzyme activity. The prepared supernatant fractions were added into a reaction mixture containing 100 mM Tris-HCl, pH 7.8, 3 mM spermidine, pH 7.0, 1 mM dithiothreitol and 50 µCi ^{14}\text{C} \text{-acetyl-coenzyme A and thereafter they were incubated at } +37^\circ\text{C for 10 min. The reaction was stopped with the addition of NH_2OH \cdot \text{HCl. The mixture were boiled and centrifuged. The resultant reaction mixtures were spotted onto pieces of P81-paper, which were air-dried, washed, dehydrated and dried. The samples were counted in a liquid scintillation counter 1450 Microbeta™ Plus (Wallac, Finland).}

4.4.7. HORMONE ANALYSIS FROM BLOOD SAMPLES AND TISSUE WEIGHT

Separate mice, syngenic (20) and transgenic (20) of both sex, were sacrificed for hormone analysis. Total blood samples were taken from anaesthetized (midazolam 0.6 mg/kg and fluanisone 1.25 mg/kg) mice through the heart with Microtainer™ K2E and SST tubes (Becton-Dickinson, USA). The collected plasma and serum samples were analysed either in a commercial laboratory (Medix, Helsinki) or by us using radioimmunoassay.

At the end of blood sampling, the mice were sacrificed for the tissue preparation. Testicles and uteri of both transgenic and syngenic mice were excised and weighed.
4.4.8. MRI ANALYSIS (II)

We used Dormicum-Hypnorm (midazolam 0.6 mg/kg and fluanisone 1.25 mg/kg) cocktail as the anaesthetic in brain metabolite analysis, which was conducted with $^1$H nuclear magnetic resonance spectroscopy ($^1$H NMR). The sampling procedure of brain tissues was carried out according to that described by Halonen et al. (1993) and the analytical protocol for the MRI analysis was based on Laatikainen et al. (Laatikainen et al., 1996).

4.5. OTHER ANALYSES

4.5.1. SEIZURE SCORING (I-II)

The development of seizures and their duration were recorded for at least 4 hours after KA treatment and after a single dose of PTZ. The seizure scoring scale used for the behavioural changes in seizure inducing experiments was modified for the mice from that described by Halonen et al. (Halonen et al., 1995). The scoring scale was: 1 = jaw clonus or head nodding, 2 = focal convulsions of one forelimb, 3 = mild bilateral forelimb clonus or rearing and severe forelimb clonus lasting at least 10 seconds, 4 = severe forelimb clonus, rearing, back fully extended, 5. rearing and falling.

4.5.2. CELL COUNTS (I-II)

Neuron counts were carried out from Nissl-stained sections to quantify the numbers of surviving pyramidal neurons in hippocampal CA1 and CA3 regions after KA and PTZ treatments. Neuronal profiles with a visible cell body and the entire outline of the cell, excluding glial cells, were counted from every 10th section (leaving 100 µm in between) of each hippocampus with a 40x objective on a Leica 3000 RB. Cell counts at all levels (-1.34 to -2.06 mm behind the bregma) were averaged and the mean numbers were used for statistical analysis. The count of neurons was performed in a blind manner.
4.5.3. IMAGE ANALYSING METHOD (I)

The exposed \textit{in situ} hybridization films were quantified with video-based image analysing software MCID\textsuperscript{TM} (Micro Computer Imaging Device System M4) from Imaging Research (St. Catherines, ON, Canada). The quantified brain regions were determined according to a rat brain atlas (Paxinos and Watson, 1986). The evaluation of autoradiograms was shown as relative optical density (ROD) values: $\log_{10}(\text{maximum possible number of grey levels/observed grey levels})$, i.e. $\log_{10}(1/\text{grey level transmittance or reflectance}).$

Background correction was made for each film before densitometric measurements. The grey level corresponding to the $^{14}$C-plastic standards (Amersham, UK) lying within the exposure range of the film was determined and used as a fifth degree polynomial approximation to construct a grey level to activity transfer. Densitometric measurements were carried out from four sections per animal and the obtained data was further analyzed statistically. The mean values of treated and untreated animal groups are presented as kBq/g.

4.6. STATISTICAL ANALYSES (I-III)

The statistical analyses were conducted either by using Microsoft Excel program for Windows (Microsoft Inc., USA), Graph Pad Prism software for Windows (Graph Pad Software Inc., San Diego, USA) or SPSS software for Windows (SPSS Inc., Chicago, USA) of which the first two programs were used mainly for graph production. SPSS software was the most versatile program for behavioural analyses. When appropriate, two-tailed Student's $t$-test, one-way ANOVA, or Fisher's protected least significant difference was used. In the behavioural analysis, the data gathered from the primary screen of SHIRPA test battery and 8-RAM was compared between sex and genotype. The SHIRPA data was compiled into six groups and analysed by non-parametric independent Kruskall-Wallis method. The data from 8-RAM was analysed with general linear model as repeated-measures ANOVA as well as electrophysiological recordings. All the data are presented as mean $\pm$S.E.M.
5 RESULTS

5.1. POLYAMINE CONCENTRATIONS AND SSAT ACTIVITY IN THE BRAIN OF SYNGENIC AND TRANSGENIC MICE (I-II)

5.1.1. POLYAMINE CONCENTRATIONS IN DIFFERENT BRAIN REGIONS

The polyamine concentrations are presented from a total of 10 different brain regions of SSAT syngenic and transgenic mice (Table 1, publication I/Table 1. and II/Table 1.). The basal levels of polyamines were determined in three separate experiments and hence there is some variation between the results. Transgenic animals overexpressing the SSAT gene showed marked changes in polyamine concentrations. The most prominent changes were an overaccumulation of PUT in every brain area, this being most pronounced in pons (20-23-fold), cerebellum (15-36-fold), midbrain (18-fold), hippocampus (10-16-fold), cerebral cortex (14-17-fold) and white matter (25-fold). The appearance of N\textsuperscript{1}acetylspermidine, not normally present in rodent tissues, is indicative of an activation of polyamine catabolism. In addition, there was also a decline in the SPD concentrations in pons (3.3-6.3-fold), cerebellum (2.3-5.1-fold), thalamus (4.1-5.2-fold) and medulla (15.2-fold) in transgenic mice although the concentration of SPD appeared to vary in these areas. The SPM concentration showed a slight insignificant increase in transgenic mice.

SSAT activity was assayed in the PTZ experiment. There were 6 different brain regions measured in both syngenic and SSAT transgenic mice (publication II/Table 1.). The SSAT activity was slightly, though significantly, increased in pons (2.2-fold), midbrain (2.4-fold), cerebral cortex (2.0-fold) and hippocampus (1.4-fold) in transgenic mice. It is generally recognized that SSAT has an extremely short half-life and its activity is difficult to detect at low enzyme levels.

5.1.2. THE EFFECT OF KAINIC ACID ON POLYAMINE CONCENTRATIONS IN VARIOUS BRAIN REGIONS

Table 1. presents the polyamine concentrations in the brain of syngenic and SSAT transgenic mice which were sacrificed 6 days after KA treatment (34 mg/kg). KA treatment caused an increase in the PUT concentration in cerebral cortex, hippocampus, cerebellum and medulla of

Table 1. Polyamine concentrations (pmol/mg wet wt.) measured from different brain regions of SSAT transgenic (tg) and syngenic (sg) mice, which were treated with kainate (34 mg/kg) and saline.

<table>
<thead>
<tr>
<th>Brain region</th>
<th>Putrescine</th>
<th>N(^\text{-})acetyl spermidine</th>
<th>Spermidine</th>
<th>Spermine</th>
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<tr>
<td>C. cortex</td>
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<tr>
<td>Saline</td>
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<td>162.9±5.8</td>
<td>232.0±8.1</td>
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<td>8.2±1.4</td>
<td>117.5±10.5**</td>
<td>243.7±19.8</td>
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<td>9.6±1.9</td>
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<td>197.2±28.5</td>
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<td>220.0±4.8</td>
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<td></td>
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<td>204.0±31.3</td>
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<td>1.2±0.5</td>
<td>864.7±288.2</td>
<td>245.0±15.7</td>
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<td>70.0±17.5</td>
<td>41.5±7.6</td>
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</tbody>
</table>

The values are ±SEM. From 6 to 11 animals per group. ***p<0.001; **p<0.01; *p<0.05. The statistical significance in the saline treated group refers to the difference between syngenic and transgenic mice and in the kainate treated group the first symbol refers to the difference induced by the drug and the latter shows the difference between the treated groups.
transgenic mice. However, the increase in the PUT concentration was significant only in medulla 
(2-fold) of transgenic mice. PUT concentrations in syngenic mice were unaltered. The 
concentration of $N^1$-acetylspermidine showed a slight increase in most of the measured areas of 
transgenic mice. Levels of acetylated spermidine were increased in hippocampus (1.5-fold), 
cerebellum (1.6-fold) and medulla (2.3-fold). With one exception, the SPD concentration had a 
tendency to decrease in different brain areas of transgenic mice, with the most distinct decrease 
seen in pons (6.4-fold) and cerebellum (1.8-fold). In medulla, the SPD content was 6.3-fold 
increased. Syngenic mice showed an increase in the SPD concentration in cerebral cortex, 
thalamus and white matter whereas there was a decline in the SPD level in hippocampus, 
cerebellum, pons and medulla. The SPM concentration was virtually unaltered in both groups. The 
molar ratio of PUT to SPD after KA administration was 0.03±0.03 in syngenic mice and 0.80±0.28 
in transgenic mice. The molar ratio of SPD to SPM was 7.74±8.88 in syngenic mice, being highest 
in pons-medulla (21.33±2.19) and 2.13±3.02 in transgenic mice.

5.1.3. THE EFFECT OF PENTYLENETETRAZO L ON POLYAMINE CONCENTRATIONS 
AND SSAT ACTIVITY IN VARIOUS BRAIN REGIONS

PTZ treatment i.p. (35mg/kg) did not increase the SSAT activity in the syngenic group 4 h 
after injection, but clearly enhanced the enzyme activity in all brain areas in transgenic mice 
(publication II/Table 1.). The PUT concentrations of transgenic mice increased most extensively in 
pons (2.8-fold) and slightly in midbrain and hippocampus. However, in cerebellum and cerebrum, 
PUT tended to decrease in response to the drug, but in comparison with syngenic mice, the level of 
PUT was still extremely high. The $N^1$-acetylspermidine level showed an approximately 2-fold 
increase when compared with untreated transgenic mice, but was still markedly higher in 
comparison with the syngenic mice (II/Table 1.). In syngenic mice, the drug slightly elevated the 
PUT concentration in cerebellum (2.1-fold), SPD content tended to increase in pons, midbrain, 
cerebral cortex and hippocampus while there was a decrease in the SPD concentration in 
cerebellum and cerebrum. The SPM concentration did not show any alterations in response to the 
drug. The molar ratio of PUT to SPD after PTZ was 0.29 ± 0.02 in syngenic and 1.08 ± 0.48 in 
transgenic animals, i.e. the difference remained over 37-fold. The molar ratio of SPD to SPM after 
PTZ was 4.55 ± 6.79 in syngenic and 1.12 ± 0.57 in transgenic animals, respectively.
5.2. SSAT mRNA EXPRESSION IN SYNGENIC AND TRANSGENIC MICE (I)

The expression of SSAT mRNA was localized in different brain areas by in situ hybridization analysis (publication I/Fig.1.). The transgenic mice showed very high levels of SSAT mRNA all over the brain in comparison with the syngenic mice and interestingly, in transgenic mice, the cerebellum appeared to contain markedly more SSAT message than was present in transgenic mouse cerebrum. This finding is interesting since the level of SSAT activity was equally in areas.

5.3. KAINIC ACID (I)

5.3.1. SEIZURE ACTIVITY

The development of seizures was recorded after KA administration and the first signs of KA-induced behaviour were observed at fifteen minutes. Both the development of seizure activity and neurotoxic effect were dependent on the dose administered and the age of the mouse. Moreover, seizure activity was required to achieve neuronal loss. On the basis of preliminary experiments, KA doses were scaled according to neurological outcome. In BALBc X DBA/2 mice, doses lower than 20 mg/kg induced only mild seizures (jaw clonus and head nodding) and no neuronal loss was detected. The doses from 20 to 30 mg/kg caused behavioural changes ranging from brief myoclonic jerks of head, body whiskers or ears, to frank convulsions with forelimb clonus and loss of postural tone. Neuronal injury seen in the hippocampus varied from no injury to the neuronal loss concentrated mainly in CA3 region. A dose of 32 mg/kg evoked also rearing and falling of the animals as well as severe seizures with variable damage to both CA1 and CA3 regions of hippocampus. When treated with a dose of 34 mg/kg, all the syngenic and transgenic animals exhibited severe seizures within 15-30 min after the injection. No obvious difference in the development of KA-induced behaviour was seen between transgenic and syngenic mice. In BALBc X DBA/2 mice, the age between 3-6 months gave the best response between dose and neurological effect.
5.3.2. LOSS OF NEURONS AND MORTALITY

Based on the information obtained from preliminary experiments, doses of 25 mg/kg and 34 mg/kg KA were chosen to define sublethal and lethal effect, respectively. In the hippocampus CA1 and CA3 regions are the most vulnerable areas to KA toxicity in rodents. Nissl-stained brain slides showed that the high dose (34 mg/kg) of KA resulted in a moderate, though significant, loss of neurons in CA3 region in nontransgenic but not in transgenic mice (publication I/Fig. 5. and 6.). The CA1 pyramidal neurons were resistant to KA neurotoxicity which is in agreement with the previous report on KA toxicity in BALBc mice (Schauwecker and Steward, 1997).

SSAT transgenic and syngenic animals were exposed to a high dose (34mg/kg, -LD\textsubscript{50}) of subcutaneous KA. The overall mortality among the syngenic animals (n=24), was 54%, while only 22% of the transgenic mice (n=23) died after the treatment (I/Table 2.). This difference was statistically significant (P<0.01).

5.3.3. GFAP EXPRESSION AFTER KA TREATMENT

The induction of GFAP mRNA after the sublethal dose of KA (25 mg/kg) was followed for seven days at different time points (0h, 6h, 12h, 1d, 3d, 7d). Treatment with the low dose did not produce any detectable neuronal loss, but resulted in strong neuronal activity sufficient to trigger astrogliosis. As revealed by in situ hybridization, the levels of GFAP-specific mRNA increased abruptly at days 1 and 3 after the injection in the brains of syngenic mice, while the changes in the brains of transgenic mice were much less pronounced (publication I/Fig. 2.). The most striking difference between syngenic and transgenic animals was an apparent lack of GFAP induction in the cerebral cortex and thalamus of transgenic animals in response to the drug.

The quantitative imaging analysis was performed from the areas Stratum radiatum (St rad) and Stratum oriens (St or) of hippocampus (results not shown) and Cerebral cortex (I/Fig. 3.). The expression of GFAP mRNA were elevated from the pretreatment level by a factor of more than 7 at days 1 and 3 after KA in the syngenic animals, while the transgenic animals showed only a modest induction of the GFAP mRNA in the cerebral cortex (I/Fig. 4.).
5.4. PENTYLENETETRAZOL (II)

5.4.1. SEIZURE THRESHOLD

Continuous infusion of PTZ into the tail vein caused epilepsy-like seizure activity dose-dependently. The development of seizures was followed from the start of PTZ infusion until tonic seizures in both animal groups. The time points of (and the doses required) for (i) first jerk, frank convulsions with forelimb clonus, loss of postural tone and rearing, and (ii) the tonic seizure, severe tonic extension of both forelimbs and hind limbs, were recorded (publication II/Table 2.). The dose of PTZ required for the occurrence of the first jerk was more than 30 % higher (p<0.001) in the transgenic animals in comparison with their wild-type littermates. Similarly, the dose required for the tonic seizure was more than 40 % higher (p<0.05) in the transgenic animals. Moreover, the time elapsing for the occurrence of the first jerk was prolonged by 35 % (p<0.001), and the time to tonic seizure was prolonged by 47% (p<0.001) in the transgenic animals in comparison with their syngenic littermates.

5.4.2. NEURONAL DAMAGE

Based on the infusion method, we chose one dose (35 mg/kg) of PTZ, which induced convulsions in most animals and hence was expected to cause neuronal damage as well (publication II/Fig. 2.). Syngenic mice proved to be more vulnerable to PTZ than SSAT transgenic mice. As expected, there was some variation in the extent of injured neurons in both the CA3 or CA1 region of treated mice (5 sg and 5 tg). Nonetheless, PTZ caused a marked reduction in the population of surviving neurons measured from the pyramidal cell layer of CA1 and CA3 in hippocampus of syngenic mice. Only some injured neurons were observed in the CA1 region of hippocampus of the transgenic mice, however, the difference between untreated and treated animals was not significant (II/Fig. 3.). There is some controversy about whether these darkly stained cells were actually dead. It is possible that they were about to die and one can postulate that at a later time point we would have been able to observe indisputable lesions.

5.4.3. PTZ COMBINED WITH IFENPRODIL

Two separate groups, transgenic and syngenic mice, received a single dose of ifenprodil (20 mg/kg) 15 min before the start of PTZ infusions. The dose and time of administration was selected
based on preliminary experiments as described in materials and methods to give maximal protection in syngenic animals against PTZ-induced seizures. As indicated, ifenprodil remarkably increased (p<0.001) the dose of PTZ required for the occurrence of the first jerk and tonic seizure in the syngenic but not in the transgenic mice (publication II/Fig. 1.). In fact, the differences in the seizure thresholds between untreated syngenic and transgenic animals totally disappeared after ifenprodil. Accordingly, ifenprodil significantly (p<0.001) prolonged the time required for the occurrence of the first jerk in both treated groups but the time difference required for the occurrence of the first tonic seizure was significant (p<0.001) only in syngenic animals. The difference of the seizure thresholds, both with regard to the occurrence of the first jerk and tonic seizure, between untreated syngenic and transgenic animals completely disappeared after ifenprodil administration.

5.5. BRAIN METABOLITE ANALYSIS BY MRI (II)

As PUT is a potential precursor of GABA (Seiler and Bolkenius, 1985), the major inhibitory neurotransmitter, it is possible that the elevated levels of brain PUT would expand also the GABA pool. We measured the concentrations of certain brain metabolites, such as GABA, glutamate and N-acetyl aspartate, in syngenic and transgenic mice using $^1$H NMR spectroscopy (publication II/Table 3.). There were no differences between the levels of these amino acids in the analyzed syngenic and transgenic brain regions (cerebral cortex, cerebrum and cerebellum).

5.6. DIFFERENCES IN HORMONE CONCENTRATIONS BETWEEN SSAT SYNGENIC AND TRANSGENIC MICE

The results of blood sample analyses are shown in publication III/Table 1. Adrenocorticotropin (ACTH) concentration was increased by two-fold in both sexes of transgenic mice and the concentration of corticosterone was also significantly increased. In contrast, levels of thyroid-stimulating hormone (TSH) and thyroxine (T4) were remarkably decreased in transgenic mice. Similarly, the testosterone concentration in SSAT mice was reduced to undetectable levels while it was readily detectable in syngenic animals.

Tissue weights were generally in proportion to body weights (not shown). The uteri:body weight ratios in transgenic mice were only half of those found in syngenic mice (tg (5): 2.84±0.64;
sg (5): 5.61±0.49, p<0.01) whereas the corresponding ratio for testicles did not differ between transgenic and syngenic males (tg (5): 6.54±0.32; sg (5): 6.99±0.39).

5.7. BEHAVIOUR DATA (III)

5.7.1. COMPREHENSIVE BEHAVIOUR OF SSAT MICE

SSAT transgenic mice did not differ from syngenic controls in their body weight (mean±sem, tg: females (22) 22.8±0.8 g, males (47) 26.0±0.8 g; sg: females 21.1±0.7 g, males 26.2±0.7 g, all ps>0.78). However, their behaviour differed clearly from the syngenic mice (publication III/Fig. 1.). Whereas syngenic mice were animated and vigorous in the jar and the arena, SSAT transgenic mice appeared almost phlegmatic, occasionally slumping with long pauses between movement episodes. They also exhibited little aggression towards the experimenter. To quantify these observations, we conducted a statistical analysis on six categories of behavioural parameters. Activity (mean±sem, tg (34): 2.56±0.08; sg (35): 2.90±0.08), aggression (tg: 1.53±0.10; sg: 2.30±0.14), grip strength (tg: 1.97±0.06; sg: 2.41±0.06) and muscle tone (tg: 2.83±0.04; sg: 2.99±0.06) categories did not differ between the sexes and were pooled for the genotype analyses. However, defence (tg: 2.32±0.14; sg: 2.57±0.11) and rear elevation (tg: 2.38±0.08; sg: 2.51±0.11) categories were sex-dependent (in defence: females: 2.14±0.18; males: 2.60±0.09 and in rear elevation: females: 2.78±0.11; males: 2.30±0.08), and were analyzed separately for each sex. SSAT transgenic mice differed from their syngenic controls in activity (p<0.004), aggression (p<0.001), grip strength (p<0.001) and muscle tone (p<0.040). In contrast, transgenic mice showed no reduction in their defensive reactivity (in females: tg (11): 2.00±0.27; sg (11): 2.27±0.24; in males: tg (23): 2.48±0.15; sg (24): 2.71±0.11), and no change in rear elevation (in females: tg: 2.82±0.12; sg: 2.73±0.19; in males: tg: 2.17±0.08; sg: 2.42±0.13).

5.7.2. LEARNING AND MEMORY

5.7.2.1. Acquisition task

All mice reached an stable level of performance in the 8-RAM win-shift task by the seventh day of testing. Therefore, only the first seven days were included in the analysis of the task acquisition (publication III/Fig. 2.). In the combined analysis of all groups, the ANOVA revealed a
significant effect of genotype (p<0.014), and also a significant genotype by sex interaction (p<0.015) in the number of errors. In the subsequent analysis with the sexes separated, no difference between transgenic and syngenic controls was found among the males. In contrast, the female SSAT transgenic mice performed significantly worse than to female syngenic mice (p<0.002).

Furthermore, ANOVA revealed a significant effect of genotype in the time required to complete the trial in the combined analysis of all groups (p<0.001) (III/Fig. 3.). In addition the effect of gender was significant (p<0.000) in the measure of time, but there was no interaction between genotype and gender (p<0.100). When mice were separated according to gender the ANOVA revealed a difference between transgenic and syngenic mice among males (p<0.026) and females (p<0.041).

5.7.2.2. Retention

After two weeks of rest the mice were re-evaluated for three days. The data indicated that all mice had achieved on the second day the performance level they had had at the end of the acquisition period (publication III/Fig. 4.). ANOVA revealed no difference between the genotypes in the number of errors (p<0.214) or any genotype by sex interaction (p<0.424). However, it invariably took longer for SSAT transgenic mice to complete the task and this was independent of sex (III/Fig. 5.). The effect of genotypes was significant (p<0.012), while no genotype by sex interaction was detected (p<0.940).
6 DISCUSSION

6.1. THE EFFECT OF NEUROTOXINS TO THE POLYAMINE CONCENTRATIONS IN SYNGENIC AND SSAT TRANSGENIC MICE

The first step in this study was the basic assessment of regional SSAT enzyme activity and polyamine concentrations in the brain of transgenic and syngenic mice. It was clearly shown that the transgene produces a clearly elevated SSAT mRNA expression in the brain of SSAT mice. However, a high level of SSAT mRNA did not lead to similar increases in SSAT activity, which may be attributable to post-transcriptional regulation of SSAT (Fogel-Petrovic et al., 1996b; Shappell et al., 1993). On the other hand, SSAT has a very short half-life and may not be easily detectable. The altered polyamine metabolism, i.e. the increase in PUT and $N^1$-acetylspermidine concentrations and the decline in the SPD concentration, more accurately reflected long-term SSAT activation. In addition, the enhanced SSAT activity produced by the transgene was maintained in every brain areas throughout the lifespan of SSAT mice (unpublished data). The results revealed that an overexpression of SSAT enzyme in transgenic mice leads to the lifetime activation of polyamine catabolism in every brain area measured.

When mice were treated with excitotoxic drugs, KA and PTZ, the effect on polyamine concentrations was more substantial in transgenic mice than in syngenic mice. Despite the long-term upregulation of polyamine catabolism, SSAT mice were still able to enhance SSAT activity and polyamine backconversion in response to the neurotoxic stimuli. Syngenic mice showed lesser signs of activated polyamine catabolism and thus only minor changes in the polyamine pools after drug treatments were seen. Our observation is in line with the results describing an activation of the polyamine interconversion pathway in response to neuronal injury, seizure activity or neuronal damage (Baudry and Najm, 1994; Hayashi et al., 1999; Rao et al., 2000). Earlier studies have indicated that PUT and SPD are distinctly present in the areas of white matter in CNS (Kremzner, 1970; Shaw, 1994), and PUT is found in grey matter as well (Fischer et al., 1972). The major part of white matter consists of axon bundles and axon terminals. Interestingly, the influence of neurotoxins on the concentrations of polyamines in transgenic mice appeared to mainly focus on the areas rich in white matter. After KA treatment, the PUT level was highest in cerebellum, hippocampus and medulla. The $N^1$-acetylspermidine concentration was increased in the same areas.
while the SPD level was decreased in cerebellum and pons, but in medulla of transgenic mice SPD was markedly increased. After PTZ treatment, the PUT concentration of SSAT mice was highest in hippocampus midbrain and pons while decreased in cerebellum. The $N^d$-acetylspermidine concentration was increased in cerebellum, midbrain and pons. PTZ treatment increased the concentration of SPD in cerebellum, midbrain and pons.

Since polyamine metabolism is sensitive to extracellular disturbances, polyamines have been proposed to play an adverse role in neuronal damage (de Vera et al., 1991; Paschen, 1992a) and apoptosis (Xie et al., 1997) as well as being involved in neuronal death (Sparapani et al., 1997) rather than participating in repair processes. Administration of SPD or SPM induces neurotoxicity in vitro (Sparapani et al., 1997), which is explained by the stimulatory role of SPD and SPM in the activation of NMDA receptors (Ransom and Stec, 1988; Williams, 1997a). In the transgenic brain, however, the SPD content declined 1 to 6-fold (100-400 pmol/mg wet tissue) depending on the brain area while the SPM level showed minor increases in some of the measured brain areas (up to 100 pmol/mg wet tissue). Whole-cell current recordings made with Xenopus oocytes have indicated that concentrations of 10-1000 µM are needed to induce currents gated by NMDA receptors (Romano and Williams, 1994) although the stimulatory effect induced by polyamines can vary between NMDA receptors in different neurons (Durand et al., 1993). Even if results of in vitro models cannot be directly extrapolated to in vivo studies, it is quite unlikely that concentrations below normal levels of SPD would account for the KA induced NMDA receptor activation and to the neurotoxic inflow of Ca$^{2+}$ ions.

On the other hand, excitotoxins and experimental injuries have been shown to induce the polyamine interconversion pathway (Baudry and Najm, 1994; Liu et al., 2001; Rao et al., 2000), which in turn increases the concentrations of PUT and $N^d$-acetylspermidine. The contributory role of $N^d$-acetylspermidine in CNS injuries is not well known but a role as a weak polyamine inverse agonist has been suggested based on NMDA receptor potentiation and intracellular free Ca$^{2+}$ measures (Pritchard et al., 1994). An increase in the PUT concentration has been linked in the literature to neuronal damage (Baskaya et al., 1997a; de Vera et al., 2002; Henley et al., 1996) and to NMDA receptor activation (Gimenez-Llort et al., 1997) with some workers even suggesting that PUT could be used as a general marker of brain damage (Camon et al., 2001). However, transgenic mice overexpressing the ODC enzyme experience a life-long PUT overaccumulation but this does not lead neurodegeneration (Alhonen et al., 1995) and these mice also show resistance to experimental injuries (Lukkarainen et al., 1995).

Our results with SSAT mice revealed a strong protection against excitotoxic neuronal cell death, which was more prominent after KA. Interestingly, KA treatment also caused a more
marked increase in the brain PUT concentration in SSAT mice than seen with PTZ. The increase in PUT elicited by KA was reported also by Martínez et al. (1991). KA activates fast excitatory AMPA/KA receptors which are predominant in the hippocampal pyramidal neurons and in the layers of cerebral cortex. PTZ exerts its effect through inhibitory GABA<sub>A</sub> receptors which are expressed in the dentate, CA3 and CA1 pyramidal basket cells. Their axons innervate with the cell bodies of the pyramidal cells (Amaral and Witter, 1995). These divergent pathways responsible for the neurotoxic stimuli may explain the difference in the resulting activation of polyamine metabolism between brain areas.

Vera (1991) and Paschen (1992) suggested that there was a correlation between high levels of PUT and the severity of symptoms. Our results with both excitotoxic models, KA and PTZ, would lead us to quite opposite conclusions. We suggest that the higher the cellular PUT level, the more neurons survive after a neurotoxic insult. Our results are in agreement with the studies showing a protective role of increased PUT level in neurotoxicity (Gilad and Gilad, 1989) and neuronal injury (Gilad and Gilad, 1991b). It is also proposed that PUT accumulation acts as an inhibitory factor in the PTZ-induced kindling model (Hayashi et al., 1999), which actually could be attributable to a weak antagonistic effect of PUT at NMDA receptors (Sacaan and Johnson, 1990; Williams et al., 1991b). Another possibility is that polyamines can influence the activation of AMPA/KA receptors of SSAT mice. Polyamines are known to block Ca<sup>2+</sup> permeable AMPA/KA receptors (Bowie and Mayer, 1995) although PUT is the least effective polyamine in this respect (Donevan and Rogawski, 1995). The inhibitory effect of accumulated PUT is further supported by an *in vitro* electrophysiological study where the postsynaptic response in CA1 field was depressed in ODC transgenic mice (Pussinen et al., 1998).

Administration of excitotoxins, KA and PTZ, induced both biosynthesis and catabolism of polyamines in the brain tissue which resulted in an overaccumulation of PUT that lasted for several days. After KA, PUT concentration in SSAT transgenic mice was ten times higher than in syngenic mice and the accumulation was still preserved after six days of treatment. In accordance, the extracellular PUT content increased in response to KA (Vivo et al., 2002). If PUT is able to block NMDA receptors or AMPA/KA receptors or both, this is most likely to be seen in SSAT mice after KA treatment because the ratio of PUT to SPD in tissues is almost opposite to that seen in syngenic mice and hence, PUT in the brain of SSAT mice may be able to compete with higher polyamines at the NMDA receptor binding site.

SSAT transgenic mice exhibited a decline in the SPD concentration of CNS throughout their life due to the accelerated interconversion of polyamines. Surprisingly, the SPD level of transgenic mice seemed to remain fairly stable after neurotoxic stimuli. Our observation of
remarkably high SPD concentration in the brainstem of untreated syngenic mice is in agreement with earlier reports (Fischer et al., 1972; Kremzner, 1970; Shaskan et al., 1973). The brainstem includes the medulla, pons, midbrain and cerebellum is the center of ascending and descending pathways that carry sensory and motor information to and from higher brain regions. A high SPD concentration coincides with rapid proliferation nerve cells and non-neuronal cells (Dornay et al., 1986; Gilad and Gilad, 1988) already in the post-natal phase (Kremzner, 1970). In that respect, we may assume that the high basal level of SPD in the brainstem of syngenic mice was involved in the aroused neuronal activity, which could also have caused the increased SPD level in the medulla of transgenic mice. Syngenic mice showed changes in relation to the drug mainly in SPD concentration although the SPD level varied depending on the drug used. Despite the activated polyamine catabolism, which converts SPD to PUT, the SPD concentration of syngenic mice was still notably high in the frontal parts of the brain. Although lower after KA or PTZ treatment, the SPD concentration of syngenic mice was still very high when compared to the SPD concentration of treated transgenic mice. According to earlier work (Dornay et al., 1986; Gilad and Gilad, 1988), the high SPD concentration observed in syngenic mice even after noxious stimuli could be the result of enhanced biosynthesis of polyamines and a sign of proliferation of non-neuronal cells after damage. Therefore, it is tempting to speculate that the high SPD concentration seen in syngenic mice correlates in some way with the repair functions after neurochemical insults. Another, possible explanation for the high SPD levels in syngenic mice may be the straight conversion of SPM back into SPD by spermine oxidase (SMO), an enzyme recently found in peripheral tissues (Vujcic et al., 2002a). In addition to the high SPD level, the backconversion by SMO would also account for the almost static SPM concentration as described in the literature. The presence of active SMO would also explain the absence of N\(^\text{¹}\)-acetylsperrmine even when SSAT was activated. Before one can draw any far reaching conclusions, the presence of SMO in the brain and its participation in the polyamine backconversion will have to be elucidated.

6.2. NEURONAL DAMAGE AND SEIZURE ACTIVITY IN SSAT MICE

6.2.1. NEUROPATHOLOGICAL CHANGES INDUCED BY NEUROTOXINS

The hippocampal formation is one of the most vulnerable parts of the brain to extracellular stressful stimuli. Neuronal cell loss in the hippocampal pyramidal cell layers became evident only after high doses (over 30 mg/kg) of KA. The first signs of neuronal death observed varied in both
CA1 and CA3 regions. After the treatment with 34 mg/kg of KA, the CA3 region was the most severely injured and only a few neurons died in the CA1 region. Experiments with rats have produced similar results (Balchen et al., 1993) but more likely the tolerance to KA induced neurotoxicity seen in CA1 is attributable to the mouse strain, namely BALBc, which was used in our experiments (Schauwecker and Steward, 1997).

The results obtained from PTZ-induced neuronal damage were in line with KA study. PTZ caused a clear increase in the number of injured neurons in pyramidal cell layers but, contrary to KA, the most vulnerable neurons were in the CA1 region. This difference in injured areas may be due to divergent pathways of KA and PTZ in eliciting the neurotoxic stimuli. The overall less severe effect on the pyramidal cells by PTZ, showing only shrunken neurons, may also be related to the early time point (4h). The lack of clear neuronal loss and shrunken neurons in CA1 region may attributable to the low dose of PTZ used (35 mg/kg).

The hippocampal pyramidal neurons of transgenic mice were protected from the neurotoxic stimuli induced by KA or PTZ. SSAT mice had reduced neuronal loss in hippocampus and reduced glial fibrillary acidic protein (GFAP) mRNA expression in cerebral cortex when compared with syngenic mice. GFAP is an astrocyte-specific intermediate filament protein widely used as a marker of neuronal damage and is expressed after intense excitatory neuronal activity (O'Callaghan, 1992; Steward et al., 1997). It seems that the life-long activation of the polyamine interconversion pathway resulting in PUT accumulation in all brain regions provides protection against excitotoxicity. Earlier reports have indicated that the activation of polyamine synthesis shown as increased ODC activity after various brain insults is related to the cell death (Paschen et al., 1991; Reed and de Belleroche, 1990). Furthermore, the activation of polyamine backconversion (Babu et al., 2001; Zoli et al., 1996b) and the apparent PUT accumulation (Zoli et al., 1996a), was claimed to be involved in the development of neuronal damage (Camon et al., 2001; Paschen, 1994). In addition, ODC transgenic rats with overaccumulation of PUT were partially protected against transient focal cerebral ischemia (Lukkarinen et al., 1998). Stronger protection has been observed in the ODC transgenic rats after transient middle cerebral artery occlusion (Lukkarinen et al., 1999). The mechanism behind the apparent neuroprotection is not completely understood, but it may be related to a blockade of excitatory receptors (Bowie and Mayer, 1995; DiScenna et al., 1994; Williams, 1997b). That, in turn, would require an increase in the levels of extracellular polyamines, because polyamine binding sites of excitatory receptors are proposed to be located on the extracellular side of the membrane. Polyamines are released from neurons into the extracellular space (Gilad et al., 1993a; Paschen et al., 1991) and injections of polyamines following brain ischemia appear to protect neurons from delayed neuronal cell death.
Polyamines may also protect neurons after neurotoxic stimuli by inhibiting caspase-3 activation, the leading trigger signal of the apoptotic cell death pathway (Harada, 1997).

6.2.2. PENTYLENETETRAZOL-INDUCED SEIZURE ACTIVITY

The activation of excitatory receptors was demonstrated by infusion of PTZ into the animals. PTZ inhibits GABA$_A$ receptors, and thus induces epileptic like seizures in treated mice. SSAT mice had an increased threshold to PTZ-induced seizure activity. This was probably related to overaccumulation of PUT in the CNS. The possibilities that PUT under these conditions would have been converted to GABA or excitatory amino acids in brain were excluded by $^1$H NMR analysis. The PTZ-induced activation of polyamine catabolism and the resulting increase in PUT have been suggested to inhibit the development of kindling (Hayashi et al., 1992). An inhibitory role of PUT is supported by a study with ODC overexpressing mice indicating that these animals have an elevated threshold to chemical and electrical stimuli (Halonen et al., 1993). Pretreatment with MDL72527, an inhibitor of PAO, increased the seizure activity in rats (Hayashi et al., 1999). Moreover, an inhibition of polyamine biosynthesis with DFMO decreased the PUT and SPD concentrations in the brain and induced hyperactivity (Gerrish et al., 1993). Contradictory studies include a report indicating that increased hippocampal PUT and SPD concentrations lead to a reduced seizure threshold (Gimenez-Llort et al., 1998). In line with the latter view is the motor activity in response to PUT (Camon et al., 1994; De Sarro et al., 1993) and SPM's abilities to induce hyperactivity or to facilitate LTP (Chida et al., 1992; Sanger et al., 1995). 

NMDA receptor antagonists such as diazepam and ifenprodil are known to depress PTZ-induced seizure activity (Becker et al., 1997; Reynolds and Miller, 1989; Tsuda et al., 1997). Ifenprodil binds a common binding site as the polyamines in the NR2B subunit of the NMDA receptor (Perin-Dureau et al., 2002; Ransom, 1991). The neuroprotective properties of ifenprodil have been reported earlier (Baskaya et al., 1997b; Dogan et al., 1997). When our animals were treated with ifenprodil prior to infusion of PTZ, the syngenic mice showed a significantly increased threshold against PTZ while SSAT transgenic mice were virtually unaffected. This finding supports an earlier report of a weak antagonistic action for PUT at the NMDA receptor (Williams, 1997b; Williams, 1989). The strikingly expanded pool of PUT and the dramatically increased molar ratio of PUT to the higher polyamines in the brain of the transgenic animals may well create a condition where PUT becomes a physiologically relevant antagonist at the NMDA receptor.
6.3. THE INFLUENCE OF POLYAMINES ON BEHAVIOUR

6.3.1. BEHAVIOURAL EFFECTS OF POLYAMINES

Analyses of SHIRPA protocol results revealed that transgenic mice had reduced spontaneous and locomotor activity and they behaved less aggressively. Further, they had lowered levels of muscle tone and reduced grip strength. These statistically significant changes in behaviour were visible already during handling of the animals. Their rather phlegmatic appearance with clumpy movements was in contrast to the animated behaviour of the syngenic mice. SSAT mice were also slower than syngenic mice in the radial arm maze, confirming the impression of slowly moving animals.

The literature on the influence of polyamines on behaviour is rather scanty, but earlier studies with intraperitoneal (Sakurada et al., 1975) and intracerebral (Sakurada et al., 1977) injections of SPD and SPM have revealed both excitatory and attenuating effects on motor behaviour. Similar observation have been made in spontaneous climbing and wheel running behaviour experiments, where SPD and SPM induced dose-dependent inhibition of these behaviours (Hirsch et al., 1987). Moreover, both SPD and PUT were able to block the darting behaviour as well as the hyperactivity induced by microinjection of the NMDA receptor antagonist, CPP (3(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid) into prefrontal cortex at doses which did not affect locomotor activity. Binding of ifenprodil, the NMDA receptor antagonist to the polyamine site was without any effect (Crawley et al., 1992). A recent study indicated that SPM administration provoked biting and licking behaviour in mice due to the activation of NMDA receptors (Tan-No et al., 2000). Only a few reports show a correlation between increased PUT and behavioural parameters. Gimenez-Llort (1997) and coworkers found that after systemic NMDA administration nonconvulsant behavioural patterns, measured in an open-field test, were related to the PUT concentrations of hippocampus and frontal cortex whereas SPD and SPM concentrations were unaltered (Gimenez-Llort et al., 1997). Systemic injection of PUT produced motor disorders at high doses (Camon et al., 1994). PUT-induced shaking behaviour was reported to increase brain vascular permeability and this correlated with the enhanced polyamine synthesis (Koenig et al., 1983). In another study, however, systemic administration of polyamines, PUT, SPD and SPM together with NMDA was shown to induce motor depression in mice. Only very high doses of PUT has been reported to induce any reduction of motor activity (Gimenez-Llort et al., 1996a).
The high level of PUT in the brain of SSAT mice produced hypoactivity and reduced aggression. This finding is in line with earlier reported results (Crawley et al., 1992; Gimenez-Llort et al., 1996b) although it seems that systemic administration produces behaviour effects that are closer to the excitatory pathway.

6.3.2. LEARNING AND MEMORY IN SSAT TRANSGENIC MICE

The possibility that altered polyamine pools as a consequence of constitutive SSAT activation, could also affect learning and memory formation was tested in the radial arm maze. The learning performance in the maze revealed that in addition to slow movements, SSAT mice had a sex-dependent learning deficiency. However, when the mice were tested in the retention task after a resting period, SSAT mice did not show any difficulties in memory retrieval.

Reports discussing the influence of polyamines on learning and memory have yielded conflicting conclusions. The learning impairment produced by CPP, a NMDA receptor antagonist, was reversed by SPM administration (Meyer et al., 1998). Similarly, locomotor activity depressed by dizocilpine, another NMDA antagonist, was reversed by a dose of ifenprodil, suggesting that ifenprodil does not induce any working memory deficit (Fraser et al., 1996). Accordingly, SPD administration alone either enhanced (Mikolajczak et al., 2002a; Mikolajczak et al., 2002b) or had no influence on the short-term recognition memory (Kishi et al., 1998a; Kishi et al., 1998b). On the other hand, the learning impairment induced by dizocilpine was enhanced by SPD in a 14-unit T-maze (Shimada et al., 1994), SPM co-administration with benzodiazepine had an effect on path length in the Morris water maze and high doses of SPM inhibited the learning of the platform position (Conway, 1998). Altogether, the learning deficit observed in SSAT mice may derive from increased the PUT levels in the brain.

On the other hand, the sex-dependent learning impairment may also be attributable to the altered hormonal levels. The reduced weight of reproductive tissues confirmed the presence of ovary atrophy as reported earlier (Min et al., 2002). It is quite probable that altered polyamine metabolism can reduce estrogen levels, which in turn then influences NMDA receptor function in hippocampus (Gazzaley et al., 1996; Gurevicius et al., 2003). However, it remains to be studied whether female SSAT mice have alterations in the induction of long-term potentiation (LTP) and NMDA mediated neurotransmission.

We also found a significant increase in the concentrations of adrenocorticotropic (ACTH) and corticosterone in SSAT mice. Earlier studies have indicated that ODC activity and an increase in the levels of higher polyamines can stimulate ACTH (Feige et al., 1986; Scalabrino and Kuopio Univ. Publ. G. A.I. Virtanen Institute for Mol. Sci.
Lorenzini, 1991) and glucocorticoids (Ientile et al., 1983) which are involved in hypothalamic-pituitary-adrenal (HPA) axis. Corticotropin-releasing hormone (CRH) is the primary activator of the HPA axis, releasing ACTH, which in turn triggers the secretion of glucocorticoid hormones (GC) from adrenal cortex. GCs suppress the hypothalamic CRH expression by a feed-back mechanism and hence, control both the basal activity of HPA system and release the stress-activated system (Muller and Keck, 2002). It is possible that the high basal level of ACTH observed in SSAT mice may contribute to the long-term activation of HPA system, which in turn could alter behaviour and induce a learning impairment. HPA hyperactivity has been demonstrated to correlate with depression (Muller and Keck, 2002). Furthermore a deficiency of CRH and hence a decline in HPA activation has been claimed to contribute to hypersensitivity and stress (Bale et al., 2000). Hormonal analysis revealed also that the basal levels of testosterone and thyroid hormones, TSH and T4, were markedly reduced in SSAT transgenic mice. The decline in the testosterone concentration may partly explain some of the noted change in behaviour: e.g. the reduced aggression and activity as well as the reduced muscle tone. Thyroid hormone manipulations early in development have been shown to affect motor behavior and learning skills (Brosvic et al., 2002). It is known that prolonged iodine deficiency can result in a learning disability and in a decrease in the serum T4 concentration (Tiwari et al., 1996) this being also compatible with our results i.e. part of the effects may be secondary to changes in thyroid hormones seen in the SSAT mice.
The purpose of this series of studies was to elucidate the roles of polyamines in cellular functions of CNS both under normal circumstances and after the challenge with excitotoxins. Transgenic mice, having disturbed polyamine metabolism, offer a unique opportunity to study the influence of a lifetime overaccumulation of PUT formation on cellular and physiological functions. They also make it possible to observe behavioural alterations induced by accelerated polyamine catabolism.

1. The polyamine homeostasis was significantly disturbed in SSAT transgenic mice showing an enhanced accumulation of PUT and the appearance of N1-acetylspermidine in every measured brain area. On the other hand, the concentration of SPD was markedly reduced while that of SPM remained almost unaltered when compared with the polyamine concentrations of SSAT syngenic mice.

2. Enhanced accumulation of PUT, an alternative precursor of GABA, did not elevate the CNS content of the latter compound.

3. The excitotoxic drugs used in our experiments were KA and PTZ. KA induced more profound alteration in polyamine metabolism than PTZ. After PTZ administration, changes in polyamine metabolism were limited to the cellular content of PUT and N1-acetylspermidine. Transgenic mice had an increased threshold to PTZ-induced seizures in comparison with syngenic mice. Ifenprodil treatment eliminated this strain difference suggesting an antagonistic role for PUT.

4. Histological analysis revealed a protective role attributable to the enhanced PUT accumulation in the nerve cells. SSAT mice were protected from the neuronal death evoked by both drugs. GFAP, a marker of neuronal damage, was likewise downregulated in SSAT mice.

5. The behavioural profile of SSAT mice was altered in several categories. In addition, female SSAT mice exhibited a learning disability, probably due to the increase of PUT concentrations but perhaps also to disturbed hormone levels.
REFERENCES


Henley, C. M., Muszynski, C., Cherian, L., and Robertson, C. S. (1996). Activation of ornithine decarboxylase and


Pentyletetrazol-induced inhibition of recombinant γ-aminobutyric acid type A (GABAA) receptors:
Mechanism and site of action. The journal of pharmacology and experimental therapeutics 298, 986-995.


Shappell, N. W., Fogel-Petrovic, M. F., and Porter, C. W. (1993). Regulation of spermidine/spermine N\textdagger\textasciitilde\textasciitilde-
acyltransferase by intracellular polyamine pools. FEBS 321, 179-183.


