# TRANSCRIPTIONAL ANALYSIS OF SEX DIFFERENCES IN HIPPOCAMPAL PLASTICITY IN THE MOUSE

Ana Maria Raposo Antunes Simões Martins

January 2006

Thesis submitted for the degree of Doctor of Philosophy
University College London





ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

## ABSTRACT

The neuronal representation of experience as stable memories requires a process termed consolidation, which engages the hippocampus.

Sexual dimorphisms in the performance of a number of tasks requiring hippocampus-dependent memory formation have previously been described. These sex differences are generally attributed to gonadal hormone-mediated mechanisms which impact on neuroanatomy and modulate memory formation.

At the molecular level, memory consolidation requires *de novo* transcription, activating the transcription factor CREB. This activation can be accomplished by a variety of signalling pathways including the CaM kinase cascade.

Male mutant mice bearing a genetic deletion of CaMKK$\beta$, an element of this cascade, are impaired in spatial memory formation in the Morris water maze (MWM), and fail to activate CREB after spatial training. Remarkably, female mutants performed equally to their WT counterparts, indicating a sex-specific requirement for this kinase in spatial memory consolidation. This mutant line was used as a tool to investigate dimorphisms in the molecular mechanisms underlying memory formation.

First, comparison of hippocampal transcriptional profiles between WT and CaMKK$\beta$ mutants by *Affymetrix* Microarray analysis identified four CaMKK$\beta$ regulated genes in males. Second, quantitative real-time PCR was used to compare hippocampal transcriptional profiles of these genes in naïve males and females, and after training in two hippocampus-dependent tasks: the MWM and contextual fear conditioning (CFC). This study identified three genes with altered

transcription thirty minutes after spatial training in the MWM and CFC in male mice: PSF, Gaa1 and SRp20. Naïve females expressed lower levels of all three genes than naïve males, and two of them (Gaa1 and SRp20) were not regulated specifically by training in these tasks at the same time point in females.

The work described in this thesis has identified two male-specific molecular markers for hippocampal activity, and provided insights into sexual dimorphisms in the molecular mechanisms underlying memory consolidation.

## DECLARATION

I, Ana Maria Raposo Antunes Simões Martins, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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To my Mother

## ACKNOWLEDGEMENTS

First of all, I would like to express my gratitude to my supervisor, Dr. K. Peter Giese, for all his support and guidance throughout the course of this project. I would also like to thank Dr. Keiko Mizuno who guided me thorugh the first steps of this project and introduced me to molecular biology and mouse behaviour techniques.

I would like to say a "big thank you" to Jeff Vernon: for always being available no matter how busy, for always having an answer or at least, an idea, for his constructive criticism in the course of the work and production of this thesis and, above all, for his endless patience.

*Merci beaucoup* to Eve Lepicard: for teaching me how to do *in situ* hybridizations, for always being ready and willing to help, for her very wise advice on scientific and non-scientific matters, for her laugh, for the "vive le labo" compilations, and for some memorable evenings....

Thanks also to Elaine Irvine: for her help and advice on behavioural techniques, for the proofreading of this thesis and for a certain birthday party.

During the last three or so years I could profit immensely from the knowledge, expertise of other members of the lab: Anna Need, Boris Rogelj, Laura von Hertzen and Florian Plattner, to all of them a big thank you.

I would also like to express my gratitude for Prof. Chris Yeo's for technical help and insightful discussions on the *in situ* data analysis.

I am indebted to my graduate tutour, Dr. Giti Garthwaite, who was extremely supportive and helpful throughout the course of my project.

In October 2005, while still writing this thesis, I joined Dr. Georgy Koentge's lab. Georgy and my new colleagues were extremely understanding and supportive. I would like to express my gratitude to all of them.

This work was supported by Portuguese Foundation for Science and Technology through the Graduate Programme on Basic and Applied Biology. I am indebted to everyone who was involved in the organization and management of the program for giving me such a wonderful oppoprtunity.

This journey would have not also been possible with the support of my friends, some met along the way and some others staying firmly in Portugal always cheering and believing. I would like to say *Muito obrigada* to all my *uncles, aunts* and *cousins* for making each return home so pleasant.

My sister Teresa, in her very own way, was a source of essential logistic and moral support. Finally, thank you to my mother, to whom this work is dedicated, for making my life sweeter, and for the last 26 years....

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| **ADI** | PM-MM Average Difference Intensity |
| **AL** | Adjacent left quadrant in the MWM |
| **AMPA** | Alpha-amino-3-hydroxy-5-methyl-4-isooxazolepropionic acid |
| **ANOVA** | Analysis of the Variance |
| **AR** | Adjacent right quadrant in the MWM |
| ***ARIH1*** | Ariadne-Like Ubiquitin Ligase |
| **BDNF** | Brain-derived neurotrophic factor |
| **CA** | *Cornus Ammonis* |
| **$Ca^{2+}$** | Calcium ion |
| **CaM** | calmodulin |
| **CaMK** | Calcium-Calmodulin Dependent Protein Kinase |
| **CaMKI** | Calcium-Calmodulin Dependent Protein Kinase I |
| **CaMKIV** | Calcium-Calmodulin Dependent Protein Kinase IV |
| **CaMKK** | Calcium-Calmodulin Dependent Protein Kinase Kinase |
| **cAMP** | Cyclic Adenosine Mono Phosphate |
| **CBP** | CREB Binding Protein |
| **cDNA** | complementary deoxyribonucleic acid |
| **C/EBP$\beta$** | CCAT/enhacer binding protein beta |
| **CR** | Conditioned response |
| **CRE** | cAMP Responsive Element |
| **CREB** | cAMP Responsive Element Binding Protein |
| **cRNA** | complementary ribonucleic acid |
| **CS** | Conditioned stimulus |
| **DG** | Dentate gyrus |
| **DNA** | deoxyribonucleic acid |
| **dNTP** | Deoxynucleoside triphosphate |
| **dsDNA** | Double stranded deoxyribonucleic acid |
| **DTT** | dithiotreitol |
| **E** | east coordinate in the MWM |
| **EDTA** | ethylenediaminetetraacetic acid |
| **ER** | Estrogen Receptor |
| **EPSP** | Excitatory post synaptic potential |
| **ERE** | Estrogen Responsive Element |

| | |
|---|---|
| **EST** | Expressed Sequence Tag |
| **Gaa1** | Glicosylphosphatydil Inositol Anchoring Attachment Protein 1 |
| **GAPDH** | glyceraldeheide-3-phosphate-dehydrogenease |
| **HPRT** | hypoxanthine-guanine-phosphoribosyltransferase |
| **IEG** | Immediate early gene |
| **L&M** | Learning and Memory |
| **L-LTP** | Late- LTP |
| **LTM** | Long-Term Memory |
| **LTP** | Long-Term Potentiation |
| **MAPK** | Mitogen-Activated Protein Kinase |
| **MBEI** | PM Model Based Expression Index |
| **$Mg^{2+}$** | Magnesium ion |
| **MM** | Mismatched Probe Sequence |
| **mRNA** | Messenger ribonucleic acid |
| **MWM** | Morris water maze |
| **N** | North coordinate in the MWM |
| **$Na^{+}$** | Sodium ion |
| **NE** | northeast quadrant in the MWM |
| **NMDA** | N-Methyl-D-Aspartate |
| **nt** | nucleotides |
| **NW** | northwest quadrant in the MWM |
| **OP** | opposite q quadrant in the MWM |
| **PBS** | Phosphate buffered saline |
| **PCR** | Polymerase Chain Reaction |
| **PKA** | Protein kinase A |
| **PM** | Probe Match Sequence |
| **PSF** | Polypyrimidine Tract Binding (PTB) Associated Splicing Factor |
| **QPCR** | Real-Time Quantitative Reverse Transcription PCR |
| **RNA** | ribonucleic acid |
| **rpm** | rotations per minute |
| **RT** | Reverse transcription |
| **S** | South coordinate in the MWM |
| **SDS** | Sodium dodecyl sulfate |
| **SE** | southeast quadrant in the MWM |
| **SRp20** | Splicing Regulator Protein 20 |
| **ssDNA** | Single stranded deoxyribonucleic acid |

| | |
|---|---|
| **STM** | Short-Term Memory |
| **SW** | southwest quadrant in the MWM |
| **TE** | Tris-EDTA buffer |
| **TF** | Transcription factor |
| **TQ** | target quadrant in the MWM |
| **US** | Unconditioned stimulus |
| **W** | West coordinate in the MWM |
| **WT** | Wildtype |

# CHAPTER I :
# INTRODUCTION

## 1.1. SEX DIFFERENCES IN THE MAMMALIAN BRAIN

In sexually dimorphic species, becoming able to reproduce requires not only proper maturation of the reproductive organs but also the development of adequate somatosensory body maps able to adjust to sex-differentiated structures. In addition, evolution is claimed to have favoured the development of sex-specific behavioural traits to account for sexual competence, parental skills and also cognitive abilities to cope with distinct environmental challenges (Tomizawa et al., 2003; Silverman et al., 2000).

Some behavioural differences between the sexes reflect dimorphisms in neuronal function which stem from a complex interplay between biological (neuroanatomical and neurophysiological) and environmental factors.

The contributions of nature and nurture to differences between males and females lead some authors to dissociate the concepts of sex and gender, the first one biologically determined and the second one, the result of environmental factors (Rose, 2005). Some other authors, however, maintain that only a complex interplay of factors of biological and social origin, can account for dimorphisms and hence, sex and gender can not be dissociated (Hines, 2004). For the remaining of this thesis the terms sex and gender will be used as having equivalent meanings, and terms like "genotypic" and "phenotypic" sex will be used whenever the distinction is considered pertinent.

While in some species, sex differences in the central nervous system (CNS) are pronounced, in many other these are subtle and their functional significance is poorly understood (Arnold, 2004; Morris et al., 2004). As Springer and Deutsh (1993) point out: *"there are true differences that are small in magnitude and easily*

*masked by individual variability or other factors that are not controlled".* Hence, for the purpose of this thesis, the term sex difference will correspond to*: "any anatomical, molecular, biochemical psychological or behavioural characteristic that differs on average for males and females of a particular species"* (Hines, 2004).

### 1.1.1. SEX DIFFERENCES IN BEHAVIOUR AND COGNITION IN HUMANS

A number of studies report sexual dimorphisms in susceptibility to neurodevelopmental disorders, mental disabilities and their phenotypic manifestations. Typical examples of such conditions include autism (typically affecting men) or depression (affecting predominantly women; Baron-Cohen et al., 2005; Cahill, 2005; Noble, 2005; Halari et al., 2004; Zechner et al., 2001).

Sex differences in cognition have been widely documented. On average, men outperform women in spatial tasks including mentally rotating and matching three dimensional objects, navigation, target directed motor skills and mathematical reasoning. Women generally excel men in verbal tasks (such as recalling words) and perceptual motor tasks and tend to have a better episodic memory as long as no strong spatial component is required (Halari et al., 2005; Postma et al., 2004; Postma et al., 1999).

In addition, imaging studies of humans subjects while performing cognitive tasks and evidence from unilateral brain injuries, point to an asymmetrical specialization of the hemispheres, with the right hemisphere engaged in perceptual and spatial functions and the left mostly devoted to speech. This lateralization is much less pronounced in women than in men, which can explain, for example, easier recovery of certain cognitive faculties after strokes and more widespread patterns of

brain activation during performance of cognitive tasks (Hines, 2004; Kovalev et al., 2003). This lesser degree of lateralization in women is thought to rely on a higher connectivity between brain hemispheres due to a slightly higher number of cells in the corpus callosum (e.g. Shin et al., 2005).

Despite some controversy, most studies in the literature have failed to find sex differences in scores of tests aiming to assess "general intelligence" or "ability to learn" (Blinkhorn, 2005; Rushton et al., 2003; Snow and Weinstock, 1990; Persaud, 1987). Spatial and verbal fluency tests assess relative performances and reaction times under standardized conditions, suggesting that sex differences in the scores obtained are indicative of different task solving strategies rather than differences in cognitive abilities (Sakthivel et al., 1999; Sandstrom et al., 1998; Astur et al., 1998).

The relative contributions of biological and cultural factors to sex differences in cognitive abilities are particularly difficult to disentangle in humans. Insights into the biological causes of sex differences are provided by rare genetic syndromes affecting the number of X chromosomes, or affecting gonadal function, gonadal hormone secretion and/or receptivity, and intersex conditions (Halari et al., 2005; Cohen et al., 1997; Zhou et al., 1995) Additionally, a variety of studies have found correlations between performance in a variety of tasks and gonadal hormone concentrations, thereby shedding light on the modulatory effects of gonadal hormones in cognition (Sherwin, 2003a; Sherwin, 2003b; Postma et al., 1999).

The biological foundation of sex differences in the brain has been modelled in a number of animals, particularly in rodents. The following sections focus on the origin of sex differences in neuroanatomy and neurophysiology.

### 1.1.2. SEX DIFFERENCES IN NEUROANATOMY

In higher vertebrates, gonadal hormones are classically viewed as the agents responsible for sexual differentiation of the CNS (Morris et al., 2004). In early life, hormones are believed to organize the brain, setting up the brain circuitry as male or female. A new burst of hormones during puberty activates the pre-set sex-specific circuits and is thought to contribute to evoke the characteristics of masculine or feminine behavioural patterns.

#### 1.1.2.1. ORGANIZATIONAL EFFECTS OF GONADAL HORMONES

In vertebrates, it is classically assumed that the development of the heterogametic brain requires active mechanisms while the homogametic brain develops by "default" (Becker et al., 2005; Arnold, 2004; Morris et al., 2004; but see Bakker et al., 2002).

In mammals, testosterone secretion by the testes shortly after birth provides a signal for the brain to develop masculine features and lose feminine features, processes termed masculinization and defeminization respectively (Bakker et al., 2002). In neurons, testosterone can bind the androgen receptor to regulate transcription (Shah et al., 2004), or can be aromatized to 17$\beta$-estradiol which exerts pleiotropic effects in neuronal cells (reviewed in McEwen, 2002, discussed in section 1.1.2.4). Thus, while males are exposed to both testosterone and estradiol shortly after birth, females are not exposed to high levels of either hormone until puberty.

In the rodent brain, these distinct hormonal environments shape sexually dimorphic nuclei (SDN) which differ in terms of volume, cell number, pattern of

connectivity, hormone responsiveness and other biochemical features (Morris et al., 2004; Shah et al., 2004; Kuhnemann et al., 1994).

In agreement with the idea that sexual dimorphisms in the brain should account for sexual competent behaviours it is not surprising that SDN in the brain are predominantly found in areas involved in the control of sexual and reproductive behaviours. Due to its direct connection to the master endocrine gland (the pituitary), the hypothalamus displays multiple SDN (reviewed in Morris et al., 2004). Hypothalamic SDN comprise the preoptic area of the anterior hypothalamus (SDN-POA), the bed nucleus of the stria terminalis (BNST) and the anteroventral periventricular nucleus (AVPV) of the hypothalamus. The SDN-POA and the BNST are involved in the regulation of male copulatory behaviour and are constituted by a significantly higher number of cells in male rodents (Morris et al., 2004). A sex difference in the size of BNST has also been described in humans (Zhou et al., 1995). The AVPV, involved in the regulation of ovulatory cycles is bigger in females (Morris et al., 2004). Perinatal manipulation of testosterone levels either by male gonadectomy or exogenous administration to females abolishes or reduces neuroanatomical dimorphisms in all the three dimorphic areas (reviewed in Simerly, 2002; del Abril et al., 1987; Dohler et al., 1984).

Testosterone manipulations also have a direct impact in sexual behaviours: lordosis in females (arching of the back when receptive to coitus) and mounting in males. In a pioneering experiment Phoenix and colleagues (1959) treated female guinea pigs with testosterone *in utero* which caused a shift towards a masculinized sexual behaviour (Phoenix et al., 1959 reported by Bakker et al., 2002). However, the impacts of testosterone in the formation of SDN and sexual behaviour can not

always be related causally as, for example, lesions of the SDN-POA do not significantly affect male sexual behaviours (De Vries, 2004).

Insights into the importance of the organizational effect of testosterone in humans is provided by condtions of perinatal over and underexposure to adrenal hormones. Girls suffering from hyperactivity of the adrenal glands [congenital adrenal hyperplasia (CAH)] or exposed to synthetic steroids *in utero* display not only obvious deficits in genital morphology, but also male-typical playing habits (Halari et al., 2005; Sherwin, 2003a). Conversely, androgen insensitivity in genotypic males leads to the development of feminized bodies. These individuals have a tendency to adopt female specific behavioural traits, which can not be attributed solely to the development of the "default" feminine brain, as cultural and social effects of the rearing should also be considered.

The classical view that the mammalian female brain develops "by default" has recently been challenged. For example Bakker and colleagues (2002) generated a mouse line with a genetic deletion of aromatase (the enzyme responsible for aromatization of testosterone to estradiol). Female null mutant mice and wildtype (WT) counterparts were ovarectomized in adulthood and treated with estradiol. This treatment evoked normal sexual receptivity in WT animals, but failed to do so in aromatase null mutants. These results suggest that an active feminization mechanism should operate perinatally to account for normal female sexual behaviours in adulthood.

### 1.1.2.2. ACTIVATIONAL EFFECTS OF GONADAL HORMONES

After the perinatal testosterone surge, only in puberty do organisms experience a new burst of hormone secretions. These are crucial for the sexual arousal of the organism, pair bonding and parental behaviour and contribute to the refinement of the organizational effects developed *in utero* and shortly after birth (McEwen, 1999).

Dimorphisms in some nuclei are only detected after puberty and can be reversed or induced by hormonal manipulations in adulthood (reviewed in Becker et al., 2005; De Vries, 2004; Simerly, 2002). In rodents, well defined sexual dimorphisms in the CNS include the extent of innervation of the septal region by arginine-vasopressin fibers which contribute to pair bonding and paternal behaviour (Young and Wang, 2004) and the posterodorsal medial amygdala (MePD) which receives inputs from olfactory and pheromonal centres (Cooke et al., 1999).

In addition, a study using three dimensional imaging techniques comparing brains from adolescent and adult mice revealed sex-specific and age-related structural differences in the hippocampus, amygdala and ventricles (Koshibu et al., 2004). Because the hippocampus is engaged in the behavioural tasks used in the work described in this thesis, sexual dimorphisms in this structure will be described in section 1.2.4.

In addition to pharmacological interventions and gonadectomies, insights into the role of gonadal hormones in sexual behaviours were provided by genetic manipulation of classical estrogen receptors $\alpha$ (ER$\alpha$) and $\beta$ (ER$\beta$) which are involved in estrogen mediated transcription (discussed in section 1.1.2.4.1). While ER$\alpha$ null mutant females displayed decreased lordosis (Kudwa and Rissman, 2003), ER$\beta$ null

mutants displayed normal sexual behaviour in both sexes. Male gonadectomy, however, led to higher lordosis in ERβ mutants, suggesting an incomplete perinatal defeminization of the brain (Kudwa, 2005). These studies do not allow the discrimination between organizational and activational effects of estrogens because these animals fail to express the receptors in all stages of development.

Despite being well established and accepted, increasing evidence has challenged the dogma that gonadal hormones are the sole players in determining sexual dimorphisms. Some of the evidence will be discussed in the following sections.

### 1.1.2.3. CONTRIBUTION OF CHROMOSOMAL REPERTOIRE TO SEX DIFFERENCES IN THE BRAIN

#### 1.1.2.3.1. Y CHROMOSOME GENE EXPRESSION

Despite having been identified over 100 years ago, it was not until 1990 that the role of the Y chromosome in determining the male sex was understood. The Y chromosome contains a small number of functional genes which collectively encode 27 proteins. These are distributed among two pseudoautosomal regions (PAR) which can recombine with the X chromosome during male meiosis, and a male specific region, named MSY (Skaletsky et al., 2003). The MSY contains a coding sequence for the transcription factor that acts like a master switch for masculinization by triggering testes development: the sex-determining region of the Y chromosome (Sry).

In order to disentangle the contribution of chromosomal repertoire versus gonadal secretions, Arnold and colleagues (Carruth et al., 2002; De Vries et al., 2002) generated a transgenic mouse model bearing the Sry gene not in the Y chromosome but in an autosomal region ($XY^{-}Sry$), which generated fertile males that were crossed with normal females. The offspring of these crosses consisted of individuals with matched genetic and gonadal phenotypes (XX, $XY^{-}Sry$) and individuals where these were mismatched: genotypic females with Sry (XXSry) and genotypic males without Sry ($XY^{-}$). Evidence obtained from cell cultures from the mesencephalic dopaminergic system harvested from embryos before gonadal development points to a higher number of dopaminergic neurons in cells bearing the Y chromosome independently of the presence of the Sry transgene. (Carruth et al., 2002). In adult animals, presence of the Y chromosome determines some subtle sexually dimorphic features independently of Sry (De Vries et al, 2003) but the presence of Sry was the key determinant for other important and robust sexual dimorphisms and for normal social and sexual behaviours (De Vries et al., 2002).

### 1.1.2.3.2. X CHROMOSOME GENE EXPRESSION

The X chromosome is particularly rich in genes involved in brain development, (some of which have been associated with single gene mental retardation phenotypes), whereas only a small percentage are present in the Y chromosme (Zechner et al., 2001). In order to overcome the imbalance of X chromosomes, permanent X-chromosome inactivation mechanisms operate in females. This inactivation occurs in a mosaic pattern and different cells within the same body will display a different gene repertoire depending on whether maternally or paternally

derived chromosome have been inactivated (Gilbert, 2000). Variations in cell and tissue pattern of X chromosome inactivation account for variations in the manifestation of and susceptibility to X-linked diseases in females (Ostrer, 2001).

However, some mammalian genes escape X-chromosome inactivation therefore causing a dosage imbalance between males and females (Carrel and Willard, 2005) which can explain differences in the transcriptional levels of some X linked genes between the sexes (De Vries et al., 2002 and references within).

### 1.1.2.4. MOLECULAR MECHANISMS UNDERLYING SEXUAL DIMORPHISMS IN NEUROANATOMY

Dimorphisms in brain structure originate from differences in cell number (due to apoptosis, cell migration and neurogenesis), connectivity, synaptogenesis and axon guidance (reviewed in Simerly, 2002). Most studies aiming to understand the sex specific regulation of these mechanisms have focused on the effects of estrogens.

#### 1.1.2.4.1. MOLECULAR AND CELLULAR ACTIONS OF ESTROGENS

In neurons, estrogens can exert their actions in a genotropic fashion driving transcription, or in a non-genotropic fashion by interaction with other signalling pathways (Morris et al., 2004; Razandi et al., 2003; Cato et al., 2002; McEwen, 2002).

Classical estrogen receptors (ER) are intracellular ligand-dependent transcription factors composed by homo or heterodimers of ER$\alpha$ and ER$\beta$ subunits.

ERs become activated upon ligand binding and exert activational effects by direct interaction with estrogen responsive elements (ERE) in the regulatory sequences of target genes, or repressional effects by interaction with other transcription factors.

*In vitro* studies document rapid responses to estrogen administration incompatible with the time demands of a genotropic action. These fast actions of estrogen are most likely mediated by membrane ER (Toran-Allerand, 2004); G-protein coupled receptors (GPCR) and modulation of a number of intracellular signalling pathways (Razandi et al., 2003; Cato et al., 2002; Bi et al., 2000).

A number of *in vivo* and *in vitro* studies in rodent models and humans document protective effects of estrogens against excitotoxicity and oxidative damage (reviewed in McEwen, 2002). These actions allegedly contribute to the prevention of some neurodegenerative diseases, and may constitute one of the bases for sex differences in vulnerability to neurodegenerative disorders (Eberling et al., 2003; Sherwin, 2003a; Dhandapani and Brann, 2002).

#### 1.1.2.4.2. SEX-SPECIFIC REGULATION OF APOPTOSIS

The major way that steroids control cell numbers is by interfering with apoptotic processes either promoting it or conferring a protective effect, depending on the tissue. In the brain this is true both for developmental processes guiding the formation of sexually dimorphic nuclei and for survival processes of certain areas which differ after puberty (Madeira and Lieberman, 1995).

For example, Forger et al. (2004) provided evidence for the specific involvement of apoptotic regulators in the development of SDN. Null mutant male mice for the pro-apoptotic gene Bax, displayed a higher number of cells in the sexual

dimorphic nuclei than did their WT counterparts. More importantly, Bax genetic deletion, completely ablated sex differences in the AVPV and BNST. Because sexual dimorphisms in these areas are opposite (BNST larger in males, AVPV larger in females), these results suggest that Bax acts in a cell or region specific-manner, conferring a protective effect in the BNST and promoting apoptosis in the AVPV.

In conclusion, the biological foundations of phenotypic sex differences comprise a complex interplay of sex determining genes with hormonal effects during different stages of development and in adulthood (summarized in Fig. 1.1).

Apart from the SDN which are directly connected to control of reproductive function, sexually dimorphic features have also been described for other areas of the brain, such as the corpus callosum, the amygdala, ventricles and the hippocampus.

Memory function in a number of species displays sexually dimorphic features assessed at the behavioural, anatomical, cellular and molecular level. These sexual dimorphisms will be addressed in the following section together with an outline of the neurobiology of memory formation and experimental approaches used to study it.

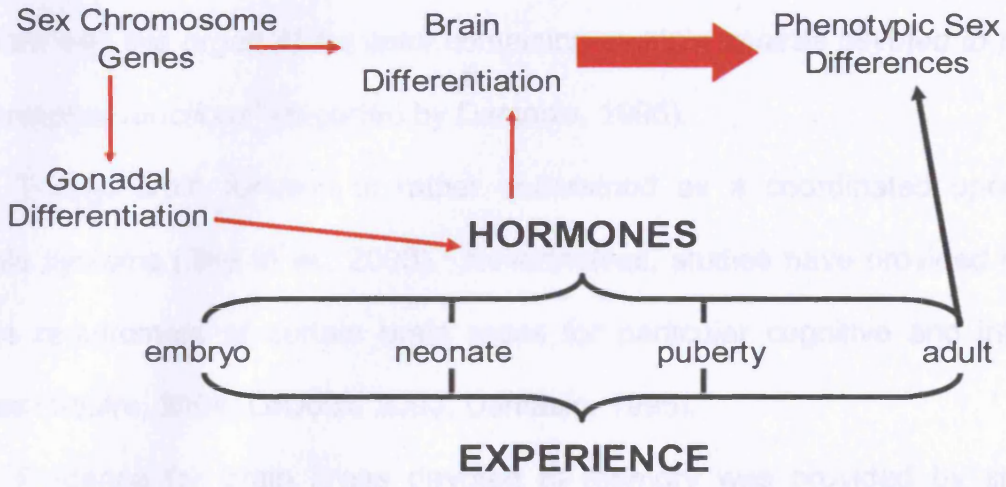


**Figure 1.1- Cascade of effects leading to phenotypic sex differences.**
Genes encoded by the sex chromosomes contribute to brain differentiation either directly or by driving gonadal hormone secretion. Gonadal hormone secretion throughout life in combination with environmental factors promote phenotypic sex differences in adulthood (adapted from McEwen, 1999).

## 1.2. MEMORY, TYPES OF MEMORY AND THE ROLE OF THE HIPPOCAMPUS

*"Life is not what one lived, but what one remembers and how one remembers it in order to recount it."*

Gabriel Garcia Marquez, "*Living to tell the tale*"

When interacting with their environment, animals face continuous challenges. The ability to learn information, recall previous experience and procedures and shape behaviours accordingly is a major adaptative advantage and a crucial survival mechanism (Kandel, 2001; Kolb and Whishaw, 2001). Understanding how this is accomplished has been one of the most challenging tasks for philosophers and scientists alike.

### 1.2.1. HUMAN AMNESIA

During the course of the 19$^{th}$ century the "phrenological theory of the mind" initiated by Franz Gall had a large number of followers. This theory postulated that the brain was *the organ of the spirit* containing multiple *centres devoted to particular psychological functions"* (reported by Damasio, 1995).

Today, brain function is rather understood as a coordinated operation of multiple systems (Shu et al., 2003). Nevertheless, studies have provided evidence for the requirement of certain brain areas for particular cognitive and intellectual abilities (Squire, 2004; LeDoux, 2003; Damasio, 1995).

Evidence for brain areas devoted to memory was provided by studies of amnesic patients, among which the most famous case is patient HM initially described by Brenda Milner and William Scoville (Scoville and Milner, 2000; Scoville

and Milner, 1957; Milner, 1954). This patient received bilateral resection of the medial temporal lobes to treat epilepsy. After surgery, HM became unable to retain new information and to retrieve memories related to events that happened during the eleven years preceding surgery, symptoms termed anterograde and retrograde amnesia respectively. However, ability to remember numbers or unrelated words for short periods of time, ability to learn and improve performance in motor tasks, and memories of very remote events remained intact (Corkin, 2002; Milner et al., 1998; Milner, 1954). Neuropathological findings and psychological studies of HM and other patients allowed the characterization of human amnesia (summarized in table 1.1) and paved the way for a field of research integrating a very wide range of techniques and approaches aiming to understand the neurobiological bases of memory (Silva, 2003; Burgess et al., 2002; Kandel, 2001; Milner et al., 1998; Silva et al., 1997).

**Table 1.1- Characteristics of human amnesia** (Squire, 2004; Eichenbaum, 1997; Squire and Zola-Morgan, 1991)

- Memory impaired on tasks requiring retrieval of facts and events
- Memory impairment exacerbated by distraction
- Memory impairment can be enduring
- Memory for events prior to the onset of amnesia can be affected
- Learning capacities for motor, perceptual, cognitive skills, sensory adaptations, and priming of perceptual and lexical stimuli are spared
- Immediate memory is spared (distinct temporal stages required for retention of new information)
- Very remote memories may be intact (temporal grading of retrograde amnesia)

### 1.2.2. MEMORY CATEGORIES

In a key study, Milner trained patient HM in a motor skill task that demanded many training sessions (Corkin, 2002; Milner et al., 1998). Like healthy controls, HM

was able to learn the task and improve performance with the number of training sessions, however he had no conscious recollection of the learning episodes. Similar ability to learn and retain on other than motor skill tasks were subsequently described in medial temporal lobe lesioned patients (reviewed by Squire, 2004; Myer et al., 2002; Kopelman, 2002). These findings provided evidence for multiple memory systems relying on dissociable brain areas.

In general, memory is broadly divided into two categories: declarative or explicit and non declarative or implicit.

Declarative or explicit memories are those that one is aware of, can express through language and trigger upon conscious recall. These include information about everyday episodic events, locations and universal factual knowledge, termed episodic and semantic memory respectively (Squire and Zola, 1998; Eichenbaum, 1997) and are dependent on the function of the medial temporal lobe (Squire and Zola-Morgan, 1991).

Memories for perceptual, motor and other skills are globally termed non declarative or implicit. These are expressed through performance, elicited in response to environmental stimuli and learned through practice. Because non declarative memory comprehends a very wide range of faculties, it also engages multiple anatomical areas mostly in the basal ganglia (Fig. 1.2; Squire and Kandel, 1999).

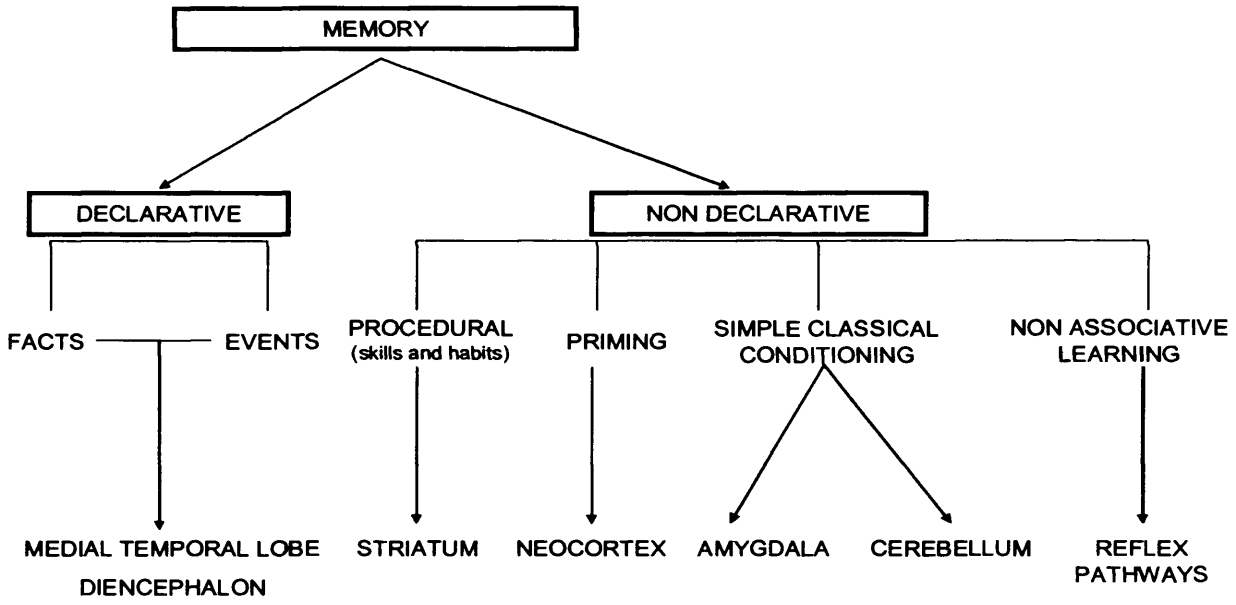


**Figure 1.2- Taxonomy of memory systems.** _Brain areas thought to be especially important for each form of declarative and non declarative memory. In addition to its role in emotional learning the amygdala exerts a modulatory effect upon declarative and non declarative memory formation (from Squire, 2004).

### 1.2.3. THE ROLE OF THE HIPPOCAMPUS IN DECLARATIVE MEMORY

The medial temporal lobe is a large region including the hippocampus proper, amygdaloid complex and adjacent areas such as the entorhinal, parahippocampal and perirhinal cortices (Amaral and Witter, 1995). Comparative studies in patients bearing medial temporal lobe lesions of different extent, some of them restricted to the hippocampus proper, established that, within the medial temporal lobes, the hippocampus is the critical structure involved in declarative memory formation (Scoville and Milner, 2000; Rempel-Clower et al., 1996; Squire and Zola-Morgan, 1991), although some authors defend that the hippocampus is only important for the episodic aspects of declarative memory but not for the semantic component (e.g. Tulving and Markowitsch, 1998)

The hippocampus is traditionally divided into two regions: the dentate gyrus (DG) consisting primarily of granule cells and the *Cornu Ammonis* (CA), further

subdivided into the CA1, CA2 and CA3 subfields, and constituted by pyramidal neurons. The DG and CA areas can be distinguished on the bases of morphology, connectivity, electrophysiological and molecular properties (Lein et al., 2004; Zhao et al., 2001; Amaral and Witter, 1995)

The hippocampal formation receives inputs and conveys outputs to multiple cortical and subcortical areas. Hippocampal intrinsic connections, other than the ones already described, include interconnections and recurrent connections within subfields (Fig. 1.3; Amaral and Witter, 1995). The trisynaptic circuit within the hippocampus proper comprises a set of three unidirectional pathways (Fig 1.3): (a) the perforant pathway (PP) that conveys inputs from the enthorinal cortex to the DG; (b) the mossy fiber (MF) pathways constituted by projections of DG granule cells to pyramidal cells in CA3 and (c) the Schaffer collaterals (SC) constituted by projection of axons from area CA3 to CA1.

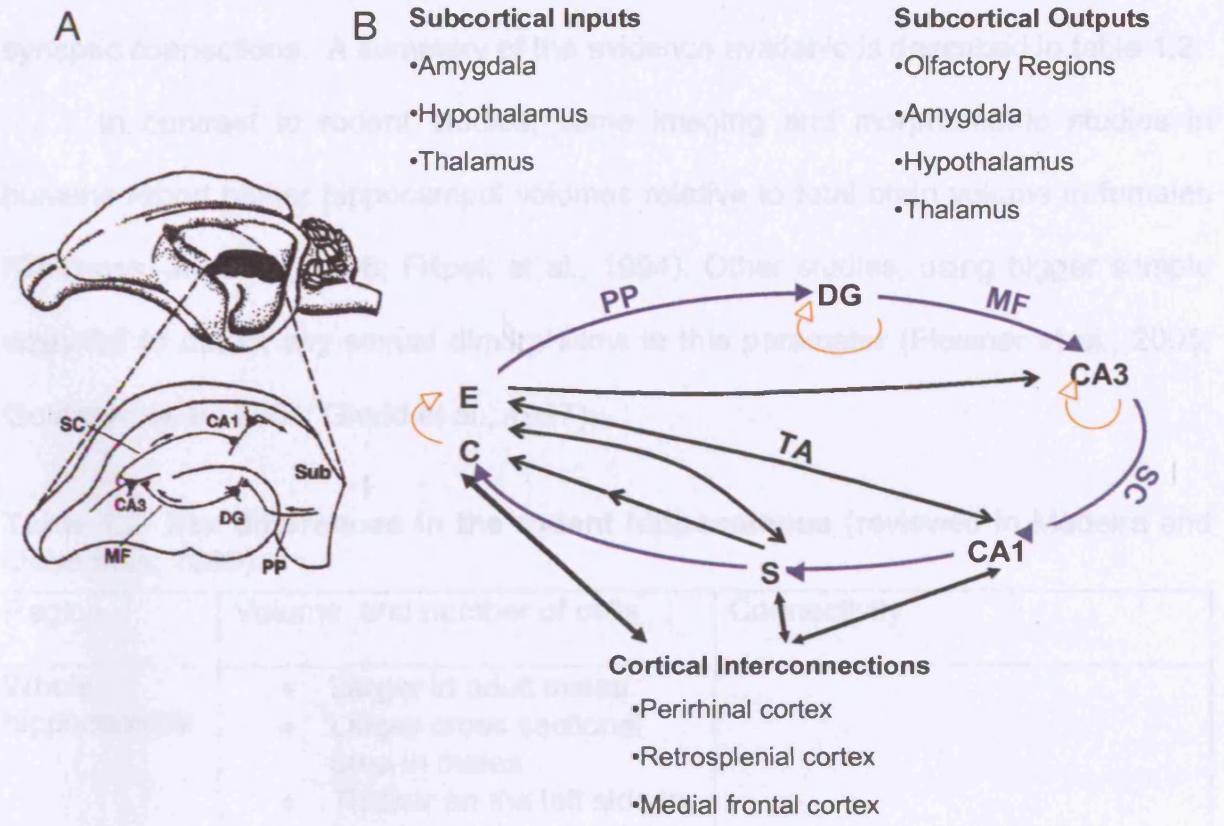


**Figure 1.3- Major intrinsic connections of the rodent hippocampal formation.** **(A)** The perforant pathway (PP), mossy fibers (MF) and Schaffer collateral (SC) pathways are unidirectional (from Anderson et al., 1971); **(B)** Some cortical and subcortical inputs and outputs of the hippocampal formation are listed; orange arrows represent recurrent connections and black arrows represent other intrinsic connections between hippocampal subfields and the subiculum with the entorhinal cortex (EC) such as the temporoammonic pathway (TA) that conveys information directly from the EC to subfield CA1 (adapted from Amaral and Witter, 1995).

### 1.2.4. SEX DIFFERENCES IN THE HIPPOCAMPAL COMPLEX

In several rodent species, the hippocampal formation is larger in males than in females either in terms of absolute volume or in proportion to the total volume of the brain (Andrade et al., 2000; Madeira and Lieberman, 1995; Jacobs et al., 1990). However, most evidence points to the fact that females compensate for a generally

smaller cell number and hippocampal volume by establishing an increased number of synaptic connections. A summary of the evidence available is described in table 1.2.

In contrast to rodent studies, some imaging and morphometric studies in humans report higher hippocampal volumes relative to total brain volume in females (Caviness, Jr. et al., 1996; Filipek et al., 1994). Other studies, using bigger sample sizes fail to detect any sexual dimorphisms in this parameter (Fleisher et al., 2005; Goldstein et al., 2001; Giedd et al., 1997).

**Table 1.2- Sex differences in the rodent hippocampus** (reviewed in Madeira and Lieberman, 1995).

| Region | Volume and number of cells | Connectivity |
|---|---|---|
| Whole hippocampus | • Larger in adult males.<br>• Larger cross sectional area in males<br>• Thicker on the left side in females and on the right side in males. | |
| Dentate Gyrus | • Wider and thicker in males of all ages<br>• Higher total number of granule cells in males | • Changes in dendritic tree formation triggered by environmental factors are more pronounced in males than in females<br>• Sprouting of perivascular sympathetic axons more pronounced in females than males |
| CA3 | • Same volume and cell number for both sexes | • CA3 has more dendrites in females |
| CA1 | • Smaller volume and total number of cells in females | |
| Mossy Fibres | • Total number of fibres smaller in females | • Each female fibre establishes a higher number of synaptic contacts.<br>• Volume of fibre buttons bigger in females |
| Subiculum | • Higher cell number in males | • Equal area of active synaptic zones |

In addition to anatomical dimorphisms, physiological and biochemical dimorphisms have also been described such as different patterns of estrogen and androgen and corticosterone receptor expression and distinct cholinergic and serotonergic activities. Additionally, hippocampal cells are capable of synthesising steroids from endogenous cholesterol (Hojo et al., 2004; Kretz et al., 2004). This local synthesis of steroids accounts for sex differences in the content of estradiol in hippocampal tissue which is higher in neonatal females than in males (Amateau et al., 2004).

## 1.3. ANIMAL MODELS TO STUDY HIPPOCAMPUS-DEPENDENT MEMORY

Although some aspects of declarative memory formation can only be studied in humans, insights into the biological mechanisms underlying memory were provided by studies in model organisms. These include invertebrates, with relatively simple nervous systems constituted by a limited number of cells and synaptic connections (Dubnau et al., 2003; Kandel, 2001), to higher vertebrates bearing nervous systems composed by a large number of cells interconnected by complex circuitries (Morris, 2001; Zola-Morgan et al., 1986).

### 1.3.1. RODENT MODELS

To study declarative memory in experimental animals such as rodents is apparently paradoxical. Lack of language does not suit the linguistic definition of declarative memory and complicates the distinction between implicit and explicit memory. Nevertheless rats and mice are able to behave as though they have built a

knowledge of their surrounding world (Clayton and Dickinson, 1998; Morris, 2001) and hence are able to form a so-called "episodic-like" memory.

The validity of using mouse models to understand the processes underlying hippocampal memory formation is supported by arguments such as the following: (a) hippocampal structure, synaptic circuitry and neighbouring regions are apparenlty well conserved between rodents and primates (Amaral and Witter, 1995; Squire and Zola-Morgan, 1991); (b) hippocampal lesions in rodents cause impairments in long-term memory formation for contextual and spatial learning tasks (Broadbent et al., 2004; Cho et al., 1999; Moser et al., 1995; Morris et al., 1982); (c) a variety of pharmacological approaches can be used in rodents (Gureviciene et al., 2003; Igaz et al., 2002; e.g. Barrientos et al., 2002; Maren et al., 1997); (d) advanced molecular tools such as targeted mutagenesis of the mouse genome allow the dissection of the molecular mechanisms involved (Silva, 2003; Giese et al., 1998; Silva et al., 1997); and (e) there are well developed and standardized memory tasks designed to explore different aspects of memory formation, some of which can test both rodents and humans (Astur et al., 2004; Sandstrom et al., 1998).

On these grounds, mouse studies have provided valious insights into the fundamental neurobiology of memory and have also been used to model human cognitive disorders (Silva et al., 2000).

## 1.4. MEMORY TASKS

In memory tasks, behavioural measures throughout training and during test sessions are used as an output of the strength of memory formation. For some behavioural paradigms, a single training episode is sufficient to trigger a robust behavioural response upon testing – single trial learning tasks. Some other tasks,

however, require several training sessions to trigger the formation of strong memories, or at least to trigger a robust response – multiple trial learning tasks.

Single and multiple trial learning tasks differ essentially in the nature of the stimulus used, biological relevance of the learning episode and cognitive demands. This distinction will be exemplified by comparison of contextual fear conditioning (a single-trial learning task) and the Morris water maze (a multiple-trial learning task) as they were used in the work described in this thesis.

### 1.4.1. FEAR CONDITIONING

Memories about basic, but significant, emotional events are generally designated "emotional memories". Most of the research into the mechanisms underlying emotional memories has focused on the neurobiology of fear. Learned fear is commonly employed to study the neural circuits and cellular mechanism underlying associative memory processes related to emotional behaviour. This concerns the ability to form associations between noxious events and the environmental stimuli that predict them, and to use this information to shape defensive behaviours (Rumpel et al., 2005; McGaugh, 2004; LeDoux, 1994).

#### 1.4.1.1. CONTEXTUAL AND CUED FEAR CONDITIONING

Beginning in the 1890, the Russian physiologist Ivan Pavlov, laid the bases for the studies of simple forms of conditioning. Several derivations of his initial paradigm have been adapted to different species (reviewed in Maren, 2001).

In Pavlovian or classical fear conditioning, a rodent is initially exposed to a novel stimulus termed conditioned stimulus (CS) which is followed by a mild foot shock, termed unconditioned stimulus (US). The animal will then form an aversive memory of the CS as a predictor of the noxious US. The formation of the aversive memories triggers conditioned responses (CR) revealed upon re-exposure to the CS. CRs include a set of defensive reactions: endocrine responses (e.g. corticosterone secretion), autonomic responses (eg. increased heart rate), potentiated startle and behavioural responses such as freezing. The majority of the studies use a readily accessible behavioural output (freezing) as an index of fear memory formation. Freezing consists of a complete suppression of locomotor activity and a refrain from all movements except for those needed for respiration (Fanselow and Bolles, 1979).

Depending on the training paradigm adopted, the CS may comprise a novel environment (background – contextual component) or sensory cues (foreground – cued component). A conditioned response can be triggered even if these components are dissociated, i.e. when the animal is exposed to the same context in the absence of cues and when exposed to the cue in a different context.

### 1.4.1.2. THE NEUROCIRCUITRY OF FEAR CONDITIONING

By using an auditory contextual fear conditioning task in rats, and by introducing lesions in different connections and areas within the sensorial input (auditory cortex) and output (motor cortex, periacqueductal gray) circuits, Joseph LeDoux and colleagues traced the "fear pathway" and identified a subcortical region – the amygdala as the locus for storage and encoding of CS-US associations (reviewed in McGaugh, 2004; Huff and Rudy, 2004). A large number of studies have

addressed the relative contribution of structures in the ''fear pathway'' to the formation of fear memories. In the majority of these studies the approaches used were to functionally inactivate elements of the pathway by means of lesions or pharmacological interventions at different time points in the course of acquisition or consolidation of the task (reviewed in Rodrigues et al., 2004). Some of the results of these studies are summarized in Table 1.3. Importantly, imaging studies in healthy and lesioned humans provide evidence for an evolutionary conservation of the fear pathways (Adolphs et al., 2005; Phelps and LeDoux, 2005; Bechara et al., 1995). In fact, lesion studies, pharmacological and genetic interventions demonstrate the crucial requirement of intact amygdala function for the acquisition and expression of fear conditioning, both in the cued and contextual variants of the task (Rumpel et al., 2005; Maren, 2005; Huff and Rudy, 2004; Nader et al., 2000; Maren and Fanselow, 1996).

#### 1.4.1.3. THE ROLE OF THE HIPPOCAMPUS IN FEAR CONDITIONING

As expected, lesions and pharmacological inactivation of the amygdala impair conditioned responses to both the contextual and cued CS, due to failure to store the CS-US associations (Kim et al., 1993; Phillips and LeDoux, 1992). Other studies have provided evidence for a participation of other brain areas, including the hippocampus in contextual fear conditioning.

In the first studies that underscored the role of the hippocampus in contextual conditioning, lesions of the dorsal hippocampus before (Kim et al., 1993; Phillips and LeDoux, 1992) and after (Kim and Fanselow, 1992) training were shown to impair freezing responses to context while sparing responses to auditory cues. However,

the involvement of the hippocampus in contextual fear conditioning was challenged by other studies. For example Rudy et al. (2002) demonstrated that damage to the hippocampus prior to conditioning does not affect memory suggesting that fear to a context can be supported by extra-hippocampal brain regions. Interpretation of these studies requires the consideration of variable factors namely the method of lesion, area of the hippocampus affected by the lesion, and time of the lesion relative to the behavioural experiment (Rudy et al., 2004).

The types of lesions used in these studies are either electrolytic (affecting the hippocampus and afferent fibres) and excitotoxic (sparing fiber integrity). This latter approach, can, however, have an impact on other elements of the circuitry causing dysfunction or over excitation of downstream structures (Sanders et al., 2003; Anagnostaras et al., 2002).

In a series of experiments, Philips and LeDoux (1994) demonstrated impaired contextual conditioning and normal cued conditioning in animals with dorsal hippocampal lesions, when trained with tone shock pairings. However, lesioned animals trained in the absence of a salient cue such as the tone or with unpaired shock tones, responded strongly to the context. Hence, in the absence of strong stimuli, the amygdala should perceive some background cues as foreground cues and build the association for the noxious event.

Determining a role for the ventral hippocampus (VH) in contextual fear conditioning has proved somewhat more difficult than demonstrating a role for the dorsal hippocampus. This is mainly due to two factors: first, damage to the ventral hippocampus has been implicated in hyperactivity which may impair the displaying of freezing responses; second, lesion of the VH may disrupt communication between the hippocampus and the amygdala. Recent studies revealed that blockade of VH

function by various means consistently causes anterograde amnesia for context and, depending on the manipulation used, also for tone (Zhang et al., 2001). Taken together, evidence from dorsal and ventral hippocampus lesions studies suggest that the contextual component of fear memories is distributed throughout the whole hippocampus (Rudy and Matus-Amat, 2005).

The hippocampus is generally believed to support the representation of context in a contextual fear conditioning paradigm. In one trial fear conditioning experiments, animals shocked very shortly after exposure to a novel environment, display low freezing upon context re-exposure, a phenomenon termed immediate shock deficit. The immediate shock deficit can be rescued by pre-exposure to the context in the absence of conditioning (Rudy et al., 2004; Frankland et al., 2004; Rudy and O'Reilly, 2001; Rudy and O'Reilly, 1999). Lesion and pharmacological inactivation of the hippocampus shortly before or immediately after context-pre-exposure prevent the repairing effect on conditioning (Matus-Amat et al., 2004; Rudy et al., 2002).

In addition to supporting contextual representations, a number of studies report that neural activity within the hippocampus can also strongly correlate with the acquisition of associations between the context and the noxious event (von Hertzen and Giese, 2005; Ressler et al., 2002; Hall et al., 2000; Impey et al., 1998) .

Despite the fact that most studies revealing the contrinbutiion of the hippocampus for contextual fear conditioning have been performed in rats, the basic findings described above have been replicated in mice (Frankland et al., 2004; Gerlai, 2001a).

A role for the hippocampus in fear conditioning has also been described for humans: a patient with amygdalar damage failed to display autonomic responses

following conditioning to a noxious stimulus, but could describe verbally the occurrence and associations related to the noxious event. Conversely, a patient with hippocampal damage displayed normal fear responses but was unable to verbalize the CS-US association (Bechara et al., 1995).

**Table 1.3- Effects of lesions and pharmacological inactivation of the dorsal hippocampus, ventral hippocampus and amygdala at different times relative to training and context pre-exposure in contextual and cued fear conditioning. (?- not tested)**

| Area | Time Point | Contextual Conditioning | Cued Conditioning | Reference |
|---|---|---|---|---|
| Dorsal hippocampus | Prior to conditioning | impaired | normal | (Phillips and LeDoux, 1992) |
| Amygdala | Prior to conditioning | impaired | impaired | (Phillips and LeDoux, 1992) |
| Dorsal hippocampus | Prior to conditioning | impaired | ? | (Kim et al., 1993) |
| Amygdala | Prior to conditioning | normal | ? | (Kim et al., 1993) |
| Hippocampus | Post-training | Impaired (assessed 7 days after conditioning) | normal | (Kim and Fanselow, 1992) |
| Ventral hippocampus | Prior to preexposure after preexposure and prior to conditioning | impaired (assessed 48 h after conditioning) | ? | (Rudy and Matus-Amat, 2005) |

### 1.4.1.4. SEX DIFFERENCES IN EMOTIONAL MEMORY

Psychological studies report better memory in women than men for emotional events. Insights from functional imaging studies point to a sex-specific pattern of activation of brain structures. For example, in a study by Canli et al. (2002), a recognition memory test for emotionally arousing images triggered a more

widespread brain activation in women than in men, despite equal rating of stimulus intensities (Canli et al., 2002). In this and other studies, a sex-specific pattern of amygdalar activity has been reported with enhanced memory correlating with increased activation of right amygdala in men and left amygdala in women (Cahill and vanStegeren A., 2003; Canli et al., 2002; Cahill et al., 2001). The Canli study also described a correlation between the emotional rating and hippocampal activation in women but not in men.

In the rodent literature, most studies tend to use male animals, and other studies, particularly with mutant animals, tend to pool data from males and females without addressing whether sex differences might be present.

Only a small number of studies report sex differences in contextual fear conditioning indicating higher freezing responses by males than by females in the contextual but not in the cued variant of the task (Mizuno et al., 2006; Kudo et al., 2004; Wiltgen et al., 2001; Anagnostaras et al., 1998; Maren et al., 1994). Apart from equipment, species and genetic background effects, a number of factors may favour or prevent the detection of sex differences. First, sex differences in rats have been detected in animals trained with one tone-shock pairing, but not after three tone shock pairings (Maren et al., 1994); Second, long placement to shock intervals (time lapse between exposure to the context and shock delivery) and context pre-exposure have been shown to eliminate sex differences (Wiltgen et al., 2001). Third, the rate of decrease of the conditioned response in animals tested on consecutive days after conditioning (extinction) is significantly faster in females (Gupta et al., 2001). The conjunction of these results suggests that sex differences in contextual fear conditioning stem from a faster rate of acquisition in males and a faster rate of extinction in females.

Some studies detected differences in contextual fear conditioning between males and randomly cycling females, from which no information about estrogen levels was available (Wiltgen et al., 2001; Maren et al., 1994). Insights into gonadal hormone influence on sexual dimorphisms were provided by monitoring of estrous cycle in intact females (Markus and Zecevic, 1997), or after gonadectomies and exogenous gonadal hormone replacements (Edinger et al., 2004; Gupta et al., 2001; Anagnostaras et al., 1998). These studies provided evidence for a deleterious effect of estrogen on contextual fear conditioning. Specifically, female rats in proestrus (when plasma levels of estrogens are higher) displayed lower levels of freezing when compared to females in estrus (when levels of estrogens were lower; Markus and Zecevic, 1997); Furthermore, gonadectomized females froze at comparable levels to males and significantly more than intact females, an effect attenuated by exogenous estrogen administration (Gupta et al., 2001).

There is no consistent evidence for a role of testosterone in the modulation of contextual fear conditioning. One study detected lower freezing responses in gonadectomized males, a deficit rescued by testosterone replacement (Edinger et al., 2004). Another study on the same strain of rats failed to detect any effect of testosterone (Anagnostaras et al., 1998). Possible causes for the discrepancies between the two experiments may be related to experimental setup and experimental procedures, concretely the time of testing (1 day in the Anagnostaras study and 5 days in the Edinger study). Because in both studies, the animals were gonadectomized in adulthood, only the activational effect of testosterone on contextual fear conditioning could be addressed, leaving opened the possibility that an organizational effect of the hormone from early life may account for the male advantage.

### 1.4.2. SPATIAL MEMORY

*"Space plays a role in all our behaviour. We live in it, move through it, explore it, defend it."* (O'Keefe and Nadel, 1978).

Being able to find our way around an environment demands the formation of spatial memories, or memories about place (Morris, 2001). The importance of hippocampal function for spatial memory was established by studies in patients bearing hippocampal lesions (Milner et al., 1998), imaging studies in human subjects while performing spatial tasks (Burgess et al., 2002) and lesion studies in rodents trained in spatial learning tasks (Broadbent et al., 2004; de Bruin et al., 2001; Cho et al., 1999; Moser et al., 1995). The discovery of place cells- pyramidal cells in the rat hippocampus with location-specific activity, provided further evidence for the involvement of hippocampal function in the encoding and storage of spatial information (O'Keefe and Dostrovsky, 1971).

Evidence from lesion studies in rodents and evidence from human patients suggest that retrieval of spatial information relies predominantly on the dorsal part of the hippocampus, while acquisition of spatial tasks may also be mediated by the ventral hippocampus (Moser and Moser, 1998; Moser et al., 1995). Recent evidence suggests that this specialization of the dorsal hippocampus is due to the fact that the dorsal hippocampus receives visuospatial inputs from the dorsolateral band of the enthorhinal cortex (Steffenach et al., 2005).

Tasks aiming to test spatial abilities in humans include mental rotation tasks (Astur et al., 2004), object relocation (Postma et al., 1999), object to position assignment, precise metric information (Postma et al., 2004) and virtual navigation

tasks (Burgess et al., 2002; Maguire et al., 1999). A variety of spatial tasks has been developed for rodents, these are generally complex tasks demanding multiple training trials. The most commonly used are the radial arm maze and the Morris water maze (MWM; Angelo et al., 2003; Need and Giese, 2003; de Bruin et al., 2001; D'Hooge and De Deyn, 2001; reviewed in Brandeis et al., 1989).

#### 1.4.2.1. THE MORRIS WATER MAZE TASK

The fact that place cells fire independently of local cues, posed difficulties in correlating place cell firing with learning processes. To circumvent the so called "local cue" problem, Richard Morris developed a new behavioural task: the Morris water maze (MWM; Morris, 2003; Morris, 1984). .

The MWM set up is composed of a round pool filled with opaque water with a submerged platform, placed in a room where several landmarks are distributed around the walls. The animal is trained to find a platform as the only way to escape the water. Because the platform is not flagged, it can not be perceived by the senses, and the animal will need to develop strategies to find it.

Once placed in the pool, animals can use two types of spatial frameworks to solve the task: egocentric and allocentric (or spatial).

Egocentric frameworks are centred with the body of the animal and move with it. Hence orientation can be provided by repetitive use of fixed motor movement, or deriving relative distances between two points. Allocentric strategies are based on a mental representation of the platform location relative to a set of extramaze cues and do not depend on the location of the animal. Hippocampal lesions cause specific impairments in the development of allocentric strategies without affecting egocentric

strategies or the ability to locate the platfrom relative to a single intramaze cue (de Bruin et al., 2001; Pearce et al., 1998).

Training produces a decrease in escape latency independently of the strategy used. In fact, even when large portions of the hippocampus are inactivated, animals are able to improve acquisition of the task with the number of training trials (de Bruin et al., 2001; Moser et al., 1995). The formation of a hippocampus-dependent strategy is probed in a transfer test or probe trial (eg. Peters et al., 2003). During this test, the platform is removed form the pool and the animals are allowed to swim freely for a certain period of time. If the animal learned the position of the platform based on the presence of extramaze cues, in other words if it has developed an allocentric strategy to locate the platform, it will spend a high percentage of time in the area where the platform was previously located. On the contrary, an animal that developed an egocentric strategy will not display a preference for any particular area of the pool.

In order to assess probe trial performance quantitatively, the pool is artificially divided in four quadrants. Quantitative measurements of spatial accuracy include the percentage of time spent in the target quadrant (where the platform was placed previously), the cumulative distance to the platform position throughout the trial (Gallagher proximity measure - GPM) and the number of times the animal crossed the platform position or equivalent positions in the pool (Peters et al., 2003; de Bruin et al., 2001; Gallagher et al., 1993).

Since it was first published, this task has been adapted to other species of rodents such as mice and used in a great number of neuronanatomical, pharmacologic and genetic studies (e.g. Peters et al., 2003; Need and Giese, 2003; de Bruin et al., 2001; Cho et al., 1999; Tsien et al., 1996)

### 1.4.2.2. THE RADIAL ARM MAZE TASK

Another hippocampus-dependent spatial task is the radial arm maze (Gresack and Frick, 2003; Bimonte-Nelson et al., 2003; Lee and Kesner, 2003; Mizuno et al., 2002), which is used in rodents and has also been adapted to humans in virtual reality environments (Astur et al., 2004; Sakthivel et al., 1999; Astur et al., 1998).

The radial arm maze (RAM) consists of a centre area with a number of identical arms radiating outwards. Some of the arms can be baited with rewards (generally food in dry versions and platforms in wet versions) which are withdrawn once found. Success in this task implies (a) being able to locate the baited arms between different training trials; (b) not re-entering arms previously visited within a training trial. In other words, animals need to learn spatial information that remained constant overtime (reference memory component), and that changed during the time of a single trial (working memory components; reviewed in Jonasson, 2005).

### 1.4.2.3. SEX DIFFERENCES IN SPATIAL MEMORY

Many studies in the sex differences literature ascribe better spatial abilities to men than to women. Men are generally better at reading maps, in mental rotation tasks, are more accurate in metric positional information and faster in the acquisition of virtual navigation tasks namely in some humanized versions of mouse tests (Postma et al., 2004; Parsons et al., 2004; Astur et al., 2004; Postma et al., 1999; Sakthivel et al., 1999; Galea and Kimura, 1993). On the other hand women are better in recalling landmarks in the map and detecting changes in landmark position (Galea and Kimura, 1993).

Some studies have reported that the male advantage in spatial tasks also occurs in rodent species (Gresack and Frick, 2003; Frick et al., 2000; Markowska, 1999; Kelly et al., 1988), however, a great number of other studies fail to report any sex difference (reviewed in Jonasson, 2005). The nature and robustness of sex differences depend on a number of factors, some of which will be discussed below.

#### 1.4.2.3.1. TASK PARAMETERS

The RAM and MWM are frequently used interchangeably to assess spatial memory and navigation abilities. In fact, robust sex differences are observed in the RAM both for the working and reference memory component (Astur et al., 2004; Heikkinen et al., 2004; Gresack and Frick, 2003; Bimonte-Nelson et al., 2003), while in the MWM these differences tend to be more subtle (Jonasson, 2005) and highly dependent on training procedures and task parameters. These tasks differ in motivation (appetitive in the RAM and aversive in the MWM) and motor demands (walking in the RAM and swimming in the MWM), with higher levels of stress imposed by the MWM procedure (Beiko et al., 2004). Additionally, in the RAM, assessment of reference memory can be biased by working memory errors within the trial, while scoring of spatial abilities in the MWM depends purely on reference memory. These factors may partially justify differences in the detection of sex differences by the two tasks.

In the MWM, differences in performance may correlate with the engagement of distinct strategies in solving the same task. In fact, a number of studies point to differences in navigation strategies based on cue utililzation. Manipulation of environmental characteristics in a virtual navigation task revealed that females rely

predominantly on landmark information, while males can more easily coordinate landmark and geometric information and are less susceptible to landmark disruption (Sandstrom et al., 1998), a finding coincident with self reported strategies (reviewed in Halari et al., 2005).

In agreement with the findings in humans, manipulation of task parameters also produces a sex-specific effect in rodents. Roof and Stein (1999) demonstrated that changes of release position across trials was significantly more detrimental for retention of platform acquisition in females, but the sex difference could be reversed if important salient cues such as the position of the experimenter remained constant throughout trials. However, no conclusions on sex differences in hippocampal memory can be extrapolated from these studies as performance was assessed by escape latencies which may not depend on hippocampal function (Moser and Moser, 1998; Moser et al., 1995). Additional evidence for the engagement of different strategies in spatial memory tasks was provided in a study by Williams et al. (1990). In this study, male and female rats were trained in the RAM until both groups reached similar levels of reference and working memory errors. Changes in the geometry of the room disrupted male performance, while female performance was more strongly disrupted by changes in landmarks.

The coordination of landmark and geometric information by the males is a more efficient strategy to locate the platform in comparison to relying on landmarks only. This efficiency is reflected in sexual dimorphisms in the rate of acquisition of the task. These difference tend to become minimal as training progresses and animals demonstrate robust spatial learning (Warren and Juraska, 1997; Perrot-Sinal et al., 1996; Frye, 1995). Therefore the time point in the course of training at which the animals are tested can be crucial for the detection of sex differences.

Additionally, pre-training in hippocampus-independent version of the MWM exerts a facilitator effect in the hippocampus-dependent version of the MWM. These effects are more pronounced in females and can therefore mask a female disadvantage (Perrot-Sinal et al., 1996).

#### 1.4.2.3.2. SOURCE OF GONADAL HORMONES

Studies performed in naturally cycling humans and rodents report impaired performance in spatial tasks during phases of high estradiol concentration (Galea et al., 2002; Postma et al., 1999; Fugger et al., 1998; Warren and Juraska, 1997), while other studies fail to find any influence of the phase of the estrous cycle in spatial memory. On the contrary, lower concentration of estrogens after estropause is related with decreased spatial abilities (Sherwin, 2003a; Frick et al., 2000).

Inter-individual variations and possible disruptions to normal cycling due to environmental conditions may bring confounds to the interpretation of behavioural outputs. To circumvent this problem many studies combine gonadectomies with hormone replacement. Yet, no consistent evidence was, so far, provided by these studies: some report advantages in gonadectomized over intact rats and a detrimental effect of estrogen replacement (Galea et al., 2002; Chesler and Juraska, 2000; Fugger et al., 1998), while others report an advantageous effect of estrogen (Sandstrom and Williams, 2004; Gureviciene et al., 2003; Markham et al., 2002; Frick et al., 2002).

Possible causes for the discrepancies between the studies mentioned above are differences in the time points of estrogen administration relative to training and testing of the animals. Sandstrom and Williams (2004) used a delayed matching to

place version of the MWM to determine the time frame of estrogen action. In this task, ovarectomized rats were given a training and test session separated by retention intervals of variable length. Generally, the longer the retention interval, the smaller the improvement in escape latency on the test trial. Estradiol injection thirty minutes prior to behavioural training caused significant improvements in performance for longer retention intervals, an effect only detectable 24 hours after the priming injection. Furthermore, these authors demonstrated that continuous estradiol replacement maintained the improvement in performance. These results suggest that the significant effects of estrogen in cognition are mediated by slow genotropic actions, rather than by fast cross talk with other intracellular signalling pathways.

Additional insights into the role of estrogen in spatial memory were provided by genetic manipulation of classic estrogen receptors. MWM training of ER $\alpha$ and ER $\beta$ null mutant female mice revealed different actions of the receptors for spatial memory acquisition: ER$\beta$ null mutants showed impaired performance when compared to their WT littermates (Kudwa et al., 2005; Rissman et al., 2002). In contrast ER$\alpha$ null mutants showed normal acquisition (Li et al., 2003; Fugger et al., 1998), while WT littermates treated with high doses of estradiol were impaired in MWM performance, suggesting that estrogen mediated activation of ER$\alpha$ receptors was detrimental for the acquisition of spatial tasks, while activation of ER$\beta$ was beneficial.

In addition to the effects of estrogen, a number of studies have also addressed the impact of progesterone in cognition (Sandstrom and Williams, 2001; Warren and Juraska, 2000). Comparison of two groups of postestropausal females with similar concentration of circulating estrogens but different concentrations of progesterone

suggested that higher levels of progesterone can be detrimental to the acquisition of spatial tasks (Warren and Juraska, 2000).

Like estrogen, testosterone is able to exert a modulatory effect in spatial abilities. For example, testosterone hypersecretion confers an advantage in mental rotation tasks in girls with the CAH condition relative to normal girls, but the opposite effect is seen in boys (Halari et al., 2005). Similarly, higher levels of circulating endogenous testosterone as well as exogenous administration correlates positively with spatial abilities in women (Aleman et al., 2004).

The studies described above focused on organisms that were either gonadally intact or that have been gonadectomized in adulthood, thereby providing insights onto the activational role of gonadal hormones in the shaping of sexually dimorphic navigation abilities. However, for example in humans, male advantages are detectable in 3-4 year old infants (Halari et al., 2005), but very little is known about the impact of organizational effects of gonadal hormones on navigational abilities in human adults. Williams and colleagues (1990) performed neonatal castration of male rats and estrogen administration to neonatal female rats, and the control groups were subsequently castrated in adulthood to prevent an activational effect of gonadal hormones. Testing of these animals in the RAM revealed the prevalence of an organizational effect of perinatal estrogen in the improvement of reference memory. Whether the organizational effects of testosterone were mediated by effects upon androgen receptors or estrogen receptors (due to aromatization) was unclear until a recent report on androgen insensitive male rats (Jones and Watson, 2005). These males secrete normal levels of testosterone, and have normal aromatase activity, but carry a mutation in the androgen receptor that renders them insensitive to testosterone. When tested in the MWM, this group of mice displayed a level of

performance intermediate between normal males and females. These results suggest that the masculinization of spatial behaviours is mediated by androgen receptors.

Only one study reports a direct impact of sex chromosome gene expression in spatial abilities. This study took advantage of a line of C57BL/6 mice in which, somegenotypic XY individuals develop as phenotypic females. XY females were significantly better than XX females in the MWM (Stavnezer et al., 2000).

#### 1.4.2.3.3. AGE OF THE SUBJECTS

An important study by Frick et al (2000) point to an age effect in sex differences in learning and memory (L&M) in the MWM. Testing of groups of young, middle aged and old mice of both sexes in the MWM pointed to an earlier impairment in females presumably due to lowering of circulating estrogen levels and cessation of cyclicity. The importance of cyclicity and its contribution for later protective effects is also provided by studies of mice in a dry version of the RAM, where the cognitive decline becomes milder as the age of ovarectomy increases, this is, for as long as the mice are allowed to cycle normally (Heikkinen et al., 2004).

## 1.5. MEMORY CONSOLIDATION IS A TIME-DEPENDENT PROCESS

Pioneering studies by Muller and Pilzecker on acquisition and retrieval of verbal material (Muller and Pilzecker, 1890, reported by Lechner et al., 1999) were the first to report the requirement of a time-dependent process for memory stabilization. Clinical studies of retrograde amnesia and use of amnesic treatments

in experimental animals (electroconvulsive shocks, hypoxia, hypothermia and inhibition of protein synthesis) provided evidence for the existence of a time frame during which memory is susceptible to disruption (Squire et al., 2001; Nadel and Bohbot, 2001; Sara, 2000; Squire and Zola-Morgan, 1991; Misanin et al., 1968; Schneider and Sherman, 1968; McGaugh, 1966; Agranoff et al., 1965; Flexner et al., 1963). Based on this and other studies, two temporal stages of memory were defined: short-term memory (STM) and long-term Memory (LTM). STM emerges within the few seconds or minutes following the learning episode and persists for short periods of time (in the minute range). LTM are stable and persist for days, months or even a lifetime. The consolidation theory of memory postulates that formation of stable, enduring and usable representations of learning experiences is a time dependent process (McGaugh, 2000).

Consolidation of hippocampus-dependent memory occurs at the cellular and systems level (Frankland and Bontempi, 2005; Dudai, 2004).

Synaptic or cellular consolidation comprises a set of molecular and cellular events triggered in hippocampal neurons by the learning experience which are thought to modulate synaptic strength and eventually contribute to the reshaping of the pattern of connectivity in the hippocampal circuitry (Lamprecht and LeDoux, 2004). These processes are initiated and occur during the first few hours following learning and will be further discussed in section 1.6.

Systems consolidation, refers to a slow process of reorganization of the memory trace within brain areas, which is thought to involve a cross talk between the hippocampus and the neocortex (Frankland and Bontempi, 2005; Dudai, 2004; Dash et al., 2004; Nadel and Bohbot, 2001). Studies of systems consolidation in human patients and lesioned rodents yielded conflicting results which gave rise to two main

theories: the multiple trace theory, and the trace transfer theory. The multiple trace theory postulates the participation of the hippocampus in the retrieval of spatial and contextual memories for as long as they persist (Winocur et al., 2005; Rosenbaum et al., 2000; Nadel and Moscovitch, 1997). On the other hand, the trace transfer theory defends that memories are only temporarily stored in the hippocampus and gradually become solely dependent on the neocortex (Clark et al., 2005; Bayley et al., 2005; Squire et al., 2001).

## 1.6. CELLULAR AND MOLECULAR BASES OF MEMORY

The studies on human patients and rodents described in the previous section provided strong evidence for a role of the hippocampus in the storage of declarative or episodic-like memories. However, finding the memory engram, the physical support for memory encoding, has proved to be a more challenging task. Nevertheless, an extensive amount of literature describes correlations between molecular and cellular mechanisms and behavioural outputs used as measures of memory formation. Some of these studies will be described in the next sections.

### 1.6.1. SYNAPTIC PLASTICITY

Neurons communicate with each other via synapses. These are specialized adhesion junctions that display highly plastic properties (Hussain and Sheng, 2005). Synapse formation, turnover and remodelling occur during development contributing to set the wiring of the brain. Pioneering ideas concerning the physical support of memory were put forward by the Spanish neuroanatomist Santiago Ramon y Cajal at

the end of the 19th century, who postulated that modifications of synaptic connections between neurons can serve as elementary components of memory storage (Cajal, 1894, reported by Bailey et al., 2000).

Following Cajal's ideas, the Canadian neuropsychologist Donald Hebb, postulated on the electrophysiological properties of synaptic transmission: "*When an axon of cell A is near enough to excite cell B and repeatedly or persistently takes part in firing it, some growth processes or metabolic changes take place in one or both cells such that A's efficiency as one of the cells firing B, is increased.*" (Hebb, 1949 reported by Bliss, 2003).

A cellular mechanism able to account for Hebbian plasticity rules, emerged from studies by Bliss and Lomo, who described a sustained increase in the efficiency of synaptic transmission in the dentate gyrus, after high frequency stimulation of the perforant path (Bliss, 2003; Bliss and Lomo, 1973). This phenomenon of increase in synaptic strength was termed long-term potentiation (LTP), and various forms of LTP have subsequently been described in all excitatory pathways of the trisynaptic circuit of the hippocampus and in other brain regions such as the amygdala (Maren, 2005; Lynch, 2004; Malenka and Bear, 2004; Sweatt, 1999). LTP is characterized by three basic properties: cooperativity, associativity and input specificity. Cooperativity corresponds to the need for a certain threshold intensity for induction; Associativity permits the potentiation of a weak input if it is active at the same time as a strong tetanus to a separate but convergent input; Input specificity prevents the occurrence of potentiation in inputs that were not active at the same time of the tetanus (Bliss and Collingridge, 1993). These three properties and the durability of LTP support a potential physiological relevance of this cellular process in L&M consolidation . (Lynch, 2004)

LTP is conventionally separated into three stages: (a) Induction, referring to the transient extracellular stimuli that trigger the potentiation effect; (b) Expression, comprising an array of molecular mechanisms that allow increased potentiation; and (c) Maintenance, pertaining to the persistent biochemical signal that lasts in the cell. Each of these stages is further divided into three temporal stages: initial, early and late LTP (L-LTP). The use of specific enzyme inhibitors and electrophysiological studies both in WT and genetically modified animals has provided evidence for the engagement of distinct biochemical and molecular mechanism in each of these stages. Among these, the dependence of L-LTP on *de novo* transcription and protein-synthesis, should be emphasized as this is also a requirement for memory consolidation (Sweatt, 1999; Nguyen et al., 1994; Malinow et al., 1988; Frey et al., 1988)

LTP is an experimental phenomenon which can be used to demonstrate the repertoire of long-lasting modifications that synapses can undergo. Many studies report a correlation between *in vitro* LTP and performance in memory tasks, while others report dissociation between these two phenomena. A conclusive demonstration that *in vivo* LTP underlies memory formation is still missing (Lynch, 2004; Malenka and Bear, 2004; Sweatt, 1999).

#### 1.6.1.1. SEX DIFFERENCES IN LTP

The magnitude of perforant path LTP induced *in vitro* was reported to be higher in hippocampal slices from male rats (Maren, 1995; Maren et al., 1994). Further studies were pursued towards understanding whether the sex difference was due to hormonal effects and, if so, which hormones were implicated in this regulation.

The degree of potentiation evoked by a single high frequency tetanus stimulation of the perforant path did not differ between castrated and intact males, indicating that, at least under this induction protocol, testosterone did not participate in the modulation of LTP (Anagnostaras et al., 1998). Under the same electrophysiological protocol, administration of estrogen to castrated female rats produced a lesser degree of potentiation in comparison to vehicle treated animals (Gupta et al., 2001). These results suggest that as far as perforant path LTP is considered, estrogen contributes to a lesser degree of synaptic potentiation.

Similarly to DG synapses, high frequency stimulation of the Schaffer collateral produces higher LTP magnitude in males than in females (Yang et al., 2004). However, contrarily to DG synapses, higher levels of estrogen both in naturally cycling and estrogen replaced animals contribute to augmented LTP in CA1 synapses (Gureviciene et al., 2003; Good et al., 1999) .

### 1.6.2. MOLECULES INVOLVED IN SYNAPTIC PLASTICITY

A number of neurotransmitters can accomplish synaptic transmission in excitatory synapses, among which the most intensively studied is glutamate. Glutamate released by the pre-synaptic terminal can act upon three kinds of ionotropic receptors embedded in the post synaptic membrane: N-methyl-D-aspartate receptors (NMDAR) and non-NMDAR such as the alpha-amino-3-hydroxy-5-methyl-4-isooxazolepropionic acid receptor (AMPAR) and kainate receptors (Cull-Candy and Leszkiewicz, 2004).

The NMDAR is a doubly gated channel permeable to calcium ions ($Ca^{2+}$). In the resting state, when neurons are polarized, the NMDAR channel is blocked by a

magnesium ion ($Mg^{2+}$). NMDAR activation depends on the temporal coincidence of two events: depolarization of the post-synaptic membrane by strong electrical stimulation and pre-synaptic release of glutamate. Thus, the NMDAR acts as a molecular coincidence detector (Tsien, 2000).

Calcium entry into the cell is the trigger for multiple events such as the process of AMPAFICATION. This corresponds to increased exocytosis of pre-formed and *de novo* synthesis of AMPAR and activation of transcription via cross talk with signalling pathways and second messengers (Deisseroth and Tsien, 2002; West et al., 2001; Berridge et al., 2000; Ho et al., 2000).

Whether strengthening of synaptic transmission relies on pre or post-synaptic mechanisms or both is still a highly debated issue. Some studies report increased neurotransmitter release and restructuring of the pre-synaptic terminal after LTP induction (Zakharenko, 2001, Antonova, 2001) and other studies reporting an engagement of the post-synaptic compartment through increased AMPAR insertion upon LTP induction and after associative learning in a fear conditioning paradigm (Malinow and Malenka, 1999; Rumpel, 2005).

### 1.6.3. MOLECULES INVOLVED IN LEARNING AND MEMORY MECHANISMS

While *in vitro* techniques may provide insights into the physiology of synaptic transmission, addressing the biological mechanisms relevant for actual L&M processes requires a combination of behavioural, pharmacologic and genetic manipulations in living animals.

The advent of mouse molecular genetic techniques provided valuable tools to unravel some of the molecules, mechanism and pathways engaged in memory

consolidation. These techniques allowed the deletion, modification and insertion of genes in the mouse genome (Giese et al., 1998; Mayford et al., 1996; Silva et al., 1992). Refinements of these techniques offered the possibility of tightly regulating the effects of the mutations introduced in a temporal and regional fashion, thus preventing developmental abnormalities and unspecific effects of the mutation on L&M (Nakazawa et al., 2003; Morozov et al., 2003; Mayford and Kandel, 1999; Tsien et al., 1996). Some of these manipulations for molecules that have been shown to be involved in L&M processes will be described below.

### 1.6.3.1. THE ROLE OF THE NMDAR IN LEARNING AND MEMORY

A combination of pharmacologic and electrophysiological techniques established that, in the majority of synapses that support LTP, the post synaptic $Ca^{2+}$ increase is mediated through activation of the NMDAR (Lynch, 2004; Malenka and Bear, 2004; Sweatt, 1999). Furthermore, pharmacologic, genetic and behavioural techniques established the critical importance of the NMDAR in L&M consolidation.

Pioneering studies by Morris and co-workers (Morris et al, 1986) revealed that pharmacological inhibition of the NMDAR with the selective antagonist D-2-amino-5-phosphonopentanoate (AP5) causes severe deficits in LTP and prevents memory formation after MWM training.

In the adult brain, the majority of the NMDAR are heteromers of NR1 and either NR2A or NR2B subunits (Tsien, 2000). Deletion of the NR1 subunit renders the receptors non functional. The relative predominance of NR2B over NR2A declines with age which is thought to be related to age-related cognitive decline (Cull-Candy and Leszkiewicz, 2004; Tsien, 2000).

Genetic deletion of the NR1 subunit in area CA1 in the adult hippocampus caused impairments in a variety of hippocampus-dependent tasks, and abolished LTP in hippocampal subfield CA1 (Tsien et al., 1996; McHugh et al., 1996). NR1 deletion in subfield CA3 caused deficits in slice LTP evoked in the recurrent-collateral CA3 synapses, but mild impairments in spatial memory in standard MWM protocols. However, impairments in a delayed matching to place version of the MWM, and upon partial removal of cues suggest that NMDARs in subfield CA3 of the hippocampus are involved in processes of pattern completion (Nakazawa et al., 2003; Nakazawa et al., 2002). Further evidence for a critical role of the NMDAR in learning processes was provided by a general improvement in performance in hippocampus-dependent tasks and enhanced LTP in mice overexpressing the NR2B subunit (Tang et al., 1999).

The studies described above, among many others, established positive correlations between NMDAR function, artificially induced LTP and memory consolidation assessed at the behavioural level. However, a number of other studies fail to find these correlations: whether this failure is related to the experimental conditions under which LTP was induced and measured, or, whether LTP-related phenomena do not always represent the cellular model of memory consolidation, is still a highly debated issue (Malenka and Bear, 2004; Morris, 2003; Tsien, 2000; Bliss and Collingridge, 1993).

### 1.6.4. MEMORY CONSOLIDATION REQUIRES *DE NOVO* TRANSCRIPTION AND TRANSLATION

Studies by Agranoff and colleagues in the 1960's demonstrated that administration of protein synthesis inhibitors produces amnesic effects in the goldfish (Davis and Agranoff, 1966; Agranoff et al., 1965). Follow up studies in rodents demonstrated that systemic and intraventricular administration of anisomycin (an agent that interferes with transpeptidation) before or shortly after training strongly affects formation of LTM, while sparing STM for a variety of tasks (e.g. von Hertzen and Giese, 2005; Barrientos et al., 2002; Schafe et al., 1999).

Additionally, the use of RNA polymerase inhibitors (like $\alpha$-amanitin) provided evidence that not only translation but also *de novo* transcription are critical for memory consolidation (Igaz et al., 2002).

An intriguing question is how the activation of cell wide mechanisms could account for input specificity, in order to strengthen particular synapses. Frey and Morris (1997) suggest that newly synthesized proteins are sequestered at potentiated synapses by an electrophysiological tag. Alternatively, Schuman and colleagues propose that strengthening of activated synapses is accomplished by local dendritic translation. In fact, mRNAs and components of translational machinery have been found in dendritic sites, and dendritic translation has been shown to be regulated in an activity-dependent manner (Sutton et al., 2004; Steward and Schuman, 2003; Steward and Schuman, 2001). No conclusive evidence points to a single agent responsible for specific marking of activated synapses for translational activation, but candidates include protein kinases, adhesion molecules, cytoskeletal elements and translational activators (Martin and Kosik, 2002).

### 1.6.5. CREB AS THE KEY MOLECULAR SWICTH FOR MEMORY FORMATION

Given the well established requirement of *de novo* transcription for memory consolidation, an intensive amount of effort attempted to find transcription factors which can enable memory formation.

The cyclic adenosine mono phosphate (cAMP) responsive element (CRE) binding protein (CREB) was identified as a molecular switch for LTM formation in different species ranging from *Drosophila* to molluscs and mammals and in a wide range of hippocampus and amygdala dependent tasks (Dubnau et al., 2003; Kimura et al., 2002; Graves et al., 2002; Waddell and Quinn, 2001; Kandel, 2001; Gass et al., 1998; Bourtchuladze et al., 1994).

Hippocampal CREB activation has been identified after training of rodents in spatial and contextual learning tasks (Kudo et al., 2004; Peters et al., 2003; Wei et al., 2002; Mizuno et al., 2002; Taubenfeld et al., 1999). Additionaly, specific activation of genes bearing CRE-elements, upon training in single trial learning tasks has also been described for amygdala and hippocampus-dependent tasks (Athos et al., 2002; Impey et al., 1998).

Moreover, blockade of CREB function by injection of antisense oligonucleotides in the hippocampus disrupted consolidation of a spatial learning task (Guzowski and McGaugh, 1997), and expression of a CREB endogenous repressor blocked consolidation of contextual fear memories (Kida et al., 2002).

These results were further complemented by mouse molecular genetics: deletions of the genes ecoding the alpha and delta isoforms of CREB (CREB$\alpha\delta$ mutations) led to strong deficits in hippocampus dependent spatial memory and contextual fear and in amygdala dependent cued conditioning, which were

accompanied by impairments in LTP in CA1 synapses (Bourtchuladze et al., 1994). Disruption of CREB function in hippocampal subfield CA1 by expression of a dominant-negative form of CREB caused impairments in performance of the MWM task (Pittenger et al., 2002) and expression of a constitutively active CREB protein facilitated the transcription dependent phase of LTP (L-LTP) (Barco et al., 2002).

Despite the evidences described above, the requirement of CREB for memory consolidation has been challenged by other studies on spatial and contextual tasks. First, impairments in the MWM have been shown to be dependent on the training protocol, as the CREB$\alpha\delta$ mutants were able to learn the task under a spaced training protocol (Hebda-Bauer et al., 2005; Kogan et al., 1997); Second, manifestation of the phenotype was strongly dependent on the genetic background of the mouse lines (Graves et al., 2002; Gass et al., 1998); Third, other mutants with a marked reduction of hippocampal levels of CREB protein displayed only mild phenotypes in the MWM (Balschun et al., 2003).

There is also controversy concerning the role of hippocampal CREB in classical conditioning tasks. Kida et al. (2002) reported impaired contextual fear memory consolidation after expression of a CREB repressor. However, expression of a dominant negative form of CREB in area CA1 did not interfere with contextual or cued fear conditioning (Pittenger et al., 2002).

### 1.6.6. CREB ACTIVATION PATHWAYS

CREB belongs to the basic-leucine zipper (bZIP) family of transcription factors that also comprises CREM and ATF. Members of this family form homo or heterodimers that can either exert a compensatory effect upon CREB loss or counteract CREB function thereby acting as transcriptional repressors (Chen et al., 2003; Pittenger et al., 2002).

CREB activation is achieved by self dimerization and phosphorylation of serine residue 133 (Gonzalez and Montminy, 1989).

Given the functional heterogeneity of genes possessing CRE elements and the broad range of stimuli that can elicit CRE-driven transcription, tissue and time specificity is achieved through: (a) Combinatorial effects in the dimerization of the bZIP family member transcription factors; (b) Recruitment of other transcriptional co-activators such as CREB-binding protein (CBP; Impey et al., 2002; Chawla et al., 1998).

In neurons, transduction of synaptic inputs to the nucleus in order to activate CREB is mediated mainly by two signalling pathways: the cAMP activated protein kinase (PKA) cascade which cross-talks with the mitogen-activated protein kinase (MAPK) cascade and the calcium/calmodulin ($Ca^{2+}$/CaM) dependent protein kinase (CaMK) cascasde (reviewed in Silva, 2003; Soderling, 1999). *In vitro* studies in cultured neurons and biochemical studies have revealed learning-specific activation of members of these pathways. Furthermore, combination of targeted mutagenesis techniques with behavioural and electrophysiological techniques have provided insights into the role of particular members of these pathways in synaptic plasticity

and L&M processes (Kelleher, III et al., 2004; Giese et al., 2001; Sweatt, 2001; Blum et al., 1999; Selcher et al., 1999; Bourtchouladze et al., 1998; Atkins et al., 1998).

Because part of the work described in this thesis was performed with mice carrying a genetic mutation for one of the members of the CaMK cascade, the biochemistry and the role of this pathway in L&M and synaptic plasticity will be addressed below.

### 1.6.7. THE CaM KINASE CASCADE

The CaMK cascade participates in many biological functions including T cell activation (Westphal et al., 1998), fertility and, as discussed below, synaptic plasticity and L&M (Wu et al, 2000a, Wu et al, 2000b).

#### 1.6.7.1. BIOCHEMISTRY OF THE CaM Kinase cascade

The CaMK cascade consists of a set of serine/threonine kinases including CaMKI, CaMKIV and a pair of upstream activating kinases, CaMKK$\alpha$ and CaMKK$\beta$ (Anderson et al., 1998; Chatila et al., 1996; Selbert et al., 1995). All members share structural similarities: they possess a catalytic domain adjacent to a regulatory region containing an inhibitory domain (AID) and a $Ca^{2+}$/CaM binding domain (CBD). A conformational constraint based on the interaction of the catalytic domain with the AID maintains the kinases in an inactive state. $Ca^{2+}$/CaM biniding to the CBD is essential for the release of the inhibitory constraint allowing activation of the protein (Anderson et al., 1998; Cruzalegui et al., 1992). The fact that upstream and downstream members of the cascade require the same allosteric activator is an

unusual feature in intracellular signalling cascades, and advantages of such a pattern of activation are not apparent (Soderling, 1999).

The mechanisms of activation of CaMKIV are well understood: after the release of the inhibitory constraint by $Ca^{2+}$/CaM binding allowing phosphorylation of multiple serine residues, CaMKK binds to CaMKIV/$Ca^{2+}$/CaM complexes and phosphorylates a threonine residue in the catalytic domain which renders the enzyme fully active (Tokumitsu et al., 1999; Anderson et al., 1998; Tokumitsu et al., 1995). Phosphorylation of this threonine residue can only be mediated by CaMKK and is essential for nuclear translocation of CaMKIV (Lemrow et al., 2004). CaMKK mediated phosphorylation is essential for full activation of the protein rendering it able to phosphorylate nuclear substrates (Impey et al., 2002; Kane and Means, 2000; Enslen et al., 1995).

Interestingly, following CaMKK-mediated phosphorylation, CaMKIV is capable of maintaining $Ca^{2+}$/CaM independent or autonomous activity, which accounts for sustained activity beyond the transient intracellular rise in calcium concentration. Notably, this autonomous activity is required for CaMKIV to drive transcription (Chow et al., 2005).

Despite the fact that both CaMKI and CaMKIV are able to activate CREB *in vitro* (Takemoto-Kimura et al., 2003; Bito et al., 1996), multiple lines of evidence suggest that CaMKIV alone is the most likely candidate for a CREB kinase *in vivo*. Firstly, CaMKIV has been found in the nucleus and cytoplasm while CaMKI has only been detected in the cytoplasm (Lemrow et al., 2004; Nakamura et al., 1995); Second, interference with CaMKIV through antisense or dominant negatives in cultured neurons, inhibits CRE-dependent transcription (Finkbeiner et al., 1997; Bito et al., 1996); Third, expression of a constitutively active form of CaMKIV promotes

increased CREB and CBP phosphorylation, thereby enhancing transcription (Impey et al., 2002).

The CaMKK family is encoded by two distinct genes giving rise to the α and β isoforms, the latter subject to alternative splicing (Hsu et al., 2001). Despite displaying structural and functional similarities and similar ability to activate CaMKI and CaMKIV *in vitro*, CaMKKs display distinct tissue intracellular distribution with CaMKKα being more widespread and CaMKKβ predominantly expressed only in brain tissue, thymus and male gonads (Vinet et al., 2003; Sakagami et al., 2000; Anderson et al., 1998; Nakamura et al., 1995). Subtle structural differences render the enzymes differentially sensitive to certain chemical inhibitors (Tokumitsu et al., 2003) and to activation by $Ca^{2+}$, as activity of CaMKKβ is not entirely dependent on $Ca^{2+}$/CaM binding, while binding of this complex is absolutely essential for CaMKKα activation (Tokumitsu et al., 2001).

No data in the literature confirm or rule out the possibility that CaMKKα can phosphorylate CaMKI or CaMKIV. It is also unclear in which subcellular compartment CaMKIV phosphorylation occurs (Chow et al., 2005).

The role of the CaM Kinase cascade in signalling transduction pathways is not limited to regulation of CREB and CBP. In fact, CaMK cascade members, crosstalk with other signalling pathways such as the MAP kinase and PKB cascades and, furthermore, CaMKIV can directly activate a number of other transcription factors (Inuzuka et al., 2002; Kane and Means, 2000; Soderling, 1999).

### 1.6.7.2. THE ROLE OF THE CaM KINASE CASCADE IN L&M

Evidence for a direct role of CaMKIV in synaptic plasticity was provided for example by the demonstration that CaMKIV is activated and involved in stimulation of gene expression during LTP in the hippocampal CA1 region (Kasahara et al., 2001). In order to investigate the role of members of the CaMK cascade in synaptic plasticity and L&M, a number of mutant mouse lines for members of the pathway have been generated.

Wu and colleagues generated a global null mutant mouse which displayed serious developmental abnormalities and was, therefore, unsuitable for behavioural testing (Wu et al., 2000). In contrast, another CaMKIV global mutant did not display obvious deficits except for abnormalities in cerebellar development (Ho et al., 2000). This mutant line displayed deficits in glutamate-induced CREB activation in the CA1 area in hippocampal slices. In addition, early LTP, a transient form of synaptic potentiation, was normal in the mutants while late LTP (L-LTP), which is dependent on *de novo* transcription, was impaired in CA1 synapses, an effect that can be partially explained by a failure to activate CREB in the mutant line. At the behavioural level the mutation affected contextual and cued fear conditioning (Wei et al., 2002), but spared performance in the MWM (Ho et al., 2000). However, the presence of truncated transcripts in the latter mouse line made it unclear whether CaMKIV function was, in fact, fully ablated.

To elucidate the role of CaMKIV while preventing the developmental deficits described, Kang et al. (2001) generated forebrain-restricted transgenic mice expressing a dominant negative transgene of CaMKIV which confered a defect in CREB phosphorylation. In agreement with the data from the global null mutant mice,

reduction of activity-induced CREB phosphorylation produced a defect in L-LTP. At the behavioural level, this mutant line displayed deficits in spatial memory formation assessed in the MWM, and contextual fear conditioning assessed 7 days after training, but not 1 day after training. In addition to the possibility that CaMKIV function was not fully ablated in the global mutant line, discrepancies in the behavioural phenotype in the MWM may be explained by the MWM setup and training protocol: in the Ho study, the swimming pool was relatively small (100 cm in diameter as opposed to 160 cm in the Kang study) and the MWM training protocol started with the visible platform version of the task which is known to facilitate subsequent learning in the hidden platform version (Warren and Juraska, 1997).

Peters et al. (2003) generated a mutant mouse line bearing a global null mutation for the gene encoding CaMKK$\beta$. These mice did not display any gross developmental abnormalities and were subsequently put through a battery of behavioural tests. Contextual and cued fear conditioning memory assessed one day after training did not differ between WT and CaMKK$\beta$ null mutants. These mice were trained in the hidden platform version of the MWM and tested in a probe trial at the end of the sixth day of training. Probe trial performance revealed impairment in spatial memory consolidation in male mice. Remarkably, probe trial performance of null mutant females was comparable to that of WT littermates (Mizuno et al., 2006). Furthermore, a deficit in hippocampal CREB activation was detected in male CaMKK$\beta$ null mutant mice sacrificed immediately after the probe trial on day 6. In agreement with the findings relative to the CaMKIV lines, deficits in CREB activation correlated with deficits in slice L-LTP in hippocampal CA1 synapses.

Probe trial data is a composite measurement of task acquisition, consolidation and retrieval of spatial memory. Hence, the findings on failure of CREB activation

and impaired L-LTP are more difficult to interpret in the light with the consolidation theory. A conclusive demonstration that lack of CaMKK$\beta$ has an impact on memory consolidation was provided by testing these mice in the social transmission of food preferences (STFP), another hippocampus-dependent task. This is an ethological task which takes advantage of the natural tendency that rodents have to prefer types of food already ingested by their peers (Need et al., 2003). The task consists of feeding a group of demonstrator mice with scented food. These mice will then interact with a group of food deprived mice, who can smell the scent of the food eaten by the demonstrator. When given the choice between two differently scented foods, mice will tend to eat preferentially the food scented with the same scent previously smelled on the breath of the demonstrator (cued food). Testing of the CaMKK$\beta$ mouse line in this task, 5 min after interaction with the demonstrator revealed a preference towards the cued food in both groups, demonstrating a normal acquisition of the task. When tested 24 hours after interaction with the demonstrator, mutant mice failed to show a preference towards the cued food. These results provided a demonstration that CaMKK$\beta$ is required for memory consolidation of, at least some, hippocampus-dependent learning tasks.

Mizuno and colleagues (2006) generated a global null mutant mouse line for CaMKK$\alpha$. This mutant mouse line displayed normal spatial learning assessed in the MWM and impaired hippocampus-dependent contextual fear conditioning in male mice but not in female mice. L-LTP was not affected in hippocampal slices from animals of either sex.

The results obtained from the Peters et al. (2003) and Mizuno et al. (2006) point to a male specific requirement for the upstream members of the CaMK cascade, CaMKK$\alpha$ and CaMKK$\beta$, in spatial and contextual memory formation respectively.

The results of the behavioural and synaptic plasticity studies of mutant mouse lines for member of the CaM kinase cascade are summarized in the table 1.4.

**Table 1.4- The impact of genetic manipulation of members of the CaM kinase cascade in synaptic plasticity and L&M tasks.**

| Mutant line | LTP | Spatial Memory | Fear conditioning |
|---|---|---|---|
| CaMKIV global mutants (Wei et al., 2002; sex of the animals not specified; Ho et al., 2000) | Normal E-LTP and impaired L-LTP in CA1 | Normal MWM; Normal RAM | Impaired contextual and cued fear conditioning assessed 1 and 7 days after training. Reduced CREB activation after contextual fear conditioning. |
| Dominant negative CaMKIV transgenic line (sex of the animals not specified; Kang et al., 2001) | Normal E-LTP and impaired L-LTP in CA1 | Impaired MWM; | Normal contextual fear conditioning assessed 1 day after training but Impaired CFC assessed 7 days after training. |
| CamKK$\beta$ global null mutant (Peters et al., 2003) | Normal E-LTP and impaired L-LTP in CA1 in males, normal in females | Males impaired in the MWM; Impaired CREB activation after spatial training Females normal | Normal contextual and cued fear conditioning assessed 1 day after training. |
| CaMKK$\alpha$ global null mutant (Mizuno et al., 2006) | Normal E-LTP and L-LTP in CA1 in both sexes | Normal in both sexes | Impaired contextual fear conditioning in males; normal in females; normal cued conditioning in both sexes |

## 1.7. MOLECULAR BASES OF SEX DIFFERENCES IN MEMORY FORMATION

The work on sex differences in spatial and contextual memory tasks has focused mostly on the effects of gonadal hormones (sections 1.4.1.4 and 1.4.2.3). In addition, the modulatory roles of gonadal hormones on LTP (section 1.6.1.1).

Furthermore estrogen treatment of cultured hippocampal neurons correlates with a number of molecular events that have been associated with memory consolidation: (a) increased CREB phosphorylation accompanied by increased density of dendritic spines (Frick et al., 2004; Murphy and Segal, 1997); (b) increased expression of synaptic proteins including the NR1 subunit of the NMDAR (Li et al., 2004; Frick et al., 2002; Gazzaley et al., 1996a; Gazzaley et al., 1996b); (c) Interaction with and modulation of signalling cascades known to participate in synaptic plasticity and memory consolidation processes (reviewed in Cato et al., 2002).

The results on the CaMKK$\alpha$ and $\beta$ mouse lines suggest that, in addition to gonadal hormone actions, sex differences in spatial and contextual memory tasks can also derive from the engagement of distinct signalling pathways in hippocampus-dependent memory consolidation. In addition, converging evidence suggests a sex-specific role for CREB in memory formation. First of all, a study of CREB activation upon contextual conditioning revealed a specific increase in phosphorylated CREB in area CA1 in male rats but not in female rats (Kudo et al., 2004). Furthermore data from our lab (K. Mizuno and K.P.Giese, unpublished) reveal lower levels of CREB activation in female WT mice after training in the MWM, when compared to male mice. Finally, a manipulation of task parameters in MWM training of CREB$\alpha\delta$ mutant mice, revealed that in males, pre-exposure to an unsuccessful learning experience prevented subsequent learning under more favourable conditions. In females, however, previous unsuccessful learning did not affect subsequent learning under more favourable conditions. Conversely, change of platform location affected female mutants more adversely than male mutants (Hebda-Bauer et al., 2005).

## 1.8. MEMORY EFFECTOR GENES

The importances of de novo transcription and translation as well as the role of some transcription factors and upstream regulatory signalling pathways in memory consolidation are well established. The relevance of these processes depends on the events ultimately triggered in the neuronal cell and that contribute to the modulation of synaptic transmission. For this purpose it is essential to identify the downstream target genes of the signalling pathways described above.

A combination of biochemistry and bioinformatic approaches has provided comprehensive data on the identity of CREB target genes (Impey et al., 2004; Conkright et al., 2003). Because CREB mediated transcription exerts pleiotropic effects in neurons (West et al., 2001; Wu et al., 2001; Finkbeiner et al., 1997; Bito et al., 1996) the identification of CREB targets is not the most efficient approach towards the identification of "memory effector genes". In addition, multiple lines of evidence challenge the requirement of CREB for memory consolidation (Balschun et al., 2003; Graves et al., 2002; Gass et al., 1998) and the importance of a number of other transcription factors in synaptic plasticity and L&M processes has been well established (eg. Ramanan et al., 2005; Levenson et al., 2004a; Guzowski et al., 2001; Taubenfeld et al., 2001). Finally, the first group of genes to be expressed after synaptic activation, termed Immediate Early Genes (IEG) comprises a number of transcription factors, such as c-fos and zif268 which drive transcription of other memory effector genes (reviewed by Guzowski, 2002). The conjunction of these factors adds a very high degree of complexity to the regulation of activity induced gene expression (Fig. 1.4).

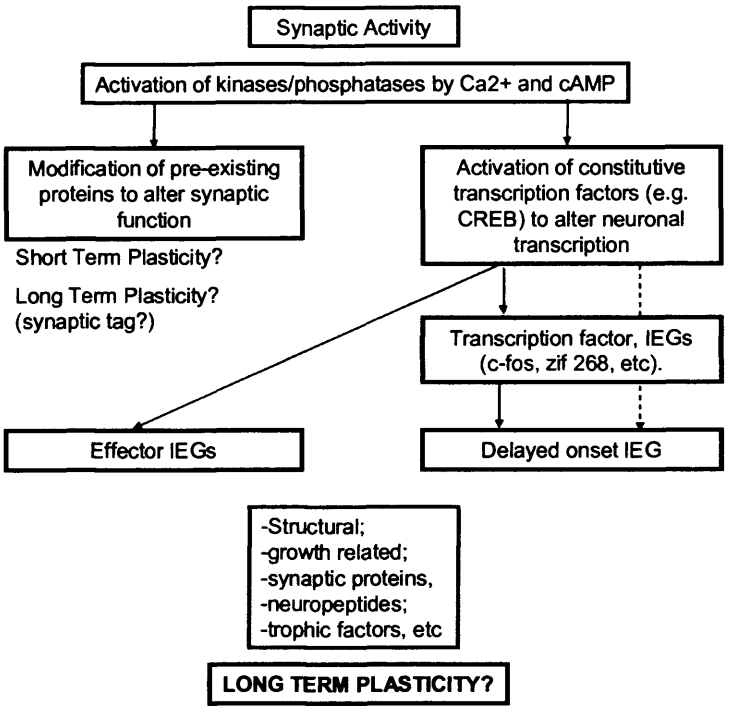


**Figure 1.4- Model of experience-dependent gene expression in synaptic plasticity and memory consolidation processes.** Synaptic activity driven by experience leads to alterations in intracellular second messenger levels, which in turn activate cellular kinases and phosphatases. These enzymes modulate the activity of a wide range of pre-existing cellular proteins, including synaptic components and transcription factors. In the nucleus, activation of CREB and related transcription factors initiates a cascade of gene expression (adapted from Guzowski, 2002).

### 1.8.1. GENOME-WIDE ANALYSIS OF GENE EXPRESSION INDUCED BY BEHAVIOURAL TRAINING

Technologies aimed at the identification of memory effector genes are based on comparative analyses of the transcriptome of whole brain or specific areas of the brain in animals subjected to different training conditions. A large number of screening technologies for gene expression in the brain have been developed (Lein et al., 2004; Proudnikov et al., 2003; Evans et al., 2002; Broude, 2002). One of the most popular ones is *Affymetrix Microarray* technology (von Hertzen and Giese,

2005; Datson et al., 2004; Levenson et al., 2004a; Cavallaro et al., 2002). A characterization of the latter technique and its general advantages and disadvantages is provided in Chapter III.

## 1.9. AIM OF THE PROJECT

Performance in tasks assessing hippocampus-dependent memory formation has been reported to be sexually dimorphic. This is classically interpreted as the effect of gonadal hormone actions in the brain.

*De novo* transcription is required for memory consolidation. Memory consolidation engages activation of the transcription factor CREB which can be activated by a variety of signalling pathways including the CaMK cascade. Peters and colleagues (2003) generated a null mutant mouse in which all the beta isoforms of CaMKK were deleted. This CaMKK$\beta$ null mutant line was subjected to a variety of behavioural tests aimed at assessing hippocampus-dependent memory formation.

Male CaMKK$\beta$ mutant animals exhibited delayed spatial memory formation assessed in the MWM. This behavioural phenotype was accompanied by an impairment in CREB activation after spatial training and in the transcription dependent phase of LTP (L-LTP) in CA1 synapses. Remarkably, CaMKK$\beta$ null mutant females were unimpaired in the MWM and in L-LTP. These studies suggested the hypothesis that the transcriptional mechanisms underlying spatial memory formation may differ between the sexes.

Starting from this hypothesis, the current project used the CaMKK$\beta$ null mutant line as a tool to investigate sexual dimorphisms in gene expression in hippocampal tissue.

The aims of this project were:

1. To identify CaMKK$\beta$ regulated genes in the male hippocampus, and to investigate whether the same genes are also regulated by CaMKK$\beta$ in female mice.
2. To investigate whether transcription of these genes in the hippocampus was regulated by training in behavioural task; and, if so, whether the pattern of regulation was sexually dimorphic

# CHAPTER II:
# MATERIAL AND METHODS

## 2.1. EXPERIMENTAL ANIMALS

### 2.1.1. ANIMAL HOUSING

Mice were housed with food and water *ad libitum* and maintained on a 12 h light-dark cycle, weaned and genotyped 3 weeks after birth and housed in groups of 2 to 5. Housing and experimental procedures were performed according to the Animals (Scientific Procedures) Act 1986.

All animals used for behavioural experiments and expression studies were aged between 8 and 16 weeks. A group of mice used only for expression studies was sacrificed on post-natal day 21 (P21).

### 2.1.2. MOUSE GENETIC BACKGROUND

CaMKKβ null mutant mice were generated as described in Peters et al. (2003). Briefly, exon 5 of the CamKK2 gene encoding the CaMKKβ protein was flanked by a recognition site for Cre-recombinase (loxP site) and a floxed Neomycin resistance cassette (NEO). This construct was injected into R1 embryonic stem cells and, after selection for neomycin resistance, the deletion of the targeted exon and the NEO cassette was achieved by transient transfection with Cre-recombinase (Fig. 2.1A). ES cells transfected with the mutant allele were injected into 129/Sv blastocysts which were then implanted in the uterus of C57BL/6 foster mothers. Male chimeras were crossed with C57BL/6 females to assess germline transmission of the mutant allele.

In the present studies, mutants and control wildtype (WT) littermates were obtained in the 129/Sv/C57BL/6F3,4,5 background by intercrosses of heterozygous

mice. WT mice used in subsequent experiments (described in chapter IV) were obtained by intercrosses of non-siblings WT offspring of the F4 generation.

### 2.1.3. GENOTYPING

The genotype was determined by polymerase chain reaction (PCR) amplification of DNA obtained from tail biopsies performed at post-natal day 21 (day of weaning). 5 mm of mouse tail were incubated overnight (ON) at 55°C in lysis buffer [100mM Tris-HCl (pH 8.3), 5mM ethylenediaminetetraacetic acid (EDTA), 0.2% Sodium dodecyl sulfate (SDS), 200 mM NaCl and 0.1mg/ml proteinase K]. DNA was extracted from the supernatant by isopropanol precipitation, washed with 70% ethanol, re-suspended in 100 μl of double distilled water and incubated ON at 55°C. 1μl of a ten-fold diluted DNA sample was used for subsequent PCR amplification. Reactions were performed in a final volume of 25μl containing 0.5 units of Taq DNA polymerase (Invitrogen, Paisley, UK), 1XPCR reaction buffer and 1.5 mM $MgCl_2$ (supplied with the enzyme), 50μM 2´-deoxynucleoside 5´-triphosphates (dNTP) and 0.2 pmol of each primer. Amplification conditions were the following: 93°C for 2 min, 35 cycles (30s at 93°C, 30s at 56°C and 45s at 72°C) and 72°C for 10 min.

A 347 base pair (bp) wildtype fragment was generated with primers (Invitrogen):

KKBETA1: 5'- CAGCACTCAG CTCCAATCAA -3'

KKBETA2: 5'- GCCACCTATTGCCTTGTTTG -3'; - spanning intron 4

and a 470 bp mutant fragment with primers KKBETA1 and

KKBETA3: 5'- TAAGCACAAGCACTCATTCC -3' (Fig. 2.1).

PCR products were loaded in 1.2% agarose gels in 1XTAE (0.04M Tris-Acetate; 0.001M EDTA) stained with 0.5 mg/ml of ethidium bromide.

All mice used for behavioural experiments were re-genotyped after sacrifice.

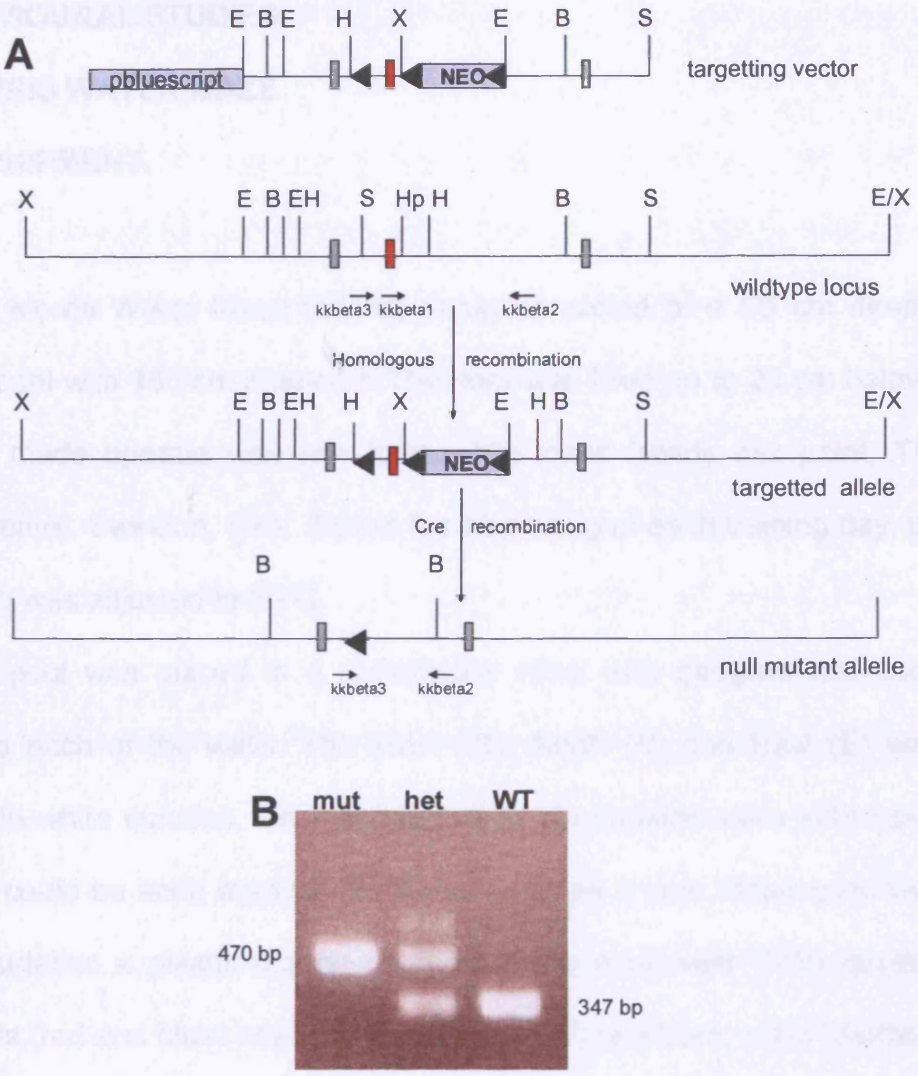


**Figure 2.1- Gene targeting strategy and genotyping reactions.** (A) Gene targeting strategy: exon 5 of the CamKK2 gene encoding the CaMKKβ protein was flanked by a loxP site and a floxed NEO, deletion of the targeted exon and the NEO cassette was achieved by transient transfection with Cre-recombinase [B-BamHI; E-EcoRI; H-HindIII; Hp-HpaI; S-SphI; adapted from Peters et al. (2003)]. Position of the primers used for genotyping is illustrated (B) PCR products of genotyping reactions (WT-wildtype band; mut- mutant band; het-heterozygote).

## 2.2. BEHAVIOURAL STUDIES

### 2.2.1. MORRIS WATER MAZE

#### 2.2.1.1. EQUIPMENT

The Morris Water Maze (MWM) setup consisted of a 50 cm deep circular swimming pool with 150 cm diameter. The pool was filled up to 20 cm below the rim with water made opaque with non toxic white paint (ready mix paint, The Early Learning Centre, Swindon, UK). Before the beginning of each training day, the water temperature was adjusted to 26°C.

The pool was placed in a rectangular room with geographical coordinates assigned to each of the walls. The West (W), North (N) and East (E) walls were covered with white curtains. On the South side (S) curtains were kept open and a violet door could be seen from the pool and used as a cue. Other cues were hung from the curtains: a plastic orange chair near the southwest (SW) quadrant, two football balls (red and blue) near the northwest (NW) quadrant, a flat "dartboard" cue placed near the northeast (NE) quadrant and a three dimensional cardboard box near the E coordinate. A video camera was placed in the ceiling of the room in the direction of the centre of the pool. The experimenter remained seated near the NE quadrant below the pool level throughout the trials, in order to prevent being used as a visible cue. All experiments were carried out under dim light provided by four upward projecting white 40 watt lamps placed in each corner of the room.

A circular platform (10 cm diameter) was submerged 1 cm below the water level, 30 cm in a straight line apart from the Southwest (SW) quadrant mark and remained in the same position throughout all training trials.

### 2.2.1.2. BEHAVIOURAL TRAINING PROCEDURE

The hidden platform version of the Morris Water Maze was previously shown to be hippocampus-dependent in this setup (Angelo et al., 2003). All experiments were performed in the afternoon, during the light phase of the cycle and all animals were naïve. The experimenter was blind to genotype.

**Handling**

Handling prior to training in the Morris Water Maze is thought to contribute to reduce fearful reactions to the experimenter and anxiety levels, which could confound the behavioural output (Need and Giese, 2003; Gerlai and Clayton, 1999). Handling consisted of holding each mouse in the experimenter's hand or lower arm for around 2 min during the same period of the day at which they were to be trained. This procedure was carried out over 10 days before the beginning of training.

**Habituation**

On each day, mice were moved in their home cages to the training room and allowed to habituate for one hour under the same light conditions in which they were to be trained and tested. Cages were placed below the pool, so that the mice could not see the pool or cues in the room.

**Pre-training**

Pre-training consisted of placing each animal on the platform (in the SW quadrant) for 30 s. The animal was then retrieved from the platform, released from the NE coordinate, and allowed to swim freely for approximately 30 s. It was then

given three practice climbs onto the platform from different angles and allowed to remain there for 30 s. If the animal jumped off the platform during these periods it was placed back in order to ensure a minimum of 2 min in total spent on the platform during the pre-training session.

**Training Trials**

Each training session consisted of a block of four trials per day. Before the beginning of each block of trials, the animals were placed on the platform for 60 s. They were then retrieved from the platform, placed in the start positions (N, S, E or W), and released facing the wall of the pool. Latency times (time spent to find the platform) were recorded for each trial. If the animals failed to find and climb onto the platform after 90 s of swimming, they were removed from the pool, placed in the platform and latency times were scored as 91 s. The time spent on the platform between trials (inter-trial interval) was 60 s. The order of start positions was pseudorandom, remaining constant for all mice on the same training day, but varying on different days.

The animals were guided to the different start positions and/or platform always using the shortest path possible and keeping the animal's eyes covered to prevent them from seeing the cues in the room.

**Probe Trial**

Because improvement in acquisition is not an accurate measure to assess hippocampus-dependent spatial memory formation, after completion of training, mice were tested for spatial preference in searching behaviour. This probe trial consisted of placing the animal on the platform for 60 s, removing the platform from the pool

and allowing the animal to swim freely for 90 s, having the NE coordinate of the pool as the starting position, as this is the most distant coordinate from the previous platform location.

Behavioural data were imaged by HVS water program (HVS image LTD, Hampton, UK)

Mice subsequently used for gene expression studies were sacrificed thirty minutes after the end of the probe trial.

### 2.2.1.3. DATA ANALYSIS

The following parameters were analysed: latency, selectivity towards TQ in the probe trial, number of platform crossings and cumulative proximity to the platform.

#### Latency

The time spent to find the platform was averaged for each training day, per group of mice. The maximum score per trial was 91 s corresponding to the trials when the mice failed to find the platform.

Acquisition curves were plotted as the average latency score per block of trials against the training day, per group of mice. Acquisition data were analysed by two-way ANOVA with repeated measures on subjects with training and genotype or training and sex as variables.

#### Probe Trial

For analysis of the probe trial data the pool was divided into four quadrants, Target Quadrant (TQ=SW), Adjacent Left (AL=SE), Adjacent Right (AR=NW) and Opposite (OP=NE).

**Percentage of Search Time Spent in Quadrant**

The relative percentage of time spent swimming in each quadrant was plotted as a bar graph.

**Number of Platform Crossings**

The number of times that the mice swam directly over the location where the platform was positioned previously was counted by the software and compared to the number of times the animals swam over equivalent "platform positions" in the three remaining quadrants.

**Gallagher Cumulative Proximity Measure**

The Gallagher cumulative proximity measure computed by HVS software is a measure modified from the Gallagher learning index (Gallagher et al., 1993). Distances between the centre of the mouse and the centre of the platform in TQ or equivalent platform positions were computed every 0.1 s (sample point). The total sum of these distances during the course of a trial corresponds to the Gallagher cumulative proximity measure. Scores obtained for this measure reflect search errors i.e., deviations from an optimal search or optimal path to the goal. Animals with an accurate search strategy swim at significantly lower cumulative distance from the platform position in TQ than from any other platform positions.

**Exclusion Criteria**

Floating and thigmotaxis are measures of anxiety which can affect performance of the task. For this reason, animals were excluded when they floated (swam at less than 5 cm/s) in more than 75% of the trials for more than 20 s, or if

they spent more than 75% of their search time in the thigmotaxis zone (outer 0.9% of the pool) in more than 85% of the trials (Need and Giese, 2003).

**Statistical Analysis**

Data were statistically analysed with Microsoft Excel or Sigmastat (SYSTAT software, SSPS Science Inc., Chicago, IL). Comparison of target quadrant preference between the groups was performed by one-way ANOVA with group as variable. Comparison of quadrant preference within each group was performed by one-way ANOVA with quadrant as variable.

### 2.2.2. CONTEXTUAL FEAR CONDITIONING

#### 2.2.2.1. EQUIPMENT

The conditioning chamber consisted of a 27.5 cm wide, 12.5 cm deep and 14.0 cm high box (Fig. 2.2; Campden Instruments, Loughborough, UK). Lateral sides and ceiling were made of aluminium and the front (door) and back of the chamber were made of transparent plexiglass. The chamber had a metal grid floor connected to a constant voltage generator (521/C, Campden Instruments, Loughborough, UK) through a shock scrambler (521/S, Campden Instruments, Loughborough, UK). The chamber was illuminated by a total of four 24 V white lights, two placed on each lateral side and two on the ceiling, which remained switched on throughout the whole training and testing procedures. A 2.8 KHz tone with an intensity of 80 dB was delivered by a speaker placed in the centre of the ceiling. The chamber was scented with 70% ethanol sprayed onto two sheets of paper, placed in a tray below the grid floor. The conditioning chamber was placed inside a sound attenuating chamber.

The conditioning room was kept under dim light (only light emmited by the computer screen) and training and testing procedures were carried out after 2 p.m. except for the "Latent Inhibition" and "Overnight Exposure" groups which were carried overnight (for approximately 14 h) with the shock being delivered in the morning.

### 2.2.2. BEHAVIOURAL TRAINING PROCEDURE

Learning of the task was shown to be hippocampus and protein synthesis dependent in this setup (von Hertzen and Giese, 2005).

#### Habituation

Unhandled naïve animals were transported from the animal rooms in their home cages and placed in the lobby of the fear conditioning room under dim light to habituate for one hour.

#### Training

Immediately before training the animals were transported individually in a cage to the training chamber and placed inside it with the lights off. The mouse was allowed to freely explore the chamber for 2 min. At the end of 2 min a 2.8 KHz tone with 80 dB of intensity (conditioned stimulus-CS) was played for 30 s paired with a 0.75 mA foot shock during the last 2 s of the tone (unconditioned stimulus-US). The animal remained in the chamber for another 30 s after which lights were switched off. The animal was immediately removed from the chamber and returned to the home cage. The chamber was washed with water and 70% ethanol between training of different animals.

**Novelty controls**

Novelty controls were exposed to the conditioning chamber under the same conditions as trained animals except for not receiving the foot shock.

**Latent Inhibition controls**

Latent inhibition controls were housed in the training chamber bedded with tissue paper for approximately 16 h, with food and water *ad libitum.* 1 h before the foot shock was delivered, the bedding and most of the food pellets were removed in order to ensure that the mouse would receive the shock. A subgroup of mice was exposed to the tone (ON exp) and another subgroup was trained without the tone (LI).

**Testing**

Animals were tested for contextual memory, by re-exposure to the conditioning chamber in the absence of the tone and foot shock, 24 hours after training. Freezing was used as the behavioural response to assess memory. The animal was considered to be freezing if it completely refrained from any kind of movement except for respiration (Ehninger et al., 2005). Freezing was sampled every 5 s for 2 s during 5 min and averaged for the 5 min period. Freezing scores were compared between the experimental groups by one-way ANOVA with group as variable, and *post-hoc* Student-Newman-Keuls tests were used when significance was found.

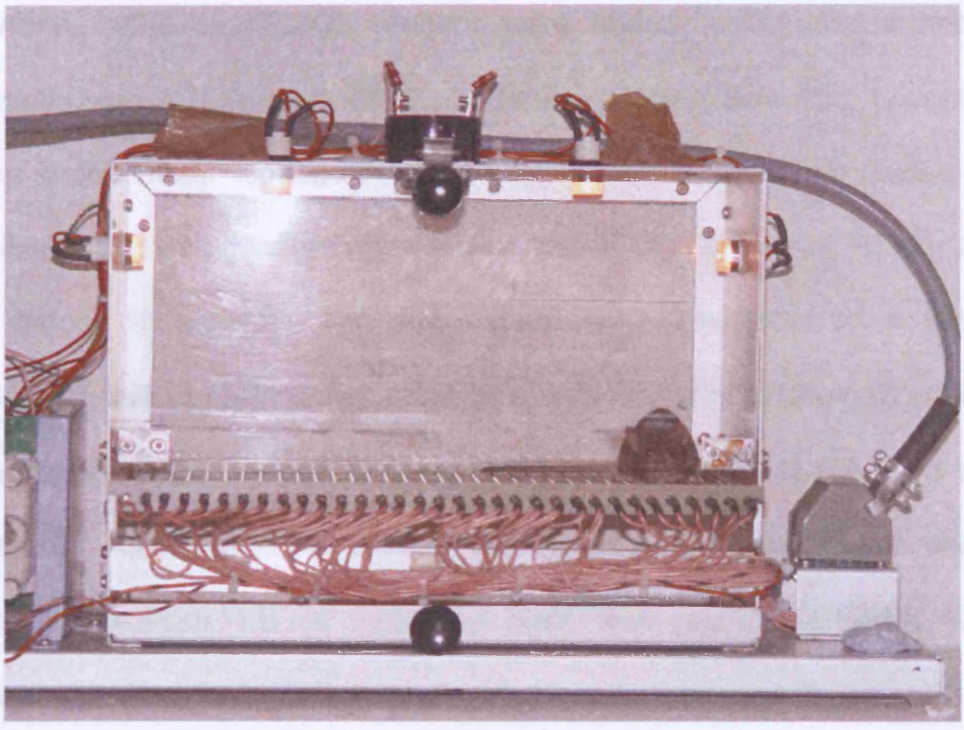

**Figure 2.2-Contextual fear conditioning equipment.**

## 2.3. MOLECULAR BIOLOGY

### 2.3.1. HIPPOCAMPAL DISSECTIONS

Mice were anaesthetized in a $CO_2$ chamber and killed by cervical dislocation. Brains were removed and placed in a sterile RNAse free dish and hippocampi were dissected from the dorsal aspect under a low magnification microscope. Hippocampal tissue was placed in an RNAse and sterile 1.5ml eppendorf tube, immediately frozen on dry ice and kept at -80°C.

### 2.3.2. RNA EXTRACTION AND QUALITY ANALYSIS

#### 2.3.2.1. RNA EXTRACTION PROTOCOL

Frozen hippocampal tissue was homogenised in TRIZOL reagent (Invitrogen Life Technologies) according to manufacturer's instructions.

Briefly, 800μl of TRIZOL reagent were added to the tissue and it was homogenised with a Powergen 125 homogenizer (Fisher Scientific, Loughborough, UK) at an approximate speed of 20,000 rpm for 20 s or until no pieces of tissue were visible. The homogenates were centrifuged at 11,000 rpm for 10 min at 4°C, to remove excess fat tissue. The supernatant was transferred to a fresh tube, incubated for 5 min at room temperature (RT) and 160 μl of chloroform were added. Tubes were vigorously shaken by hand for 15 s, incubated at RT for 3 min, and centrifuged at 13,000 rpm for 30 min at 4°C. The aqueous upper phase was transferred to a fresh 1.5 ml tube and RNA was precipitated with 400 μl of isopropanol. After incubation at RT for 10 min the tube was centrifuged at 13,000 rpm for 10 min at 4°C. Supernatant was removed and the pellet washed with 75% ethanol, vortexed and centrifuged at 10,000rpm for 5 min at 4°C. Ethanol was removed, and the pellet allowed to dry until it became transparent, when it was re-suspended in 100 μl RNAse free water and stored at -80°C. At this stage, a 10 μl aliquot was collected at this stage for subsequent analysis of RNA integrity. RNA was purified with the QIAGEN RNeasy Minikit (QIAGEN, West Sussex, UK) following the manufacturer's instructions. RNA was re-suspended in a final volume of 50 μl and a 5 μl aliquot collected for analysis of RNA integrity.

### 2.3.2.2. RNA INTEGRITY AND QUALITY

#### RNA integrity

RNA integrity was assessed using the Agilent 2100 Bioanalyzer (Agilent technologies, Palo Alto, CA, USA). To rule out the possibility of RNA degradation, the

ratio between 28S/18S ribosomal RNA present in the sample was estimated. Only samples with a ratio higher than 1.4 were processed further.

Whenever the use of the bionalyzer was not possible, RNA integrity was analysed by loading the sample in 1.2% agarose gel in 1XTAE containing 0.5 mg/ml ethidium bromide (Fig. 2.3). The 28S/18S ratio was estimated by comparing optical densities of the respective bands in the gel. Only samples where this ratio was higher than 1.4 were processed further.

**RNA Quality**

To rule out protein contamination of the samples the ratio of optical absorbances between nucleic acids (260 nm) and proteins (280 nm) were determined. Samples, in which this ratio was out of the 1.9-2.0 range were discarded.

Contamination with genomic DNA was ruled out by PCR amplification of cDNA with primers spanning two exons of the hypoxanthine-guanine-phosphoribosyltransferase (HPRT) gene and is described in section 2.3.4.1.

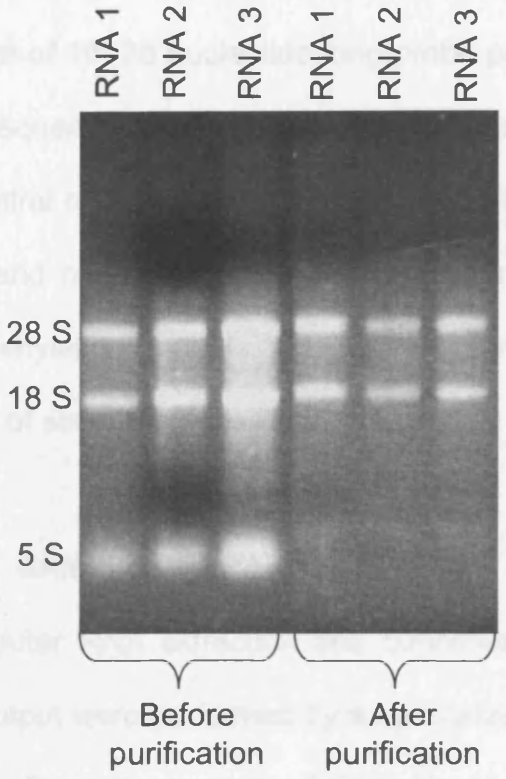


**Figure 2.3- Agarose gel of total RNA extracted from hippocampal tissue, before and after purification with the QIAGEN RNeasy Minikit.**

**RNA quantification**

RNA was quantified using the Agilent 2100 Bioanalyzer (for the samples used in the microarray analysis), or by spectrophotometric measurement of the absorbance at 260 nm (for the samples used in the QPCR reactions).

### 2.3.3. *Affymetrix* MICROARRAY SCREENING

The Genechip® Murine Genome array U74Av2 (MG-U74Av2; *Affymetrix,* Santa Clara, CA) represents 12,000 transcript sequences. Approximately half of these sequences correspond to functionally characterized genes and the other half correspond to uncharacterized expression sequence tags (EST) derived from the

Unigene database (Build 74, April 2001). Each gene or EST in the array is probed by a set of 16, 25 nucleotide long probe pairs. Each pair comprises a perfect match (PM) sequence and a mismatched (MM) sequence differing from the first one only on the central nucleotide. Subtraction of MM from PM signals accounts for background noise and non-specific interactions. Each chip also includes hybridization controls polyAdenylation controls, and probes for maintenance genes, that allow the quality control of sample processing.

### 2.3.3.1. SAMPLE PREPARATION

After RNA extraction and quantification, all subsequent steps until the final data output were performed by a specialized technician in the *Affymetrix Microarray* core facility at the Institute of Child Health, University College London who followed the instructions of the *GeneChip Expression Analysis Technical Manual (Affymetrix)*.

Briefly, 10 μg of purified RNA (from hippocampal tissue of a single animal) were primed with T7 oligo$(dT)_{24}$ for reverse transcription into single stranded cDNA. This was subsequently converted to double stranded cDNA (dsCDNA), which was *in vitro* transcribed into cRNA using T7 RNA polymerase, and biotin labelled ribonucleotides. Fragmented Biotinylated cRNA derived from one hippocampal sample were hybridized onto a U74Av2 GeneChip Array at 45°C for 16 hours. Hybridized chips were stained with streptavidin and phycoeritrin solutions and scanned for fluorescence.

Readings of "raw" fluorescence intensities for each probe set were computed by the *Microarray* analysis Suite (MAS) software. This primary output allowed a general quality control assessment of various aspects of sample preparation such as hybridization efficiency (provided by the percentage of probe sets called "present"

and the detection of pre-labelled hybridization controls included in the chip), and validation of *in vitro* transcription (provided by the signal ratio between 5' and 3' directed probes for selected maintenance genes).

### 2.3.3.2. DATA ANALYSIS

Analysis of differences in hybridization intensities across chips from different groups permits a comparison of transcriptional profiles between distinct biological samples. The *Affymetrix* Data Mining Tool 3.0 (DMT, *Affymetrix*) was used for a preliminary analysis of the data. This software performed direct pairwise comparisons between signal intensities across arrays corresponding to the WT and mutant groups.

A number of factors both in the manufacture of the chips and in sample preparation may represent sources of variability and impose biases on signal intensities (Chudin et al., 2002). Normalization of fluorescence intensities is required to bring all arrays within an experiment to a similar overall brightness, and diminish bias generated by non-biological causes. Microarray data analysis softwares use distinct normalization and mathematical analysis procedures to compare transcriptional profiles. In the present study, in addition to the output from the DMT, two alternative softwares were used: dCHIP (Li and Wong, Harvard University, USA) and Genespring (*Silicon Genetics*; Agilent technologies, Palo Alto, CA, USA).

### 2.3.3.2.1. DChip NORMALIZATION PROCEDURES AND MODELS OF ANALYSIS

dCHIP uses the invariant set normalization method, which encompasses the following steps: (a) definition of the array with median overall brightness as a "baseline" array; (b) identification of an invariant set of probes (based only on signals arising from PM), corresponding to genes that are not differently expressed between groups; (c) calculation of a normalization curve defined by the median intensity values for each probe on the invariant set; (d) fitting of signal intensities of the variant probe sets to the normalization curve (Li and Hung, 2001). Chips with low hybridization intensities (present call percentage, lower than 30%) are treated as outliers and excluded from the analysis. All eight arrays hybridized in the present analysis had present call percentages above 30%, and were used in the transcriptional analysis

Two models of comparative analysis were independently used: the PM-MM average difference intensity model (ADI) and the PM model-based expression index (MBEI) because they differ in sensitivity depending on the transcript level. The ADI presents the advantage of accounting for background noise and non-specific interactions by subtracting MM from PM signals (Chudin et al., 2002). This method discards probe sets with a PM-MM difference over three standard deviations higher than the average PM-MM difference across the array, carrying the risk of excluding probe sets for transcripts with true large responses (Li and Wong, 2001). For this reason, the MBEI model, which accounts for PM signals only, was also used in this analysis.

### 2.3.3.2.2. GENESPRING NORMALIZATION PROCEDURES AND MODELS OF ANALYSIS

GeneSpring uses a multiplicative normalization method starting with a *per Chip* normalization followed by a *per gene* normalization. The *per chip* normalization uses positive control genes, mRNA belonging to other genomes and housekeeping genes to normalize expression levels to the overall brightness of the Chip. The *per Chip* normalization has the advantage of accounting for chip-wide variations in intensity due to imperfections in the processing of the sample; however this procedure can mask high hybridization levels arising from true high transcriptional levels.

The per chip normalization was followed by a *per gene* normalization which adjusted the levels of expression of each gene to the median of the expression of the same gene throughout the experiment. Transcription level comparison started by filtering out genes having mean normalized expression levels that did not vary between the groups.

The *per chip* normalization has the advantage of accounting for chip-wide variations in intensity due to imperfections in the processing of the sample, however this procedure can mask high hybridization levels arising from true high transcriptional levels.

### 2.3.3.2.3. COMPARISON CRITERIA

Standard ANOVA and t-tests for Microarray data analysis, were used with dCHIP and GeneSpring softwares. CaMKKβ mutants and WT mice were defined as

experimental and baseline groups respectively. Genes with a detected transcriptional change higher than 30% with a significance level of 95% were further analyzed.

### 2.3.4. QUANTITATIVE REAL TIME PCR

The quantitative real-time PCR (QPCR) technique allows relative quantification of transcripts from reverse transcribed and amplified cDNA. The QPCR master mix includes a fluorescent dye which binds to double stranded DNA, and is incorporated into products as the amplification proceeds. Amplification reactions were performed in 96 well plates placed in a thermal cycler and fluorescence detector (ABI Prism 7700, Applied Biosystems, Warrington, UK). Fluorescence data were monitored as the PCR products were generated. The starting copy number of the product being monitored is considered to be proportional to the cycle at which the product is first detected. The sooner a significant increase in fluorescence is observed, the more abundant the transcript is in the original sample (Bustin, 2000).

#### 2.3.4.1. cDNA REVERSE TRANSCRIPTION FROM HIPPOCAMPAL mRNA

An estimated amount of 2.5 μg of total RNA was used for cDNA synthesis primed with 25 ng oligo$(dT)_{24}$ (Invitrogen) and incubated at 70°C. The cDNA synthesis reaction mix contained 1 X first strand buffer, 10 mM dithiotreitol (DTT), 10 nM dNTP, and 20 U of RNaseOUT™ (Recombinant Ribonuclease Inhibitor; Invitrogen). RNAse out (Invitrogen) and 200 U of Superscript™ IIRT enzyme (Invitrogen). cDNA synthesis reaction was performed for 50 min at 42°C, after which the enzyme was inactivated at 70°C for 10 min.

To rule out the possibility of contamination with genomic DNA, cDNA was amplified for 35 cycles (30s at 93°C, 30s at 59°C and 1 min at 72°C) using primers for HPRT. Primer sequences were (Invitrogen):

H1:5'-GCTGGTGAAAAGGACCTCT-3';

H2:5'-CACAGGACTAGAACACCTGC-3'

As this primers span contiguous exons, a 249 bp fragment is generated from cDNA and a 1100 bp fragment is generated from genomic DNA (Fig. 2.4).

Each cDNA tested aliquot was diluted tenfold for subsequent QPCR.

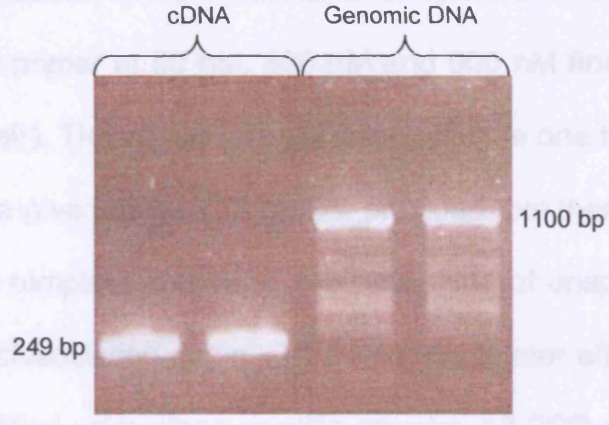


**Figure 2.4- Amplification of cDNA and genomic DNA with primers for HPRT.**

### 2.3.4.2. PRIMER DESIGN FOR QPCR

mRNA sequences for the transcripts detected in the microarray analysis were downloaded from the ensemble database (http://www.ensembl.org; Build 33). Specific primers were designed using *Primer Express Software* (Applied Biosystems) and chosen according to either of the following criteria: (a) Spanning exon/exon

boundaries in order to exclude the possibility of amplification of traces of genomic DNA; (b) spanning the 3'UTR of the transcript to assure ability to capture shorter cDNAS.

Primer sequences are listed in appendix I.

### 2.3.4.3. OPTMIZATION OF PRIMER CONCENTRATIONS

Different combinations of concentrations of forward and reverse primers were tested, using either primer at 50 nM, 300 nM and 900 nM final concentrations (ie. 6 permutations per pair). The combination chosen was the one that yielded the earliest threshold cycle for a given amount of cDNA, provided that there was no primer dimer formation in the no template control or any other kind of unspecific amplification, as determined by the dissociation curve. To determine primer efficiency, serial dilutions of cDNA were amplified using gene-specific primers. All PCR products exhibited high linearity amplification plots ($r^2$>0.98) and the slope of the standard curves was approximately -3.2, which is indicative of a two fold amplification per cycle (efficiency close to 1, according to *SYBR Green PCR Master Mix and qPCR protocol booklet*).

Primer concentrations were selected under the following criteria: (a) earlier cycle of detection for a fixed fluorescence threshold; (b) no primer dimmers detected in the dissociation curve of the melted final PCR products.

### 2.3.4.4. CHOICE OF INTERNAL CONTROLS

Internal control transcripts HPRT (primer concentrations 300nM forward primer/300nM reverse primer and 900 nM forward primer/ 900nM reverse primer) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; primer concentrations 900 nM forward primer / 900 nM reverse primer) were tested for each gene. Validation of internal controls was performed by plotting differences in cycle threshold between pairs of genes ($\Delta$Ct) vs. log RNA concentrations in arbitrary units. The internal control chosen corresponded to the trendline displaying a slope as close to zero as possible (<0.1) for a 1 to 1/16 fold range of concentrations. Whenever distinct internal controls were valid for the same PCR product, the one displaying a smaller cycle threshold difference was chosen as this is indicative of a similar abundance between the gene tested and the internal control.

### 2.3.4.5. AMPLIFICATION REACTIONS

Amplification reactions contained 1XSYBR green PCR Master Mix [SYBR Green I Dye, AmpliTaq Gold DNA polymerase, dNTPs with dUTP, passive reference and optimized buffer components; Applied Biosystems], optimized primer concentrations (appendix 1) and 1 $\mu$l of cDNA (approximately 2.5 ng) in a final volume of 25 $\mu$l. Each sample was run in triplicates both for the gene and the internal control, and a no template control reaction was performed also in triplicate per reaction mix used. Micro-Amp 96-well plates were used (capped with an ABI prism optical adhesion cover and run on ABI PRISM 7000/7700 Sequence detection system (Applied Biosystems). The qPCR conditions were 95$^{\circ}$C for 10 min followed by 40 cycles of 95$^{\circ}$C for 15sec and 62$^{\circ}$C for 1min. Dissociation curves were generated after

amplification in order to monitor the purity of the product formed, and the discard the possibility of contamination.

PCR product levels were monitored by measuring the increase in fluorescence with the ABI PRISM 7000/7700 Sequence detection system (Applied Biosystems).

### 2.3.4.6. DATA ANALYSIS

The quantification procedure followed the following steps: (a) a constant fluorescence threshold was defined; (b) the cycles of detection between replicates were averaged; (c) the average cycle threshold for the internal control was subtractedl from the average cycle threshold for the target transcript (ΔCt); (d) The ΔCt for each experimental group was averaged (at this step samples with a ΔCt deviating more than two standard deviations from the average of the group were excluded); (e) The average cycle threshold difference for the calibrator group (ΔCt cal) corresponding to naïve male mice, unless otherwise stated, was calculated; (f) ΔCt cal was subtracted from each ΔCt to obtain a (ΔΔCt) in order to normalize cycle threshold differences to the average ΔCt of the calibrator group; (g) The ΔΔCt values were subjected to logarithmic transformation to obtain a percentage of expression; (h) The relative percentage of expression were compared between the groups using one-way ANOVA.

For the experiments described in chapter III, each cDNA sample was loaded in triplicates for both the target transcript and internal control, the cycle threshold was averaged per triplicate and whenever one of the triplicates differed in more than 0.4 cycles from the average of the other two it was excluded from the analysis. For the experiments described in chapter IV no replicates were used in each plate,

expression values were normalized in each plate and these were averaged for 3 to 5 plates.

#### 2.3.4.7. PRODUCT SPECIFICITY AND PURITY

After confirming from the dissociation curve that each well presented a single PCR products were cloned into pCR2.1 TOPO vector (Invitrogen), used to transform XL10 competent cells and plated in LB agar containing 100 μg/ml ampicillin and grown ON at 37°C. White colonies were inoculated in liquid LB containing 100 μg/ml ampicillin and grown ON at 37°C. Plasmid DNA was isolated using QIAprep Spin Miniprep Kit (QIAGEN) according to manufacturer's instructions. PCR products were digested with EcoRI (supplier) and the digestion products loaded in 1.2% agarose gels in TAE. DNA from clones containing the insert of the expected size were sequenced by the WIBR sequencing service using vector-specific primers (M13 fwd and M13 rev, Invitrogen). The sequences obtained were submitted to Basic Local Alignment Search Tool (BLAST) from the National Centre for Biotechnology Information (NCBI) and identified.

### 2.4. *IN SITU* HYBRIDIZATIONS

#### 2.4.1. TISSUE PREPARATION

Mice were anaesthetized under $CO_2$ and killed by cervical dislocation and brains scooped out from the ventral part of the skull and frozen in -20 to -30°C isopentane (Merck, Harlow, UK), placed in a dry-ice chilled 15 ml falcon tube and stored at -80°C.

**Sectioning**

Coronal sections (15 μm thick) were cut in a cryostat at -40°C and thaw-mounted onto polilysine coated superfrost slides (Invitrogen). Slides were fixed in freshly prepared and filtered ice cold 4% parafromaldeheyde (PFA) for 5 min, rinsed in phosphate buffered saline (PBS) for 1 min and 70% ethanol for 5 min and stored in 95% ethanol at 4°C.

### 2.4.2. PROBE DESIGN

Antisense DNA probes complementary to the transcripts were designed to span exon/exon boundaries having around 50 nucleotides length (nt) and a 50% GC content. Probe sequences for each transcript are listed in appendix II.

### 2.4.3. IN SITU HYBRIDIZATION PROTOCOL

#### 2.4.3.1. PROBE LABELLING

Probe oligonucleotides were diluted to a final concentration of 5 ng/μl. 10 ng of oligo were labelled with 500 nM [$\alpha$-$^{35}$S] dATP (1000 Ci/mmol; Amersham Biosciences, Bucks, UK) in a reaction containing 1 X terminal transferase buffer and 22.5 U terminal deoxynucleotidil transferase (TdT). Reactions were performed at 37°C for 30 to 45 min, and stopped by adding 40μl of 10 mM Tris-EDTA buffer.

To remove unincorporated radiolabelled nucleotides, the reaction mix was purified through a sephadex column, centrifuged for 1 min at 2,000 rpm. 2 μl of flow through were diluted in scintillation fluid and the counted in a scintillation counter. 1 μl of 1M

dithiotreitol (DTT) were added to the reaction. Labelled probes were kept on ice until being used.

### 2.4.3.2. HYBRIDIZATION

In situ hybridization buffer [50% formamide, 4XSSC (0.15M Sodium Chloride and 0.015M Sodium citrate), pH 7.0, 25 mM Sodium phosphate pH 7.0; 1 mM sodium pyrophosphate; 5X Denhardt's solution (0.2 g/L Ficoll; 0.2g/L polyvinylpyrrolidine; 0.2 g/L bovine serum albumin (BSA), herring sperm DNA; 0.1mg/ml polyadenlylic acid and 0.1g/ml dextran sulphate] was previously prepared filtered and stored at -20°C. Slides were removed from 95% ethanol and allowed to air-dry for 30 min. 100,000 to 300,000 cpm of labelled probe and 2 μl of DTT were diluted per 100 μl of hybridization buffer. An unspecific labelling mix was set up by adding 10 ng of the unlabelled probe to the above mix. 60 μl of hybridization buffer were applied to a glass coverslip, and the slides were gently pushed against the coverslip and placed in the hybridization chamber. The hybridization chamber was kept moist by covering the chamber edges with pieces of Wattman paper embedded in "chamber buffer" (50%formamide, 4XSSC) and sealed with NESCO film (Fisons Scientific Apparatus, Loughborough, UK) to prevent evaporation. Hybridization was performed ON at 42°C.

### 2.4.3.3. WASHING

Slides were washed in 1X SSC for 10 min at RT, 2 times in 1XSSC at 55°C , and briefly rinsed in 0.1XSSC, 70% ethanol, 95% ethanol at RT and allowed to air dry for 1h.

### 2.4.3.4. EXPOSURE

Slides were exposed to a $^{35}S$ sensitive autoradiographic film (KodaK, Biomax) together with $^{14}C$ microscale standards (Amersham Sciences, Bucks, United Kingdom). Exposures were performed at RT and times of exposure varied according to the transcript being probed as indicated appendix II.

### 2.4.4. IMAGE ANALYSIS

The autoradiograph of every brain section was imaged with a monochrome camera. Image densities were calibreted with reference to the $^{14}C$ microscale standards (nCi/g tissue equivalent). Two to three animals were used in each condition and, for each animal, the measurements obtained represented the average of measurements taken from 8 to12 different sections.

Image analysis was performed using MCID $M5^{+}$ analysis software (Imaging Research, St Catherine's, Ontario, Canada). Two alternative measurements were used: densitometry and "estimated counts above threshold".

**Densitometry**

Densitometry readings were sampled with a single 1 pixel-wide ribbon covering the central longitudinal area of each hippocampal subfield of interest. These measurements are proportional to the average amount of transcript expressed per cell within the hippocampal subfield sampled.

**Estimated counts above threshold**

When comparing levels of expression between different conditions, an overall density measurement per hippocampal subfield may dilute true differences restricted

to a small number of cells. To circumvent this problem, an alternative method was used: First, a line limitating the edges of each hippocampal subfield was drawn for each section and the average density for each subfield in the control condition was defined as the threshold density. The number of pixels, with a density above threshold levels were measured as " estimated counts above threshold". This analysis provides a measurement proportional to the number of cells that express a number of copies of the transcript above a pre-determined level.

Densities and "estimated counts above threshold" within each hippocampal subfield for each condition were averaged for all the sections considered and are presented in bar graphs. Data were not statiscally analysed, as the number of animals (2-3 per condition) was very low.

# CHAPTER III:
# RESULTS I

## 3.1. INTRODUCTION

Memory consolidation is a time dependent process requiring *de novo* transcription (Igaz et al., 2002), engaging activation of transcription factors such as CREB (Bourtchuladze et al., 1994). One of the signalling transduction pathways involved in CREB activation is the CaM Kinase cascade (Takemoto-Kimura et al., 2003; Soderling, 1999; Bito et al., 1996). Known members of this cascade are CaMKI and CaMKIV and two upstream activating kinases CaMKKα and CaMKKβ (Vinet et al., 2003; Hsu et al., 2001; Soderling, 1999; Anderson et al., 1998).

Evidence from mutant mouse models points to a direct engagement of components of this cascade in synaptic plasticity processes and memory formation (Peters et al., 2003; Wei et al., 2002; Kang et al., 2001; Ho et al., 2000).

Peters et al. (2003) described an impairment in hippocampus-dependent spatial memory in the MWM in male mice carrying a global deletion of CaMKKβ. This behavioural phenotype was accompanied by a deficit in spatial training induced CREB activation in the hippocampus - a molecular correlate of spatial memory formation (Mizuno et al., 2002); and an impairment in the transcription dependent phase of LTP - a cellular model of long-term memory formation (Nguyen et al., 1994). Remarkably, lack of CaMKKβ did not affect spatial memory in the MWM, CREB activation or L-LTP in female mice (Mizuno et al., 2006; K. Mizuno and K.P. Giese, unpublished). These data suggest a male specific requirement for this kinase in the mechanisms engaged in the consolidation of spatial memories. Thus, the CaMKKβ null mutant line represents a tool for the identification of plasticity regulated genes, and potentially provides an insight into molecular dimorphisms in the processes underlying memory formation.

## 3.2. AIM

The observations on male CaMKK$\beta$ null mutant mice suggest that lack of CaMKK$\beta$ in male mice is responsible for a transcriptional deregulation in the hippocampus that may account for the behavioural, molecular and cellular phenotypes described.

The aims of the work described in this chapter were (a) to identify CaMKK$\beta$ regulated genes in the male hippocampus and (b) to investigate whether the same genes are also regulated by CaMKK$\beta$ in the female hippocampus.

## 3.3. EXPERIMENTAL APPROACH

In order to identify genes regulated by CaMKK$\beta$ after spatial training that may account for the phenotypes described by Peters et al. (2003), male CaMKK$\beta$ mutant and WT male mice were trained in the MWM in a 4 trials per day training protocol and tested in a probe trial at the end of the third day of training. Animals were sacrificed 30 mins after the probe trial, hippocampal tissue was dissected, mRNA extracted, reverse transcribed into cDNA, in vitro transcribed into cRNA and hybridized with high-density oligonucleotide array chips (*Affymetrix)*. The differences in transcriptional levels identified were further investigated and confirmed by quantitative real time PCR (QPCR; Fig. 3.1).

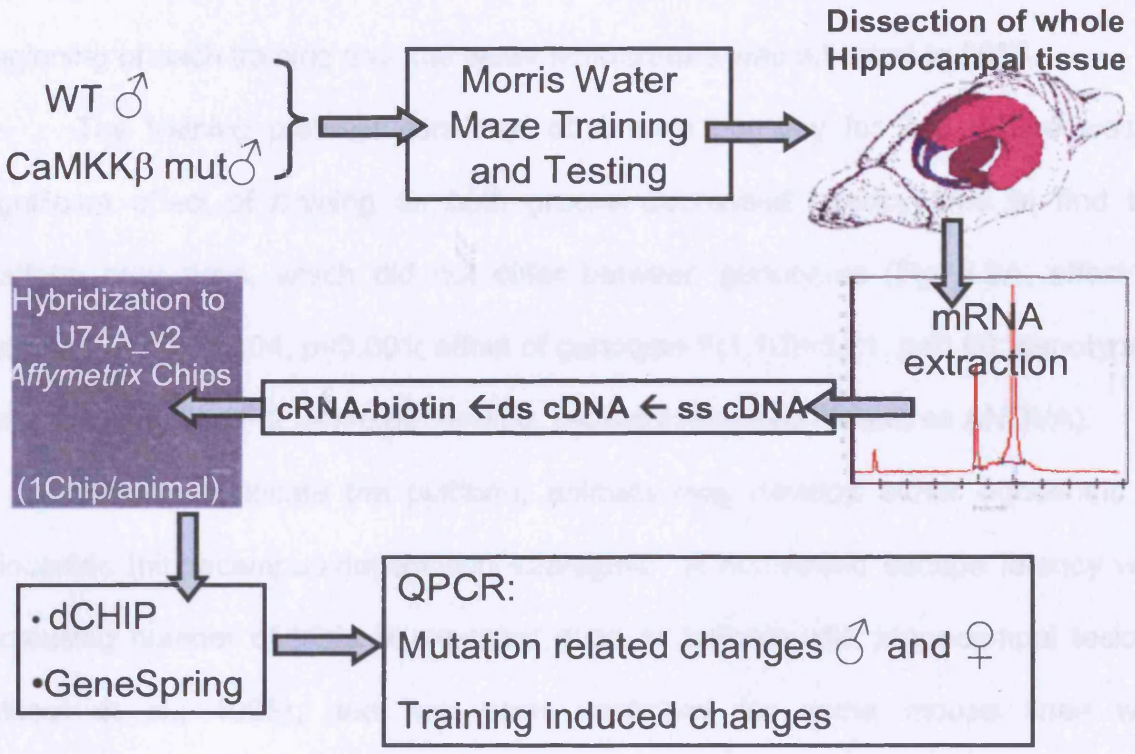


**Figure 3.1- Different steps in the experimental procedure used to identify CaMKK*β* regulated transcripts.** Widtype (WT) and CaMKK*β* null mutant (mut) male mice were trained and tested in the MWM, hippocampal tissue was dissected, mRNA extracted and processed into cRNA which was hybridized to *Affymetrix* Gene Chips to generate gene expression profiles. Results were analysed using two different softwares and confirmed with quantitative real time PCR (QPCR).

## 3.4. EXPERIMENTAL RESULTS

### 3.4.1. TRAINING OF MALE CaMKK*β* NULL MUTANT AND WT MICE IN THE MORRIS WATER MAZE

Naïve male CaMKK*β* null mutant (n=6) and WT littermates (n=6) between 10 and 16 weeks old in the C57BL/6/129/Sv F3, F4 and F5 backgrounds were trained in the hidden platform version of the Morris Water Maze, in a hippocampus

dependent set up (Angelo et al., 2003). All training trials were performed in the morning (2 hours after the beginning of the light phase of the cycle), and, at the beginning of each training day, the water temperature was adjusted to 26°C.

The training protocol consisted of 4 trials per day for 3 d. There was a significant effect of training as both groups decreased latency time to find the platform over days, which did not differ between genotypes (Fig 3.2A, effect of training $F(2,34)=12.04$, $p<0.001$; effect of genotype $F(1,10)=0.21$, $p=0.66$; genotype x training interaction $F(2,34)=0.62$, $p=0.55$, two-way repeated measures ANOVA).

In order to locate the platform, animals may develop either egocentric or allocentric (hippocampus-dependent) strategies. A decreased escape latency with increasing number of trials is expected even in animals with hippocampal lesions (Moser et al., 1995), and has been described for some mouse lines with hippocampus-dependent spatial memory impairments (eg. Peters et al., 2003). In order to assess the formation of a hippocampus-dependent spatial strategy, trained animals were tested in a probe trial during which the animals swim in the pool in the absence of the platform (D'Hooge and De Deyn, 2001; Morris, 1984). The use of a spatial strategy to solve the task is revealed by a preference for the previous platform location.

At the end of the third day of training, mice were tested in a 90 s probe trial, being released in the pool from the most distant coordinate from the location where the platform was previously placed.

During probe trials there was no difference in average swim speed between the groups (Fig. 3.2B, WT: 25.2 ± 0.7cm/s; mut: 25.2 ± 0.5 cm/s; $F(1,10)=0.00$ ; $p=1.00$, one-way ANOVA with genotype as variable). Furthermore, the percentage of time spent in the thigmotaxis zone, defined as an annulus of 10 cm near the rim of

the pool, did not differ between genotypes (Fig. 3.2C, WT: 2.6 ± 0.8%; mut: 3.0 ± 1.7%; F(1,10)=0.03; p=0.86, one-way ANOVA with genotype as variable) and was significantly lower than the 19% time expected if the animals swam randomly. The percentage of time spent swimming at a speed lower than 5 cm/s, considered as floating, was also similar between the two genotypes (Fig 3.2D, WT: 6.6 ± 2.0%; mut: 8.9 ± 1.9%; F(1,10)=0.69; p=0.43; one-way ANOVA with genotype as variable).

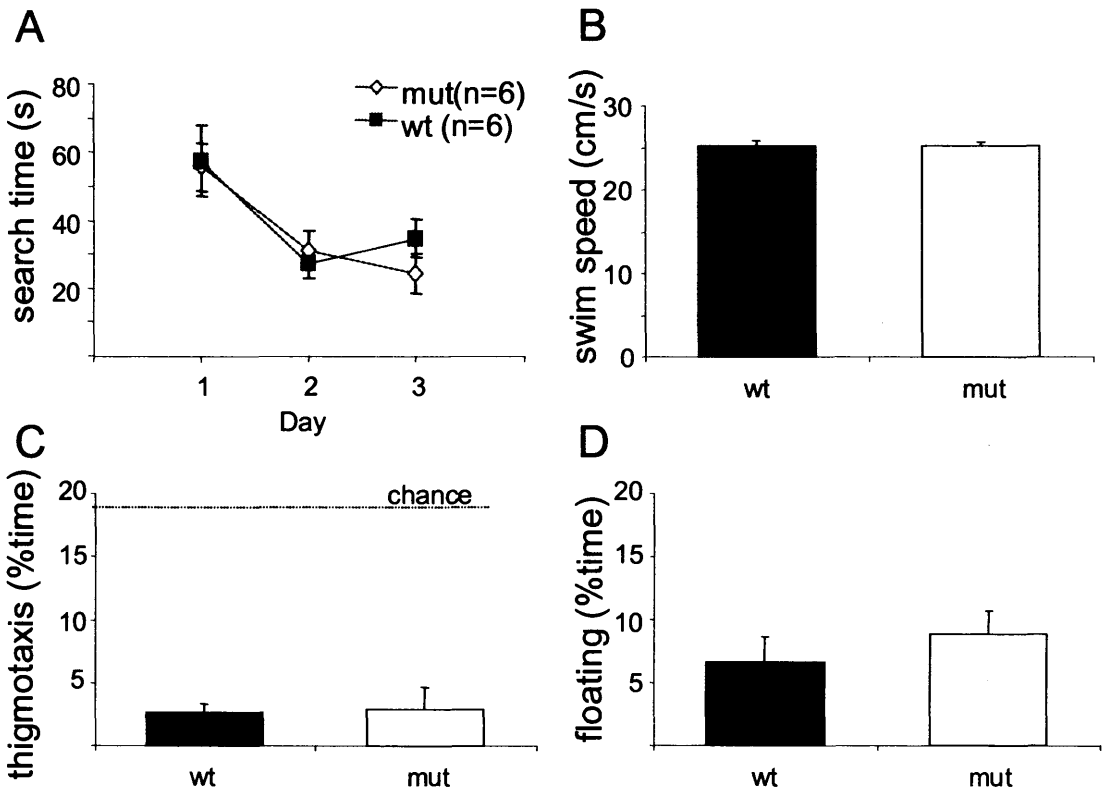


**Figure 3.2- Normal acquisition and swimming abilities in CaMKK$\beta$ male null mutant mice.**
**(A)** Both genotypes decreased their latency times to find the platform with the number of training trials; **(B)** During the probe trial, swim speeds did not differ between genotypes; **(C)** The percentage of time spent swimming in the thigmotaxis zone did not differ between genotypes and was lower than expected by chance. **(D)** The percentage of time spent floating did not differ between genotypes. (Mean±SEM)

In order to assess the spatial preference, the pool was artificially divided into four quadrants: the target quadrant (TQ), where the platform was previously located and adjacent right (AR), adjacent left (AL) and opposite (OP) quadrants relative to

TQ. Measurements included percentage of time spent in quadrants and platform crossings (corresponding to the number of times the animal swam directly over the location where the platform was placed previously and equivalent positions in the remaining three quadrants). Another measurement used was the Gallagher cumulative proximity measure modified from the Gallagher learning index (Gallagher et al., 1993). Distances between the centre of the mouse and the centre of the platform in TQ or equivalent platform positions were computed every 0.1 s (sample point). The total sum of these distances during the course of a trial corresponds to the Gallagher cumulative proximity measure. The lower the cumulative distance relative to TQ, the more accurate is the mouse's "knowledge of the platform position" (HVS Water 2020, instruction manual).

Animals were considered selective, if the percentage of time spent in TQ was higher than 35% and higher than the percentage of time spent in any other quadrant by at least 10%. Analysis of quadrant search times showed no significant difference between genotypes in percentage of time spent in TQ (Fig. 3.3A, WT: 34.5 ± 3.9%; mut: 38.3 ± 2.4%, $F(1,10)=0.72$; $p=0.42$, one-way ANOVA with genotype as variable). During the probe trial WT mice spent a significantly higher percentage of search time in TQ (Fig. 3.3A, $F(3,20)=3.46$, $p<0.05$; one-way ANOVA with quadrant as variable, $p<0.05$ for all TQ *post-hoc* Student-Newman-Keuls comparisons), and the same was true for the mutants (Fig. 3.3A, $F(3,20)=15.42$; $p<0.001$, one-way ANOVA with quadrant as variable, $p<0.01$ for all TQ *post-hoc* Student-Newman-Keuls comparisons).

Despite being selective towards TQ at this stage of training, neither of the groups displayed accurate search behaviour as assessed by the number of platform crossings and cumulative proximity to platform. Number of platform crossings in TQ

did not differ between genotypes (Fig. 3.3B, WT:1.83 ± 0.22, mut: 2.50 ± 0.48, $F(1,10)=1.62$, $p=0.23$, one-way ANOVA with genotype as variable) and TQ over OP preference quadrant preference was only detected in the mutants (Fig. 3.3B, WT: $F(3,20)=0.99$; $p=0.42$; mut: $F(3,20)=6.67$; $p<0.01$, one-way ANOVA with quadrant as variable, $p<0.01$ for TQ vs OP, *post-hoc* Student-Newman-Keuls comparison).

Analysis of cumulative proximity revealed that both genotypes swam at similar distances from the previous platform location (WT: 25.6 ± 2.1 m; mut: 30.0 ± 1.9 m, $F(1,10)=1.56$; $p=0.24$; one-way ANOVA with genotype as variable). Only mut animals swam at closer proximity to TQ than to AL or OP (Fig 3.3C, WT: $F(3,20)=1.61$; $p=0.21$; mut: $F(3,20)=13.35$; $p<0.001$; one-way ANOVA with quadrant as variable, OP vs TQ $p<0.001$ and AL vs TQ $p<0.01$, *post-hoc* Student-Newman-Keuls comparison).

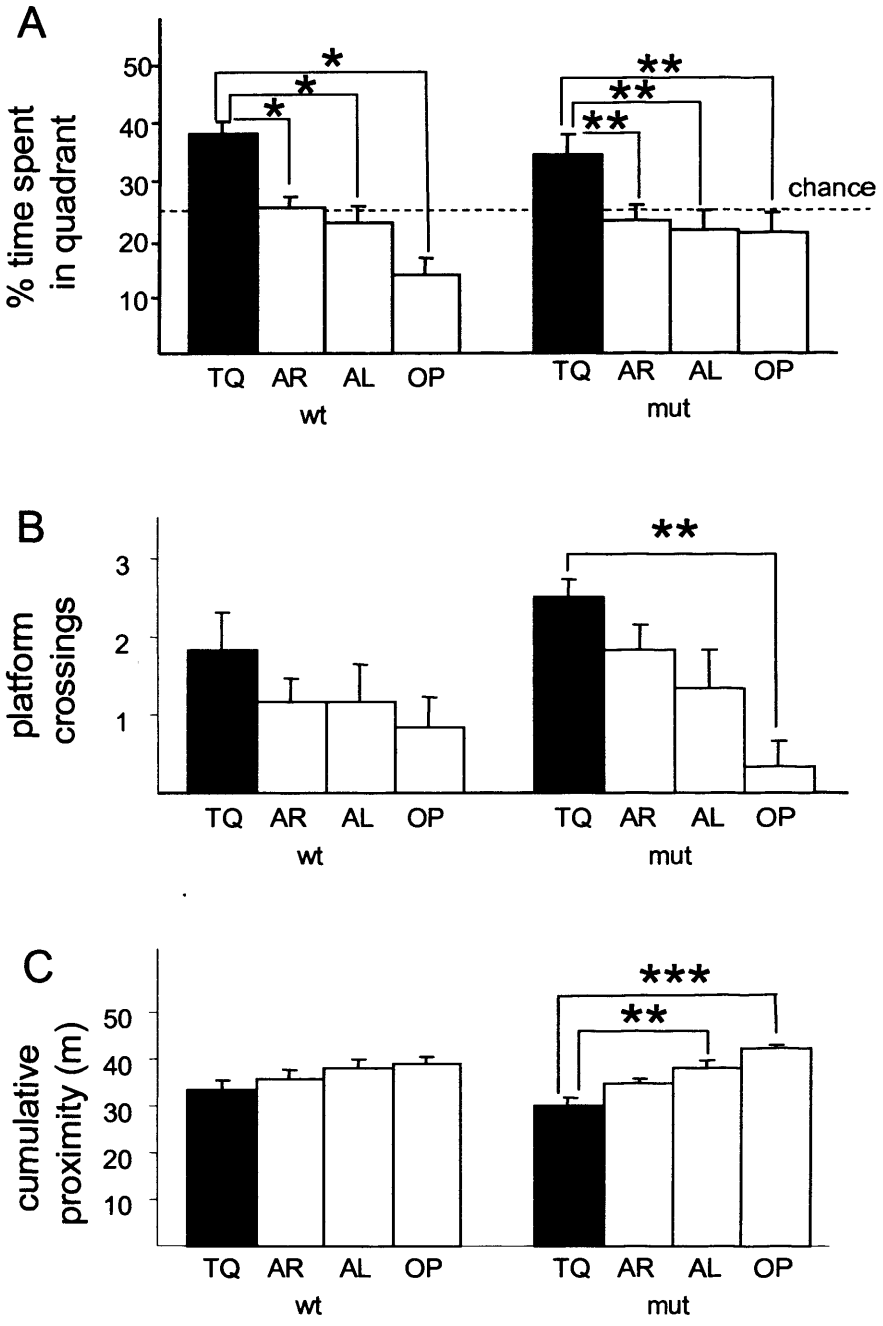


**Figure 3.3- CaMKK$\beta$ male mutant mice displayed normal spatial memory assessed in a probe trial at the end of the third day of training.**
**(A)** Both genotypes (WT: n=6, mut n=6) spent a significantly higher percentage of their search time in TQ, and this selectivity did not differ between genotypes; **(B)** WT mice crossed all platform positions equally often, mut performed more platform crossings in TQ than in OP, but number of platform crossings in TQ did not differ between genotypes; **(C)** Cumulative distance to platform position in TQ did not differ between genotypes, WT mice searched equally close to the platform positions in every quadrant and mutants searched significantly closer to TQ than to AL and OP. (Mean±SEM, *p<0.05, **p<0.01, ***p<0.001, TQ- target quadrant; AR- adjacent right; AL- adjacent left; OP- opposite)

### 3.4.2. BEHAVIOURAL DATA FOR THE ANIMALS USED IN THE TRANSCRIPTIONAL ANALYSIS

In this study, high density oligonucleotide arrays (*Affymetrix)* were used to screen a proportion of the murine genome for differences in hippocampal gene expression between two groups of animals: male CaMKK$\beta$ null mutant mice and WT littermates both trained in the MWM as described in section 3.4.1.

Peters et al. (2003) describe impaired spatial memory formation in male CaMKK$\beta$ null mutant mice. This was observed in a probe trial given at the end of the sixth day of training in a 4 trials per day training protocol. Based on this observation CaMKK$\beta$ mutants would not be expected to be selective at the end of the third day of training. However, in the present study, both genotypes were selective and training was stopped at this stage (Fig 3.3). Nevertheless, analysis of individual performance scores revealed a high intra-group variation in selectivity. Animals were considered selective when they spent at least 35% of their search times in TQ during the probe trial, and non selective if they spent similar percentages of time in every quadrant, or at least similar amounts of time in two different quadrants (Fig. 3.4).

A number of studies report a correlation between levels of performance in the MWM, levels of CREB activation and expression of some "memory effector genes" (Brightwell et al., 2004; Balschun et al., 2003; Guzowski et al., 2001; Gass et al., 1998). In order to gain an insight into the global differences in the transcriptional profiles between the genotypes, whilst avoiding possible biases related to performance levels, two selective and two non selective animals were used per group to generate four biological replicates.

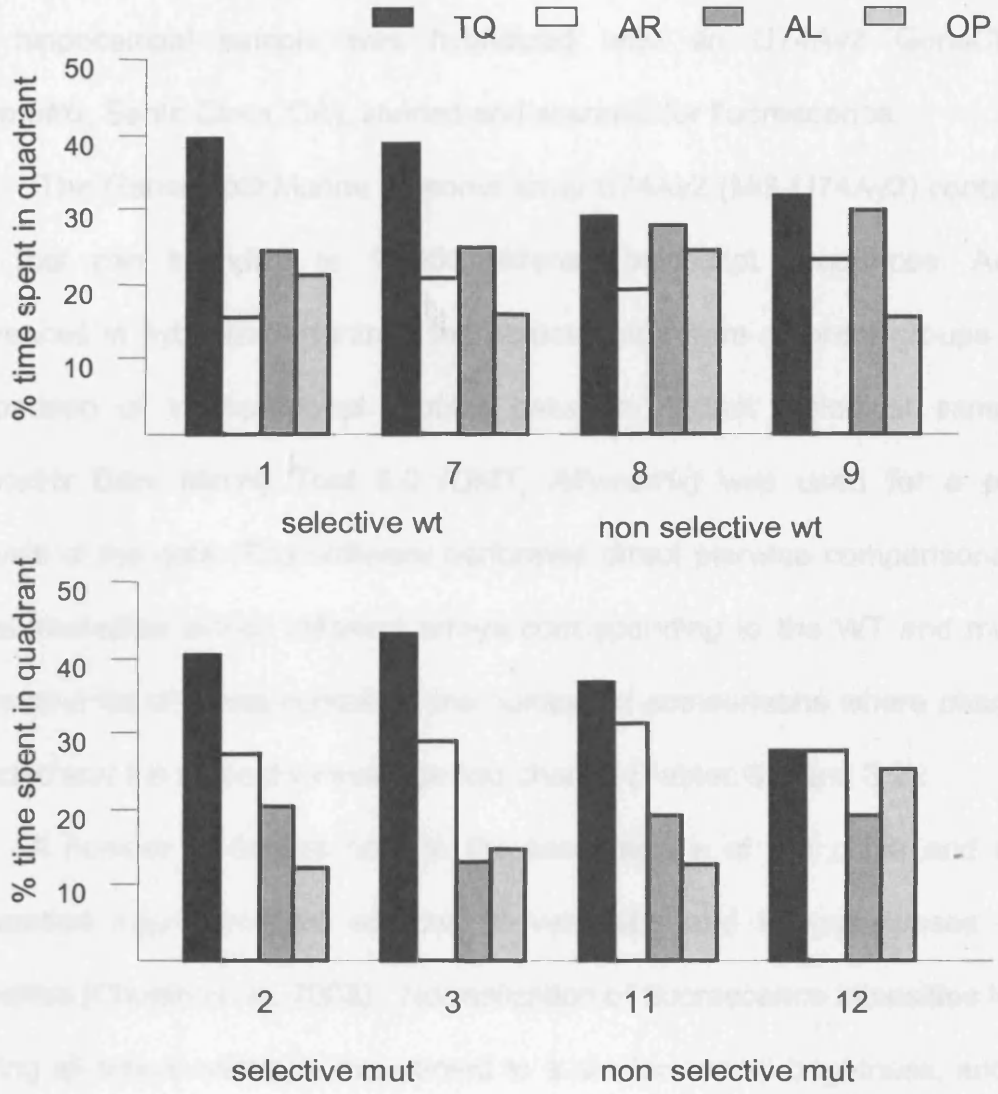


**Figure 3.4- Individual probe trial performance data for the animals used in the transcriptional analysis.**
Animals were classified as selective and non selective according to percentage of time spent in TQ during the probe trial (Top panel, WT animals; Bottom panel, mut animals).

### 3.4.3. MICROARRAY ANALYSIS OF CaMKK$\beta$ REGULATED GENES AFTER TRAINING IN THE MORRIS WATER MAZE

Thirty minutes after the probe trial, mice were sacrificed and hippocampi dissected. Hippocampal mRNA was reverse transcribed into cDNA which was *in vitro*

transcribed into cRNA as described in chapter II. Biotynilated cRNA derived from one hippocampal sample was hybridized onto an U74Av2 GeneChip Array (*Affymetrix*, Santa Clara, CA), stained and scanned for fluorescence.

The Genechip® Murine Genome array U74Av2 (MG-U74Av2) contains probe sets that can hybridize to 12,000 different transcript sequences. Analysis of differences in hybridization intensities across chips from different groups permits a comparison of transcriptional profiles between distinct biological samples. The *Affymetrix* Data Mining Tool 3.0 (DMT, *Affymetrix*) was used for a preliminary analysis of the data. This software performed direct pairwise comparisons between signal intensities across different arrays corresponding to the WT and mut groups. The output list of genes contained the number of comparisons where changes were detected and the respective average fold change (Tables 3.1 and 3.2).

A number of factors both in the manufacture of the chips and in sample preparation may represent sources of variability and impose biases on signal intensities (Chudin et al., 2002). Normalization of fluorescence intensities is required to bring all arrays within an experiment to a similar overall brightness, and diminish bias generated by non-biological causes. Microarray data analysis softwares use distinct normalization and mathematical analysis procedures to compare transcriptional profiles. In the present study, in addition to the output from the DMT, two alternative softwares were used: dCHIP (Li and Wong, Harvard University, USA) and Genespring (*Silicon Genetics*). The normalization procedures and models of analysis used by both softwares are described in Chapter II.

For statistical group comparisons, it was firstly ensured that only probe sets with normally distributed signal intensities were considered. Probe sets were discarded if normalized signal intensities for any of the probes were out of the 95%

confidence interval for the distribution of the mean. A parametric t-test, not assuming variances equal, was used for statistical group comparison procedure, as this is the test advised for standard experiments where the number of replicates per group is small (according to dCHip and GeneSpring user's manuals).

### 3.4.3.3. COMPARISON CRITERIA

Comparison of transcriptional profiles between the WT and mut groups was performed on normalized data. The WT group was defined as a baseline group, and the genes with a fold change between the group means above a 30% threshold, with a p-value of less than 5% for the null hypothesis, were designated "candidate genes".

Within the lists of candidate genes that fulfilled the criteria described above, a subset, listed in Tables 3.1 and 3.2, was chosen for further investigation. The criteria for the choice of these genes were: (a) described function in synaptic plasticity and learning and memory processes; or (b) described or putative participation in sexually dimorphic molecular mechanisms in the CNS; (c) known biological function with potential relevance to synaptic plasticity processes; (d) if the function was unknown, coincident detection by more than one software tool. Candidate genes included: neurotransmitter receptors, signalling molecules, apoptosis regulatory factors, transcription factors and splicing factors.

The group of neurotransmitter receptors included: The GluR1 subunit of the AMPA receptor, the proper function of which is critically required for LTP and LTD expression and retention of spatial memories (Lee et al., 2003); the alpha subunit of the $GABA_A$ receptor, a receptor involved in the modulation of anxiety related behaviours (Holmes, 2001) and context learning in classical fear conditioning

paradigms (Huff and Rudy, 2004); and the neuropeptide Y receptor 2 (NPYR2), which is known to play a role in spatial memory assessed in the MWM (Redrobe et al., 2004).

In the signalling molecules group the following genes were investigated: the IQ motif containing GTPase activating protein (IQGAP1), a protein involved in modulation of cytoskeletal organization (Li et al., 2005), which interacts with the Ras/Raf signalling pathway involved in memory formation (Giese et al., 2001). Finally, the transcriptional profile of the, GPI-anchor attachment protein 1 (Gaa1), involved in post-translational processing of transmembrane proteins (Tsui-Pierchala et al., 2002; Hiroi et al., 2000) was also investigated.

Proteins involved in regulation of apoptosis were selected on the basis of a number of reports connecting differences in regulation of apoptosis to sexual dimorphisms in brain anatomy (Morris et al., 2004; Forger et al., 2004). This group included an anti-apoptotic factor, the serine protease inhibitor serpin 3n, which regulates the activity of a number of pro-apoptotic proteases (Horvath et al., 2005), and a pro-apoptotic factor, the bcl-2-related ovarian killer protein (Bok), which is prominently expressed in reproductive tissues and thought to participate in hormonally regulated cyclic cell turnover mechanisms (Hsu et al., 1997).

*De novo* steroidogenesis has been identified in the brain (Hojo et al., 2004), and given the modulatory role of steroids in cognition (Sherwin, 2003a; Sherwin, 2003b), the transcriptional profile of the steroidogenic acute regulatory protein (Star; Manna et al., 2003), was further investigated. This was a particularly promising candidate as it was previously identified as a CREB target (King et al., 2002).

The group of splicing factors included: the splicing factor arginine/serine rich 3 (Srp20; Jumaa et al., 1997), the brain specific isoform of the polypyrimidine tract

binding protein (brPTB; Lillevali et al., 2001), the PTB associated splice factor (PSF; Chanas-Sacre et al., 1999; Patton et al., 1993) and the U2 small nuclear ribonuclear protein (U2AF, Kielkopf et al., 2004). Interest in these factors was based on a number of reports on activity-dependent regulation of mRNA splicing in neurons (eg. O'Connor et al., 2004).

The ubiquitin ligase Arih1 was selected on the bases of a description of an important role of ubiquitination factors on the tuning of synapse formation (DiAntonio et al., 2001) and on the fact that lack of the Arih1 orthologue in *D*rosophila is associated with a neurodegenerative phenotype (Aguilera et al., 2000).

Other genes investigated included: the ATP binding cassette PMP70, encoding a peroxisomal transmembrane protein which is implicated in the pathogenesis of Zelwegger syndrome, a mental retardation syndrome (Collins and Gould, 1999); the transcription factor, pre-B cell leukaemia transcription factor (Pbx3), which is induced during neuronal differentiation in development (Qin et al., 2004); an FMS-like tyrosine kinase, a transmembrane receptor involved in the modulation of diabetogenesis and the pathogenesis of leukaemia(Advani, 2005; Bates et al., 2002); and Synaptotagmin 4, a post-synaptic membrane protein which acts as a calcium sensor and is thought to mediate post-synaptic vesicle trafficking (Yoshihara and Montana, 2004).

**Table 3.1-** Candidate genes detected as being expressed at lower levels in the hippocampus of CaMKK$\beta$ null mutant male mice after training in the MWM, when compared to WT controls (*p<0.05; **p<0.01; ***p<0.001; nd- not-detected).

| Probe Set | Gene name | % transcript level in CaMKK$\beta$ trained male mice (normalized to WT trained male mice ) | | | | Biological Function |
|---|---|---|---|---|---|---|
| | | Affy DMT | dCHIP | | Gene Spring | |
| | | | ADI | MBEI | | |
| 104374_at | Serpin 3n (serine protease inhibitor) | 50%* (2 comp) | nd | 50%* | 38%** | Endopeptidase inhibitor activity; anti-apoptotic factor |
| 92943_at | GluR1 (Glutamate receptor, ionotropic, AMPA1, $\alpha$ subunit). | 70%* (1comp) | nd | 67%* | 67%* | Neurotransmitter receptor subunit |
| 161784_f_at | Star (steroidogenic acute regulatory protein 1) | 50%* (1 comp) | nd | nd | 60%* | Neurosteroid synthesis<br><br>Known CREB target |
| 100561_at | IQGAP1 (IQ motif containing GTPase activating protein 1) | nd | 67%* | nd | 67%* | Neuronal cell adhesion |
| 161682_f_at | Gaa1 (GPI-anchor attachment protein 1) | nd | nd | nd | 67%* | Anchoring of GPI moieties to proteins |
| 98031_at | Bok (Bcl-2-related ovarian killer protein) | nd | nd | nd | 61%* | Regulation of apoptosis |
| 101004_f_at | SRp20 (Splicing factor arginine/serine rich 3 ) | nd | nd | nd | 56%** | Regulation of alternative splicing and nucleocytoplasmic transport of mRNA |

**Table 3.2-** Candidate genes detected as being expressed at higher levels in the hippocampus of CaMKK$\beta$ null mutant male mice after training in the MWM, when compared to WT controls (*p<0.05; **p<0.01; ***p<0.001; nd- not-detected).

| Probe Set | Gene name | % transcript level in CaMKK$\beta$ trained male mice (normalized to WT trained male mice ) | | | | Biological Function |
|---|---|---|---|---|---|---|
| | | Afffy DMT | dCHIP ADI | MBEI | Gene Spring | |
| 94088_at 160374_r_at | brPTB (polypirimidine tract binding protein- brain specific isoform) | nd | 250%* | nd | 160%*** | Regulation of alternative splicing and nucleocytoplasmic transport of mRNA |
| 104046_at | Arih1 (ariadne-like E3 ubiquitin ligase) | nd | 170%* | 140%* | 300%* | Targeted protein degradation |
| 93045_at | PMP70 (ATP binding cassette subfamily) | nd | 140%* | ND | 200%* | Peroxisome biogenesis Zelwegger syndrome |
| 95559_at | FMS-like tyrosine kinase; Riken cDNA 6330403k07, | 135% (1comp) | 140%* | 140%* | 150%** | Transmembrane receptor |
| 160190_at | Synaptotagmin 4 | 125%* | nd | 160%* | nd | Ca2+ sensors of vesicular traffic and endocytosis |
| 93615_at | Pbx3 (Pre B-cell leukemia transcription factor 3) | nd | nd | 200%* | 150%* | Transcription |
| 100703_at | NPYR (Neuropeptide Y receptor Y2) | nd | nd | nd | 173%* | Neurotransmitter receptor |
| 97486_at | U2AF (small nuclear ribonuclear protein) | nd | 149%* | nd | 149%* | Alternative splicing |
| 99620_at | PSF (PTB associated splicing factor proline/glutamine rich) | nd | nd | nd | 190%* | Alternative splicing and transcriptional co-activator |
| 92938_at | GABA_A receptor subunit $\alpha$1 | nd | 140%* | nd | nd | Neurotransmitter receptor subunit |

Microarray analysis is a widely used tool for high throughput screening of transcriptional differences, and has been used in a number of studies aiming to identify genes regulated by behavioural training (e.g. Levenson et al., 2004a; Dubnau et al., 2003; Leil et al., 2002). Results from these and other studies underscore the importance of using independent follow up assays to confirm differences in gene expression between groups, as artifactual false positives are inevitably present (Dubnau et al., 2003).

### 3.4.4. MUTATION RELATED TRANSCRIPTIONAL DIFFERENCES IN MALE MICE

The transcriptional analysis described above provided an insight into the patterns of hippocampal gene expression of CaMKK$\beta$ mut and WT male mice. Due to the high cost of this type of experiment, the initial microarray screening could only be performed for a limited number of conditions. The initial screening procedure focused on hippocampal transcripts from male mice trained in the MWM, using four biological replicates per genotype: CaMKK$\beta$ mut and WT.

As CaMKK pathway members are involved in transcriptional regulation, differential levels of expression of certain genes are expected between WT and mut mice in the naïve hippocampus; furthermore, based on the observation that male CaMKK$\beta$ mutant mice fail to activate CREB after spatial training and display an impairment in the transcription dependent phase of LTP in hippocampal CA1 synapses (Peters et al., 2003), it is also possible that differences in gene expression induced by behavioural training may also account for the transcriptional differences identified. Hence, at least two factors may account for the transcriptional differences

identified: (a) the lack of CaMKK$\beta$ *per* se (baseline differences); (b) the lack of spatial training-induced CaMKK$\beta$ activity.

The first follow-up step used in this transcriptional analysis, aimed to identify baseline differences in gene expression, i.e, CaMKK$\beta$ regulated genes in the naïve hippocampus. For this purpose, hippocampal transcriptional levels of candidate genes were compared between WT and mut naïve mice using a relative quantification method: QPCR. These animals were sacrificed at the same time of the light cycle at which trained animals were sacrificed, to avoid potential confounds due to circadian changes in levels of expression of certain genes (Ronnback et al., 2005).

The assay consisted of measuring target gene and internal control gene expression in the same samples. Internal controls act as loading controls accounting for between sample variations arising from subtle differences in start RNA concentration, RNA quality and reverse transcription that inevitably occur. Data were subject to normalization and logarithmic transformation assuming a perfect doubling of PCR products per cycle, as described for example, by Leil et al. (2002),.

The results of this confirmatory step are summarized in figures 3.5 and 3.6.

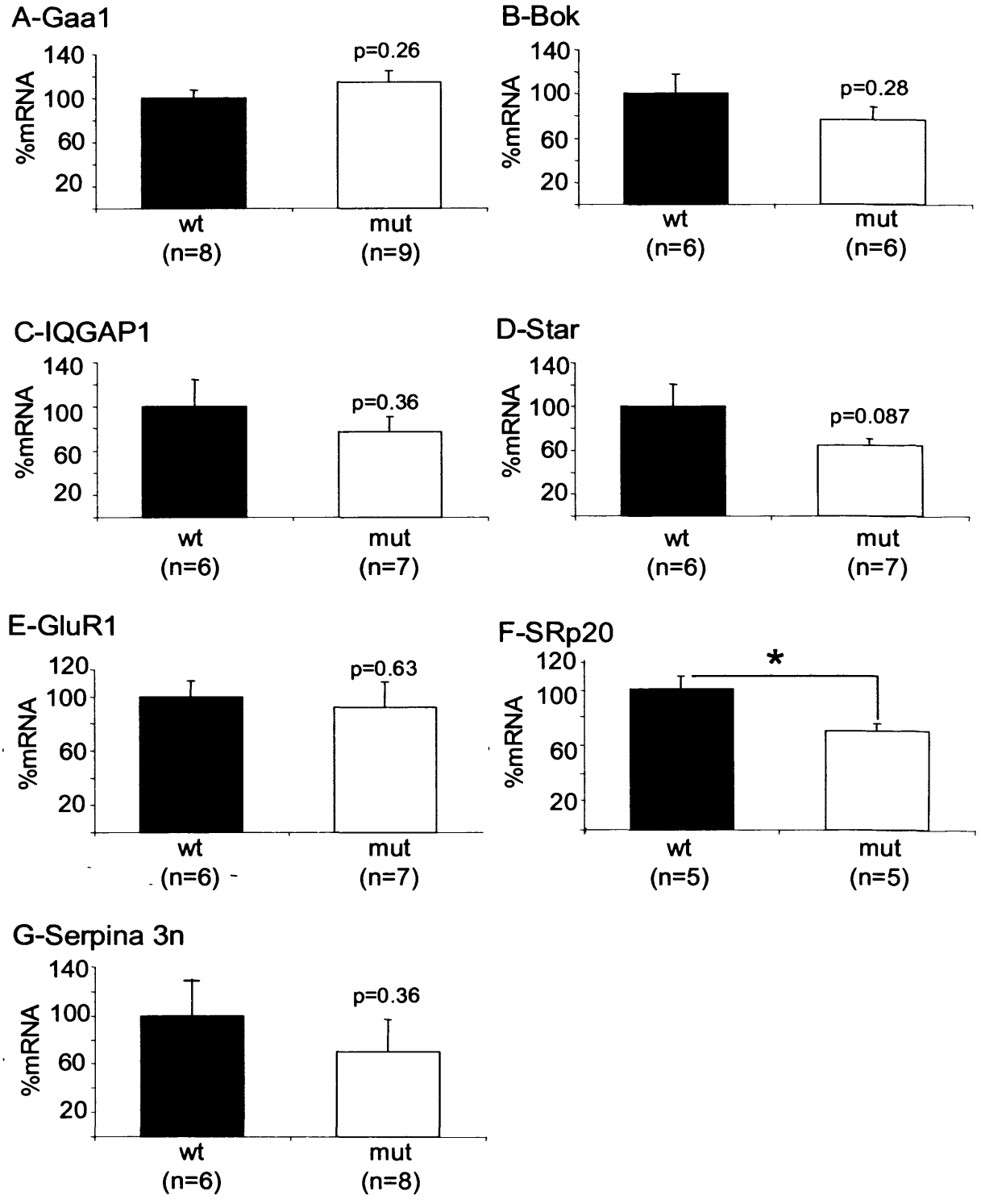


**Figure 3.5- QPCR comparison of transcriptional levels of candidate genes in the naïve hippocampus (I).** These genes were detected in the Affymetrix microarray screening as being expressed in lower levels in trained CaMKK$\beta$ null mutant males and are listed in table 3.1. The only gene detected as being expressed in lower levels in the naïve hippocampus was SRp20 (F, WT: 100 ± 10%; mut: 69.6±5.5%; $F(1,8)=7.37$; $p=0.026$, one-way ANOVA with genotype as variable). (Mean±SEM, p-values are indicated above the mut bar, *$p<0.05$, all values were normalized to the average WT levels of expression).

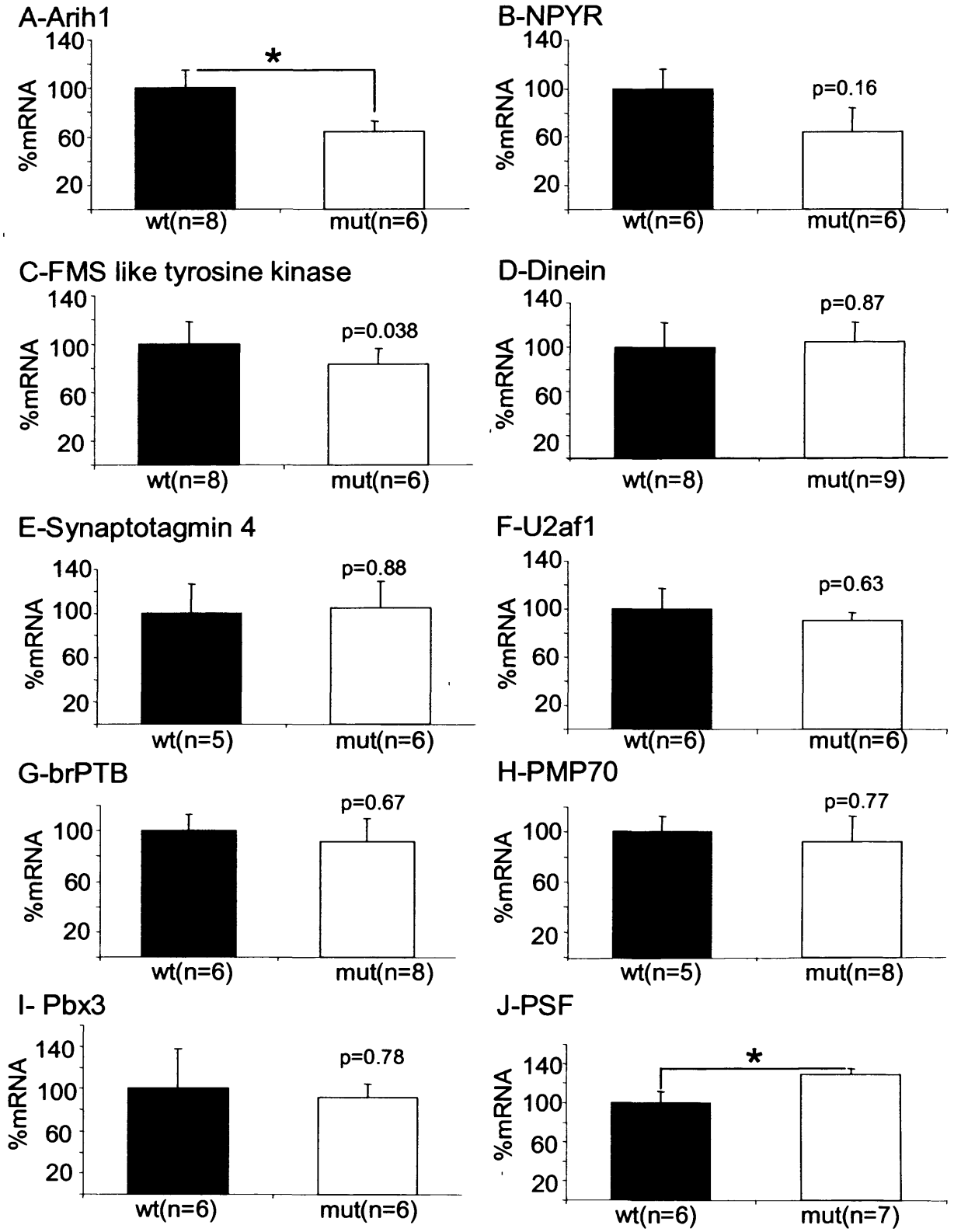


**Figure 3.6- QPCR comparison of transcriptional levels of candidate genes in the naïve hippocampus (II).** These genes were detected in the Affymetrix microarray screening as being expressed in higher levels in trained CaMKK$\beta$ null mutant males and are listed in table 3.2. Two genes were detected as being differently expressed (A-Arih1; WT: 100 ± 15%; mut: 64.4 ± 8.2%; $F(1,12)=5.43$; $p=0.038$; J-PSF; WT: 100 ± 11%; mut: 131.9 ± 6.2%; $F(1,11)=5.95$; $p=0.033$; one-way ANOVA with genotype as variable). (Mean±SEM, p-values are indicated above the mut bar, *$p<0.05$, all values were normalized to the average WT levels of expression).

Analysis of transcriptional levels of candidate genes in the naïve hippocampus, revealed three genes as being differently regulated between the genotypes in male mice (Table 3.3). The direction of variation for PSF and SRp20 was the same for naïve and trained animals. For Arih1 an opposite direction of variation was detected with these two independent methods: in trained mice, mutants expressed higher levels of the transcript than WT; in naïve mice, mutants expressed lower levels of the transcript than their WT counterparts.

**Table 3.3-** Comparison of hippocampal transcriptional levels of candidate genes after MWM training and in naïve animals(*p<0.05; **p<0.01; ***p<0.001; nd- not-detected).

| Gene name | % transcript level in male trained CaMKK$\beta$ mutants (normalized to male trained WT ) AFFYMETRIX MICROARRAY ANALYSIS | | | % transcript level in male naïve CaMKK$\beta$ mutants (normalized to male naïve WT naïve) QPCR ANALYSIS |
|---|---|---|---|---|
| | Afffy DMT | dCHIP | Gene Spring | |
| Arih1 (ariadne-like E3 ubiquitin ligase) | nd | 170% | **300%*** | 67.00% F(1,12)=5.43; p=0.038 |
| Splicing factor arginine/serine rich 3 (SRp20) | nd | nd | 56%** | 69.55% F(1,8)=7.37; p=0.026 |
| Splicing factor praline/glutamine rich (PTB associated) (PSF) | nd | nd | 190%* | 128.65% F(1,11)=5.94; p=0.033 |

### 3.4.5. KNOWN CREB TARGET GENES

CaMKK$\beta$ is a member of the CaM kinase cascade known to mediate CREB phosphorylation, an essential event for the activation of CREB mediated transcription (Gonzalez and Montminy, 1989). The hippocampal levels of expression of four

known CREB target genes with functions in synaptic plasticity processes were compared between WT and CaMKK$\beta$ mut male mice. The genes tested were: the brain derived neurotrophic factor (Barco et al., 2005; Blanquet et al., 2003), members of the orphan receptor family of transcription factors: nuclear orphan-receptor 1 (Nor1), Nur77 and Nurr1 (von Hertzen and Giese, 2005; Darragh et al., 2005; Inuzuka et al., 2002). No differences in hippocampal levels of expression of either of these genes were detected between genotypes (Fig. 3.7).

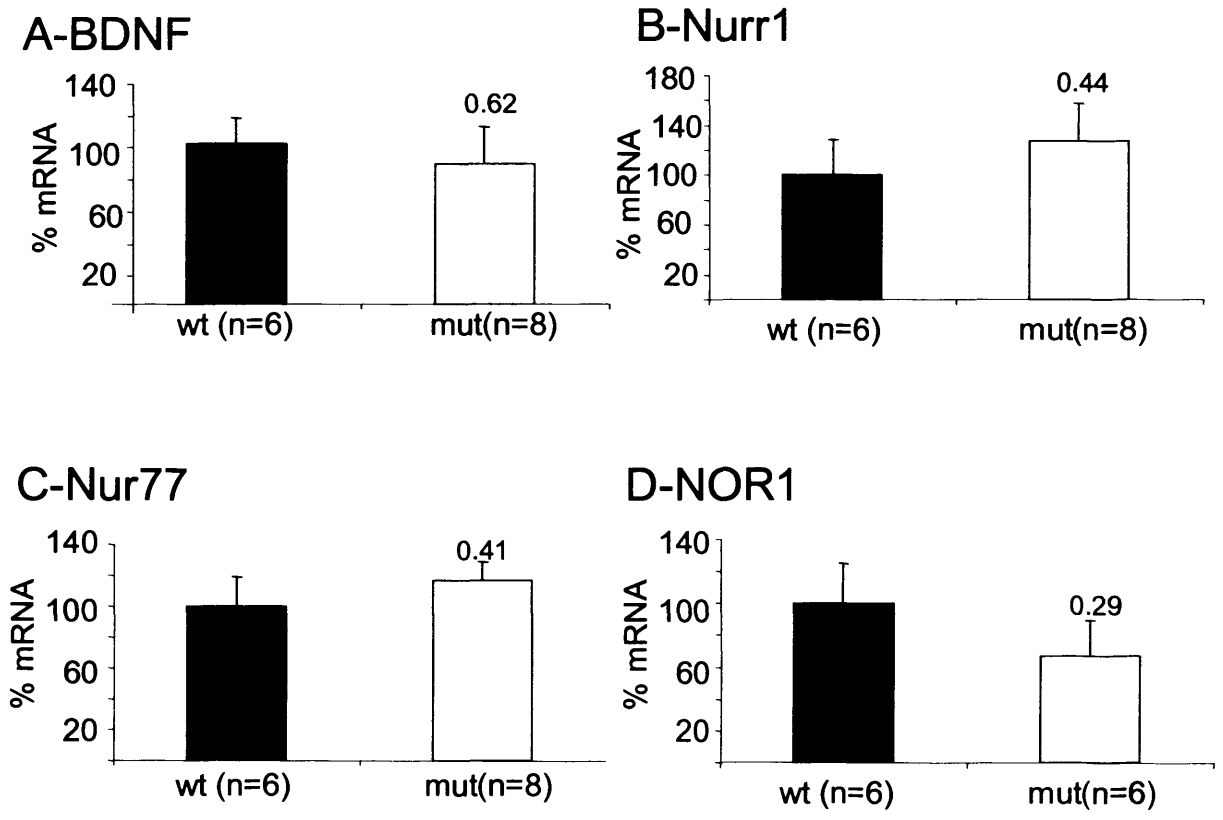


**Figure 3.7- QPCR comparison of transcriptional levels of known CREB and CaM kinase cascade targets in the naïve male hippocampus.** None of the genes screened was differently regulated between WT and CaMKK$\beta$ null mutant mice. (Mean±SEM, p-values are indicated above the mut bar, all values were normalized to the average WT levels of expression).

### 3.4.6. TRAINING of CaMKK$\beta$ NULL MUTANTS AND WT MALE MICE IN THE MWM FOR 6 DAYS

An anaysis of the videotapes of the behavioural training procedures for the cohort of mice described in section 3.4.2, suggested that the mice could have been exposed to extramaze cues while being moved around the pool in between trials. This could have eased the learning of the task and justifiy the selectivity in the probe trial at the end of the third day of training. In addition, the small number of animals in each group could have also prevented the detection of the phenotype. In an attempt to replicate the experimental conditions and use a number of animals comparable to the Peters et al. (2003) study, two new cohorts of CaMKK$\beta$ mut and WT male mice were trained in the MWM using a four trials per day training protocol for six days. However, the desired effect was not achived because the behavioural data from the two experiments could not be pooled. This was due to a significant difference in swim speed during the probe trial between the two cohorts (effect of cohort: $F(1,25)=6.86$, $p<0.05$; effect of genotype: $F(1,26)=0.312$, $p=0.312$; genotype x cohort interaction $F(1,24)=0.334$, $p=0.57$, two-way ANOVA, with genotype and cohort as variables). Hence, behavioural data pertaining to each cohort are presented separately.

#### 3.4.6.1. FIRST COHORT

The first cohort of mice consisted of WT (n=7) and mut (n=11) male mice in the C57BL/6/129/Sv F4,5 genetic background. A decreased latency was observed with the number of training days in both groups [Fig. 3.8A, effect of training: $F(5,107)=24.88$, $p<0.001$; effect of genotype: $F(1,16)=0.30$, $p=0.59$; genotype x training interaction $F(5,107)=0.85$, $p=0.52$, two-way repeated measures ANOVA on

transformed values (square root of the escape latency) because the data were not normally distributed].

During probe trials there was no difference in average swim speed (Fig. 3.8B, WT: 24.8 ± 1.4 cm/s; mut: 22.4 ± 1.2 cm/s; $F(1,16)=1,92$; $p=0.19$, one-way ANOVA with genotype as variable), percentage of time in the thigmotaxis zone (Fig. 3.8C, WT: 0.7 ± 0.3%; mut: 3.5 ± 1.1%; $F(1,16)=3.94$; $p=0.065$, one-way ANOVA with genotype as variable), or percentage of time floating (Fig. 3.8D, WT: 1.59 ± 0.60%; mut: 3.02 ± 0.97%; $F(1,16)=1.19$; $p=0.29$, one-way ANOVA with genotype as variable).

Analysis of quadrant search times showed no significant difference between genotypes in percentage of time spent in TQ (Fig, 3.9A, WT: 38.3 ± 4.9%; mut: 41.0 ± 4.5%; $F(1,16)=1.57$; $p=0.70$, one-way ANOVA with genotype as variable).

Additionally, both genotypes were selective towards TQ (Fig, 3.9A, WT: $F(3,24)= 6.20$; $p<0.01$, one-way ANOVA with quadrant as variable and $p<0.05$ for all TQ *post-hoc* Student-Newman-Keuls comparisons; mut: $F(3,40)=9.96$; $p<0.01$ for TQ vs OP and $p<0.05$ for the remaining quadrants *post-hoc* Student-Newman-Keuls comparisons).

Number of platform crossings did not differ significantly between genotypes (Fig. 3.9B, WT: 7.3 ± 1.3; mut: 4.7 ± 1.0, $p=0.13$, $F(1,16)= 2.61$, one-way ANOVA with genotype as variable). Both genotypes crossed significantly more times the platform position in TQ than in any other quadrant (WT: $p<0.05$, $F(3,24)=3.88$, mut: $p<0.05$, $F(3,40)=2.98$, one-way ANOVA with quadrant as variable; WT: $p<0,01$ for TQ vs AL comparison and $p<0.05$ for the remaining comparisons; mut: $p<0.05$ for TQ vs AL and AR, *post-hoc* Student-Newman-Keuls comparisons).

Both genotypes swam at a similar cumulative proximity to TQ (Fig. 3.9C, WT: 43.7 ± 3.6 m, mut: 43.6 ± 3.3 m, p=0.83, F(1,16)=0.046, one-way ANOVA with genotype as variable). Each genotype swam significantly closer to TQ than to any other quadrant (WT: F(3,24)=3.01; p<0.05; mut: F(3,40)=2.84; p<0.05, one-way ANOVA with quadrant as variable, WT : p<0.05 for TQ vs OP and TQ vs AL; mut: p<0.01; TQ vs AL p<0.05 and TQ vs OP, *post-hoc* Student-Newman-Keuls comparisons).

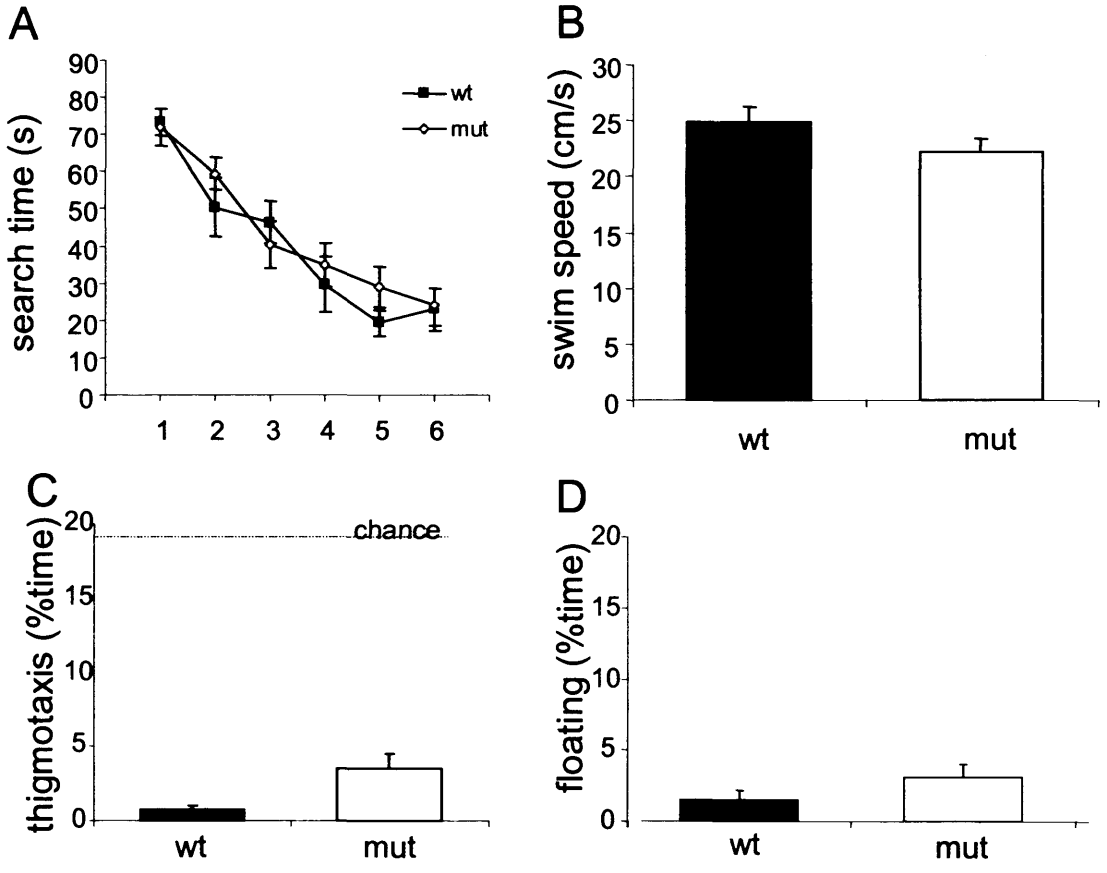


**Figure 3.8- Normal acquisition and swimming in CaMKK$\beta$ male null mutant mice after a 6 days training protocol (cohort I).**
**(A)** Both genotypes decreased their latency times to find the platform with the number of training trials; **(B)** During the probe trial, swim speeds did not differ between genotypes; **(C)** The percentage of time spent swimming in the thigmotaxis area did not differ between genotypes and was lower than expected by chance. **(D)** The percentage of time spent floating did not differ between genotypes. (Mean±SEM).

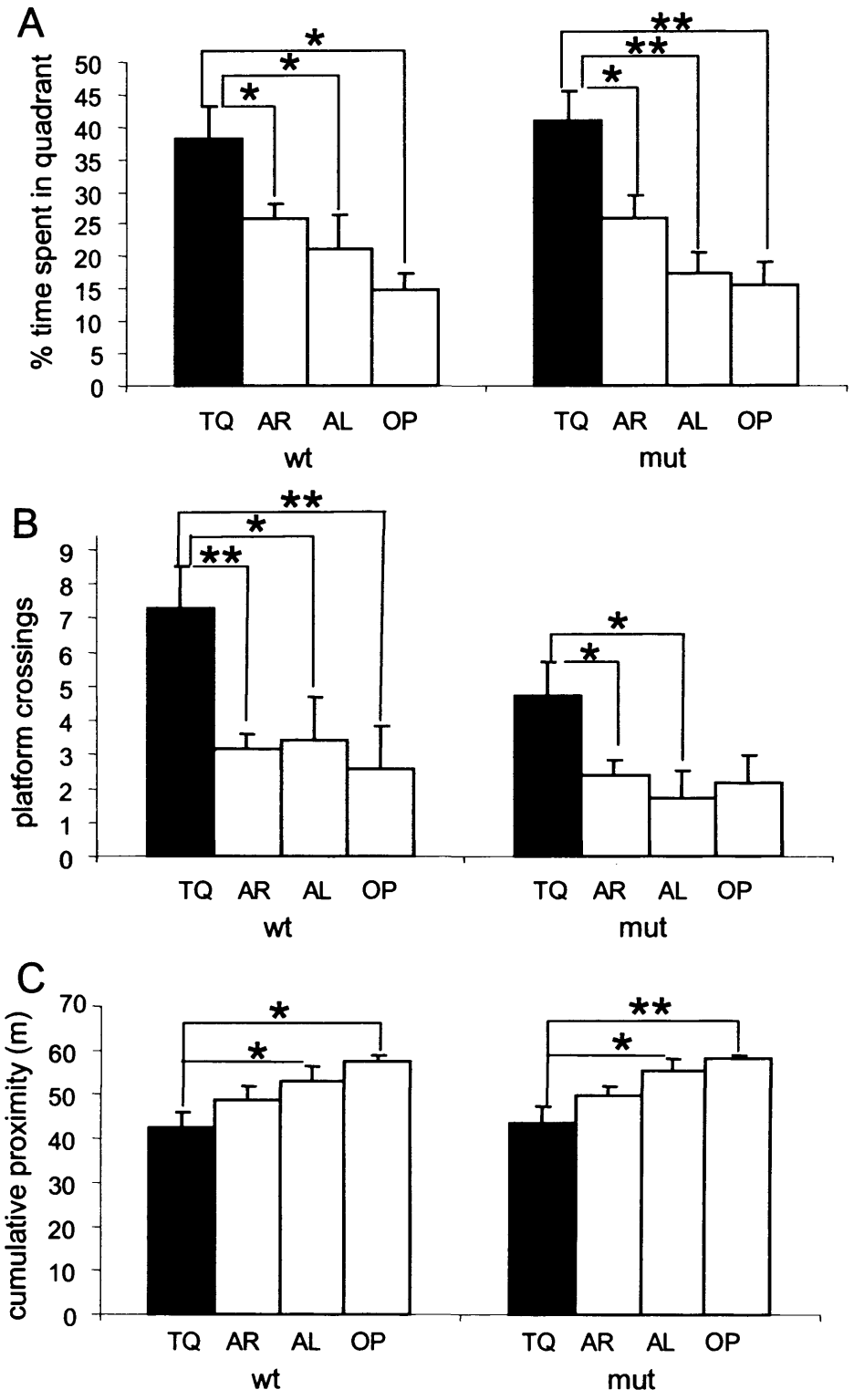


**Figure 3.9- CaMKK$\beta$ male mutant mice displayed normal spatial memory assessed in a probe trial at the end of the sixth day of training (cohort I).**
**(A)** Both genotypes (WT: n=7; mut: n=11) spent a significantly higher percentage of their search time in TQ, and this selectivity did not differ between genotypes; **(B)** WT mice crossed the platform in TQ more often than any other platform positions, mut mice crossed the platform position in TQ more often than in AR or AL, but number of platform crossings in TQ did not differ between genotypes; **(C)** Cumulative proximity to platform position in TQ did

not differ between genotypes, and both groups swam at a closer proximity to TQ than to AL or OP (Mean±SEM, *p<0.05, **p<0.01, ***p<0.001).

### 3.4.6.2. SECOND COHORT

This cohort of mice included WT (n=4) and mut (n=7) male littermates in the C57BL/6/129/Sv F5 genetic background. During the six day training period, there was a significant effect of training for both genotypes, no difference between genotypes, and no genotype-training interaction (Fig. 3.10A, effect of training $F(5,45)=12.18$, $p<0.001$; effect of genotype: $F(1,9)=3.09$, $p=0.113$; genotype x training interaction $F(5,45)=1.09$, $p=0.38$; two-way repeated measures ANOVA, with genotype and training as variables). Because, a difference in the escape latencies between genotypes, was apparent in days 3 and 4, one-way ANOVA planned comparisons on escape latencies were performed for each of these days. A significantly higher latency in the mut group was detected on the third day of training ($p<0.05$) and a trend towards a significant difference on the fourth day ($p=0.083$). In order to address whether this difference could be related to differences in swim speed, these were compared throughout the whole training procedure, with no differences between genotype being found (Fig 3.10B, effect of genotype $F(1,9)=0.15$; $p=0.71$; effect of training $F(5,45)=0.20$; $p=0.46$; genotype x training interaction $F(5,45)=1.45$; $p=0.22$ two-way repeated measures ANOVA). In fact, the differences in acquisition were reflected in the average path length (Fig 3.10C, effect of genotype $F(1,9)=1.77$, $p=0.22$; effect of training $F(5,45)=14.07$ , $p<0.001$; genotype x training interaction $F(5,45)=0.93$, $p=0.47$ two-way repeated measures ANOVA). Planned comparisons on path length for days 3 and 4 revealed significantly longer path lengths in the mutant group on day 4 ($p<0.05$) and a trend towards a higher path length on day 3 ($p=0.071$).

During probe trials, the genotypes did not differ in average swim speed (Fig. 3.10D, WT: 20.0 ± 1.3 cm/s; mut: 19.3 ± 1.7 cm/s, $F(1,9)=0.80$; $p=0.78$, one-way ANOVA with genotype as variable), percentage of time spent in the thigmotaxis zone (Fig 3.10E, WT: 9.7 ± 5.4 %; mut: 2.1 ± 1.7 %; $F(1,9)=2.79$; $p=0.13$, one-way ANOVA with genotype as variable), or percentage of time spent floating (Fig. 3.10F, WT: 7.9 ± 4.0 %; mut: 3.4 ± 1.0 %; $F(1,9)=1.97$; $p=0.19$, one-way ANOVA with genotype as variable).

Analysis of quadrant search times showed no significant difference between genotypes in percentage of time spent in TQ (Fig. 3.11A, WT: 50 ± 17 %; mut: 49.5 ± 7.9 %; $F(1,9)=0.0019$; $p=0.97$, one-way ANOVA with genotype as variable). Both genotypes spent a significantly higher percentage of time swimming in TQ than in any other quadrant (Fig. 3.11A, WT: $F(3,12)= 3.63$; $p<0.05$; mut: $F(3,24)=10.37$; $p<0.001$, one-way ANOVA with quadrant as variable; WT: $p<0.05$ for all TQ comparisons, mut: $p<0.01$ for all TQ *post-hoc* Student-Newman-Keuls comparisons).

Analysis of platform crossings revealed no significant difference between number of TQ crossings between genotypes (Fig. 3.11B, WT: 6.0 ± 3.2; mut: 4.00 ± 0.98; $F(1,9)= 0.55$; $p=0.48$, one-way ANOVA with genotype as variable). Although a higher number of platform crossings in TQ was apparent for the WT group (Fig 3.11B), there was no significant difference in the number of platform crossings in the different quadrants ($F(3,12)=1.71$; $p=0.21$, one-way ANOVA with quadrant as variable). Mutants showed a preference for TQ ($F(3,24)=3.01$; $p<0,01$; one-way ANOVA with quadrant as variable, Student-Newman-Keuls *post-hoc* comparisons revealed a preference for TQ vs AR and OP , $p<0.01$).

Analysis of cumulative proximity revealed no difference between genotypes (WT: 40.7 ± 3.2 m; mut: 37.1 ± 3.9 m; $F(1,9)=0.17$; $p=0.69$). No significant lower

global proximity to TQ was detected for WT ($F(3,12)=1.86$; $p=0.18$, one-way ANOVA with quadrant as variable) presumably due to the high variability of performance within the group. Mutant mice swam significantly closer to TQ than to any other quadrant ($F(3,24)=9.40$; $p<0.001$; one-way ANOVA with quadrant as variable; TQ vs OP ($p<0.001$), *post-hoc* Student- Newman Keuls comparisons).

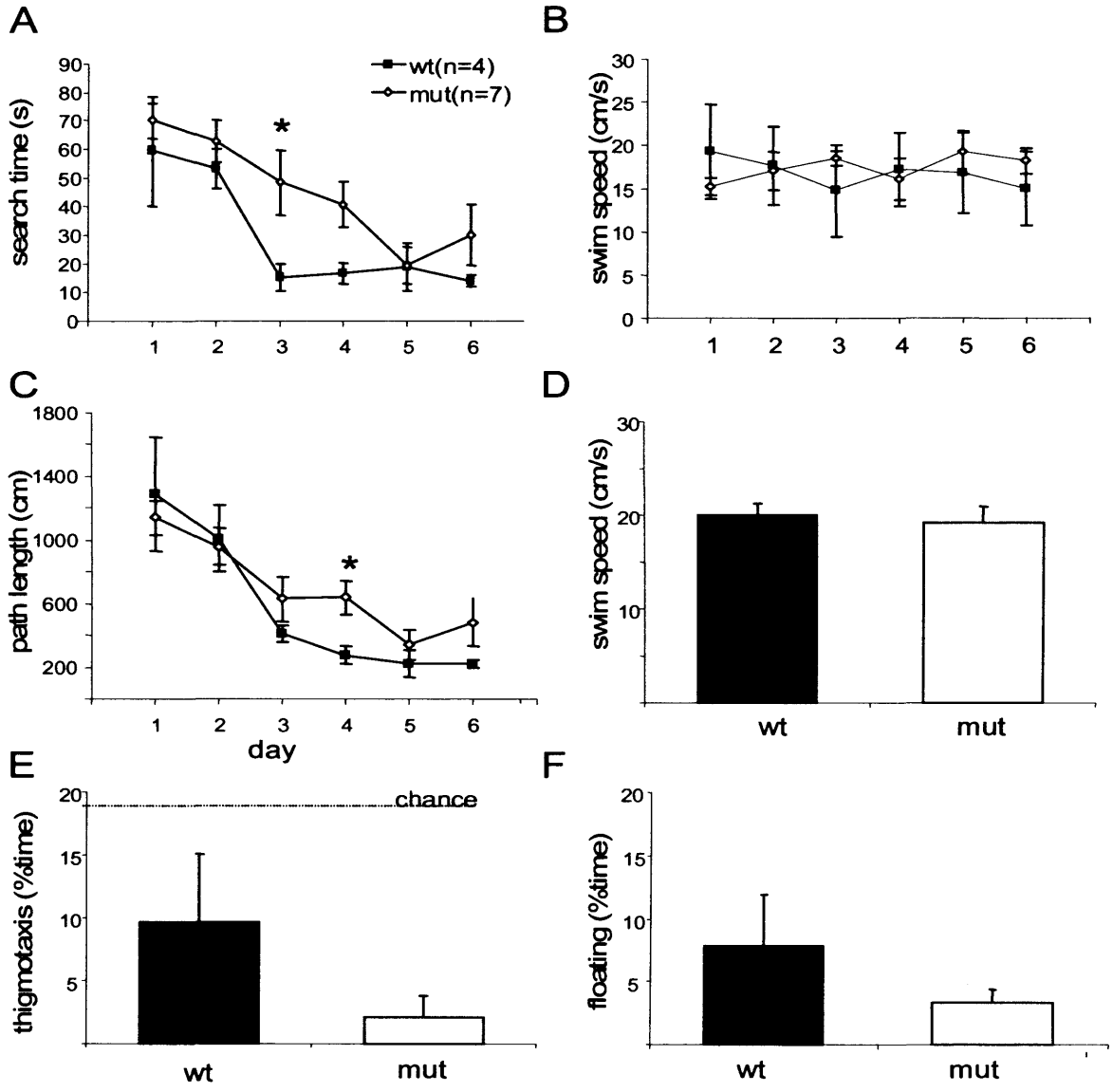


**Figure 3.10- Acquisition and swimming abilities in CaMKK$\beta$ male null mutant mice after a 6 days training protocol (cohort II).**
**(A)** Both genotypes decreased their latency times to find the platform with the number of training trials, but there was a difference in escape latencies on the third day of training; **(B)** Swim speeds did not differ between genotypes during the acquisition of the task; **(C)** The path length of the mut group was significantly longer on the 4$^{th}$ day of training; **(D)** During the probe trial, swim speeds did not differ between genotypes; **(E)** The percentage of time spent swimming in the thigmotaxis area did not differ between genotypes and was lower than expected by chance. **(F)** The percentage of time spent floating did not differ between genotypes. (Mean±SEM, *$p<0.05$).

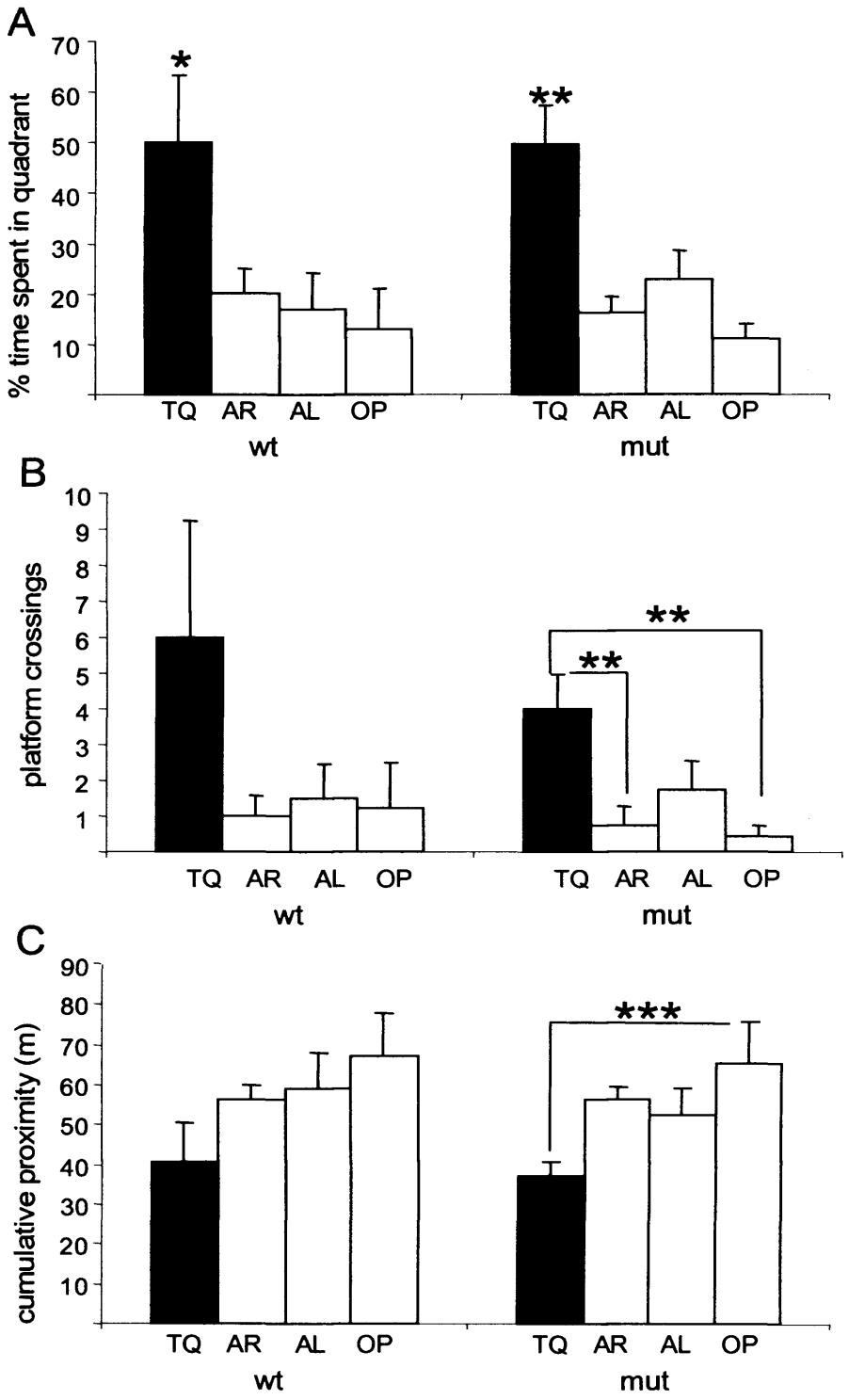


**Figure 3.11- CaMKK$\beta$ male mutant mice displayed normal spatial memory assessed in a probe trial at the end of the sixth day of training (cohort II).**
**(A)** Both genotypes (WT: n=4; mut: n=7) spent a significantly higher percentage of their search time in TQ, and this selectivity did not differ between genotypes; **(B)** No significant difference between number of platform crossings in TQ and the remaining quadrants was detected for the WT group; Mutant mice crossed the platform position in TQ more often than in AR or OP, but number of platform crossings in TQ did not differ between genotypes; **(C)** Cumulative proximity to platform position in TQ did not differ between genotypes, but only mut mice swam at a closer proximity to TQ than to OP (Mean±SEM, *$p<0.05$, **$p<0.01$, ***$p<0.001$).

### 3.4.7. COMPARATIVE ANALYSIS OF SPATIAL TRAINING INDUCED TRANSCRIPTION BETWEEN MALE CaMKK$\beta$ MUT AND WT MICE

The initial *Affymetrix* transcriptional comparison screening was performed in hippocampal tissue from trained animals and the first follow up steps were performed in samples from naïve animals, in order to account for mutation related changes (Table 3.3). Hence, the possibility that some of the genes detected in the microarray analysis (listed in Tables 3.1 and 3.2) are regulated by CaMKK$\beta$ after training in the MWM still remained untested.

This possibility was only tested for one of the candidate genes encoding the glycosylphosphatydilinositol (GPI)-anchor attachment protein (Gaa1), a protein that catalyzes the attachment of GPI moieties to membrane proteins (Hiroi et al., 2000a). This gene was chosen because preliminary data (described in chapter IV) pointed to a transcriptional regulation after training in the MWM in male WT mice. To address whether Gaa1 expression is regulated by CaMKK$\beta$ in an activity-dependent manner, the hippocampal levels of expression were compared between naïve mice (WT: n=6; mut: n=6) and mice that were trained in the MWM for six days (WT: n=4; mut:n=7), and shown to be selective in a probe trial given at the end of the sixth day of training. (Figs. 3.9 and 3.10).

According to the previous finding (Fig. 3.6), transcriptional levels did not differ between genotypes in naïve animals (Fig. 3.12, WT: 100.0 ± 6.4%; mut 111 ± 11%, $F(1,9)=0.71$, $p=0.42$, one-way ANOVA with genotype as variable). A spatial-training induced up regulation of the gene was detected in naïve but not in mutant animals (planned comparisons: WT naïve: 100.0 ± 6.4%; WT trained: 124.6±4.7%, $F(1,8)=0.49$, $p=0<0.05$; mut naïve: 111 ± 11%, mut trained: 108.2 ± 8.6%; $F(1,11)=0.051$, $p=0.82$; one-way ANOVA with genotype as variable). These findings

suggest that activation of CaMKKβ induced by training in the MWM regulates Gaa1 transcription.

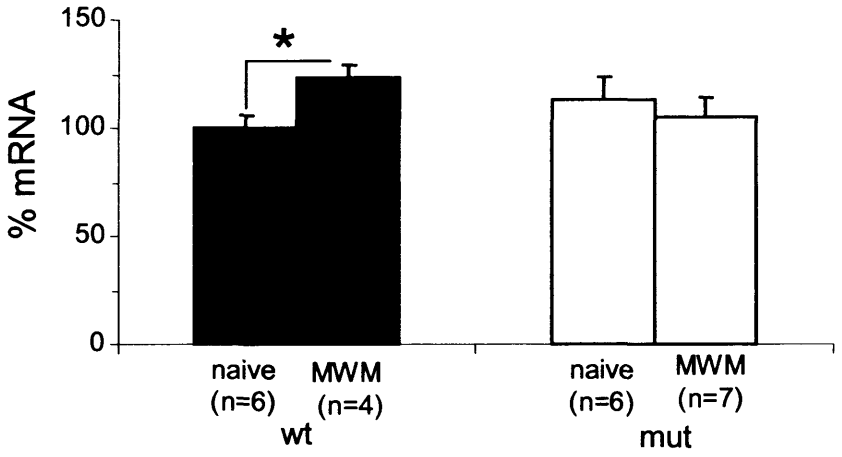


**Figure 3.12- CaMKKβ mutation dependent transcriptional output in males trained in the MWM.** Levels of expression of Gaa1 did not differ between genotypes in naïve mice, however a spatial training induced upregulation was found for WT mice, but not for mut mice. (Mean±SEM, *p<0.05, all values were normalized to the average WT naive levels of expression).

### 3.4.8. TRANSCRIPTIONAL LEVELS OF CANDIDATE GENES IN FEMALE NAÏVE MICE

Mizuno et al. (2006) describe a sex-specific role of CaMKKβ in hippocampal learning and memory processes. In order to assess whether CaMKKβ regulates the expression of the candidate genes in females, the transcriptional profile of these genes was compared between naive WT and CaMKKβ null mutant female mice (Fig. 3.13). Lack of CaMKKβ in naïve females did not have an impact on Gaa1, PSF or SRp20 expression levels (Fig. 3.13, B: Gaa1, WT:100.0 ± 7.7%; mut: 94.5 ± 9.7%; $F(1,13)=0.21$; $p=0.65$; C: PSF, WT: 100±11%, mut: 124.9±8.6%; $p=0.11$; $F(1,13)=2.82$; D: SRp20, WT: 100.0±5.0%, mut: 108.4 ± 8.9%, $p=0.40$, $F(1,12)=0.75$; one-way ANOVA with genotype as variable). However, Arih1 expression is

significantly higher in null mutant females than in the WT counterparts (WT: 100 ± 16%, mut 180 ± 43%, p=0.047, F(1,13)=4.80, one-way ANOVA with genotype as variable) which was opposite to the pattern of expression in males.

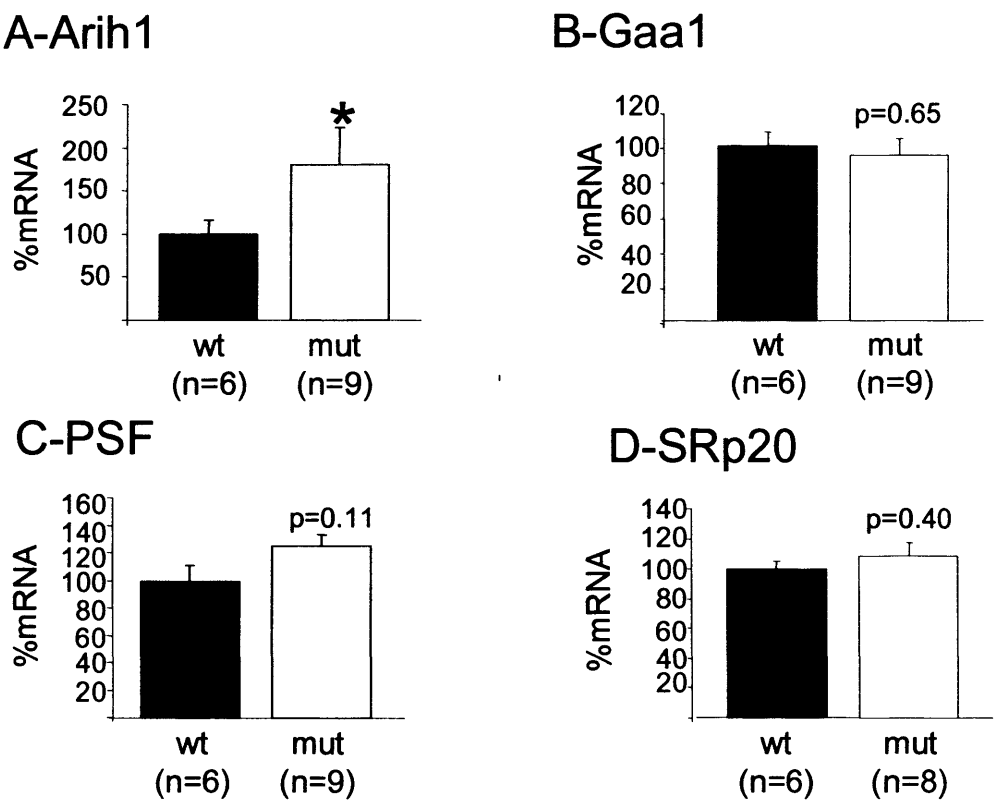


**Figure 3.13- QPCR comparison of transcriptional levels of candidate genes in the female naïve hippocampus.** All these are CaMKK$\beta$ regulated in the naïve male hippocampus, but only Arih1 (A) is differently regulated in the naïve female hippocampus of WT and mutant animals (MEAN±SEM, p-values are indicated above the mut bar, *p<0.05, all values were normalized to the average WT levels of expression).

## 3.5. DISCUSSION

Peters et al. (2003) demonstrated a role for the CaMKK$\beta$ isoforms in hippocampal learning and memory and synaptic plasticity processes. Male CaMKK$\beta$ mutant mice were impaired in spatial memory in the MWM, assessed in a probe trial given at the end of the sixth day of training, a deficit that was overcome by four additional days of training. The behavioural phenotype was accompanied by impaired spatial training induced CREB activation and deficits in L-LTP. These observations were the basis of the project described in this thesis.

The work described in the present chapter aimed at identifying genes that displayed distinct transcriptional profiles between WT and CaMKK$\beta$ mutant male mice in response to spatial training. However, the delayed spatial memory formation previously described was not replicated in three independent cohorts of mice (one of which was trained for three days only). Possible causes for these discrepancies include experimental and biological factors, which will be addressed below. Nevertheless, an *Affymetrix* Microarray analysis revealed a number of genes that displayed dinstinct levels of expression in the hippocampus of WT and CaMKK$\beta$ null mutant mice after training in the MWM. A QPCR follow up assay on a number of target genes detected in the Microarray analysis, pointed to differences in regulation of four of these genes purely due to the mutation (Arih1, SRp20 and PSF) or due to training in the MWM (Gaa1).

### 3.5.1. EXPERIMENTAL CONDITIONS

In the Peters *et al.* (2003) study, a delayed spatial memory formation was reported for CaMKK$\beta$ null mutant male mice. This impairment was assessed by three independent measurements of spatial learning. These were the percentage of time spent in TQ, the number of platform crossings (which reflects precise knowledge of the platform position) and the Gallagher cumulative proximity. While the first two reflect spatial biases in the search path, the third one is a measurement of accuracy as it reflects deviations from the optimal path. This is considered one of the most sensitive and informative methods to assess spatial memory formation (Gallagher et al., 1993). In the probe trial at day 6 in the Peters *et al.* (2003) study, all WT animals displayed a preference towards TQ while CaMKK$\beta$ failed to do so, as assessed by all three measurements. In the present studies, no differences in preference towards TQ were detected between genotypes and within genotype comparisons of percentage of time spent in each quadrant, revealed a global preference for TQ in WT and mutant animals. Surprisingly, and contrarily to the Peters et al. (2003) study, in two of the cohorts tested, the number of platform crossings and the cumulative proximity measure indicated a preference towards TQ in mutants but not in WT suggesting higher spatial accuracy in mutants.

Learning and memory paradigms applied to rodents aim to assess learning abilities and/or memory formation based on behavioural outputs. When applied to animals subject to anatomical, pharmacological or genetic manipulations, these behavioural outputs are generally interpreted as direct consequences of these interventions. However, a number of studies report a strong dependence of behavioural results on laboratory environment factors which include training

apparatuses and procedures; mouse housing conditions and experimenter effects (Wahlsten et al., 2003; Gerlai, 2001b; Crabbe et al., 1999).

Effects of differences in training apparatus and experimental setup, can be ruled out when comparing the present study and the study by Peters et al (2003) as they were performed in the same facility. However, effects of animal housing and rearing conditions are prone to vary between testing periods of different animal cohorts. This can explain, not only the discrepancies between the published and the present study, but also the differences in swim speed between the first and second cohorts trained by the same experimenters as reported in sections 3.4.6.1 and 3.4.6.2.

A strong influence of laboratory environments on behavioural outcomes for a number of tests has been reported even when variability in mouse rearing conditions and experimental apparatuses are minimised (Wahlsten et al., 2003; Crabbe et al., 1999). Nevertheless, a number of studies report that one of the factors that can account for the highest variability in behavioural responses in a number of behavioural tests is differences in handling by the experimenter (Chesler et al., 2002a; Chesler et al., 2002b; Wahlsten, 2001; Gerlai and Clayton, 1999). However, it is important to mention that the impact of the variability of experimental conditions or experimenter effects in the hidden platform version of the MWM has never been systematically addressed.

Pre-training handling was longer in the experiments described in this chapter (ten days), than in the published study (six days; Peters et al., 2003). Pre-training handling is known to contribute to reducing stress and fearful responses to the presence of the experimente. For this reason, it is possible that a longer period of handling in the current study has contributed to differences in stress responses and

consequent differences in performance. Furthermore, handling of the animals during the training procedure is also known to strongly influence anxiety related behaviours such as thigmotaxis and escape from the platform (Gerlai, 2001a; Gerlai and Clayton, 1999). Subtle differences in handling during the training procedure may have contributed to differences in the behavioural outputs obtained. The training procedure described in this chapter may have contributed to a faster learning by the mutant group when compared to the cohorts trained by Peters et al. In fact, analysis of acquisition data in the second cohort of mice trained in the MWM (Fig. 3.10) revealed longer escape latencies and path lengths during the third and fourth day of training. Although, as discussed above, these are not accurate measurements of spatial memory formation, it is possible that a difference in probe trial performance could have been found on these particular training days, at least for this cohort of mice.

Another important factor to bear in mind when interpreting the behavioural data is the statistical power of the sample sizes used (Wahlsten, 2001). While the data presented in Peters et al (2003) refers to a high number of animals (WT: n=11; mut n=12), the present data refers to rather smaller sample sizes. Larger sample sizes confer not only an increased statistical power to the analysis but can also buffer intrinsic individual differences in learning abilities (Matzel et al., 2003).

### 3.5.2. BIOLOGICAL EFFECTS

The gene targeting technique used to generate the CaMKK$\beta$ null mutant mice, produced a general deletion of the functional transcript affecting all the body in all developmental stages. Lack of the functional CaMKK$\beta$ protein may have triggered

alterations in a set of developmental, physiological and even behavioural processes in order to compensate for the effect of the mutation (Gerlai, 2001b). These compensatory mechanisms may operate differently or vary across generations. Furthermore, polymorphisms in the genetic background and general differences in the chromosomal constitution between littermates in a hybrid background may account for large biological variations in the genetic repertoire, which are prone to mask mutation-related phenotypes (Gerlai, 2001b; Dubnau and Tully, 1998). The behavioural study by Peters et al. (2003) used mice in the F2–F4 generations while the data reported in this chapter concerns mice in the F4-F5 generations. Therefore, the discrepancies between the behavioural phenotypes described in the Peters *et al.* (2003) study and the current study may be due to genetic background effects. In addition, animals used in the Peters et al. (2003) were between 2 and 6 months old, while in all the studies performed in this thesis, no animal was older than 4 months. Therefore, the contributuion of age effects in the phenotype described in the Peters *et al.* (2003) study, can not be ruled out.

Another important factor to bear in mind is that overtraining can overcome the behavioural deficit described, a feature common to other mouse lines such as the CREB$\alpha\delta$ mutants (Hebda-Bauer et al., 2005; Kogan et al., 1997; Bourtchuladze et al., 1994). A likely hypothesis is that, in these lines, a developmental or physiological compensation may determine changes that account for the molecular requirements of the specific training paradigm.

Despite the failure to replicate the behavioural phenotype previously described, the transcriptional screening reported in this chapter revealed genotype effects, in the levels of expression of a number of genes. Whether these differences can be attributed directly to CaMKK$\beta$ function, or are a consequence of

compensatory mechanisms triggered in response to the mutation can not be established at this stage. For the sake of simplicity, the candidate genes identified were designated "CaMKK$\beta$ regulated genes" due to differences in transcriptional levels between WT and CaMKK$\beta$ null mutant mice.

### 3.5.3. ARRAY ANALYSIS

The present study aimed to identify CaMKK$\beta$ regulated genes in the hippocampus of male mice. As explained in section, 3.4.3, the first screening process was performed in mice that had been trained in the MWM. Thus, both the mutation and the training procedure may have contributed to the regulation of gene expression.

Microarray data may display a very high degree of intra-group variation which may be derived from true biological differences or differences in sample preparation and technical limitations (Chudin et al., 2002; Pritchard et al., 2001).

As described in chapter II, preparation of the mix of cRNAs to be hybridized with the array is a complex process encompassing multiple steps. Despite monitoring of sample quality after each of these steps, small differences in the handling of different samples may compromise sample quality and introduce non systematic errors which can not be accounted for in the analysis. Furthermore, the array manufacture conditions may also impose constraints: non specific interactions due to combinatorial complexity; thermodynamic equivalence of probes; accuracy and spatial uniformity of probe synthesis onto the glass slide (Chudin et al., 2002). Importantly, as well, known immediate-early genes with functions in L&M processes

such as Arc (Guzowski et al., 2000) are not probed in these arrays and were obviously missed in this transcriptional analysis.

The use of microarrays to characterize transcription profiles in total brain or brain tissues such as the hippocampus has been reported in a number of studies (Datson et al., 2004; Lein et al., 2004; Zhao et al., 2001). However, detection of change in gene expression in the brain is complicated by a series of factors like the complexity of the tissue analysed, transcript abundance, magnitude of transcriptional changes and time point addressed which is particularly important for the detection of transcriptional changes induced by behavioural training.

**Nature of the tissue analysed**

The hippocampus is a heterogeneous tissue, composed by a variety of cells with different molecular and electrophysiological properties (Zhao et al., 2001; Amaral and Witter, 1995). The use of alternative techniques such as microdissection of hippocampal subfields (Datson et al., 2004) or mRNA *in situ* hybridization (ISH) (eg. Zhao et al., 2001) analysis may help to narrow down the subset of cells where the changes occur and provide a more accurate estimation of the magnitude of the fold change. In addition, transcriptional analysis at the single cell level, for example by single cell laser capture (Tietjen et al., 2003) may provide valuable insights into the magnitude and cell-specificity of the transcriptional changes elicited by behavioural training.

**Magnitude of transcriptional changes**

The differences in transcription between genotypes, either in the baseline or in an activity induced manner, may be subtle and close to the level of "noise"(Barlow

and Lockhart, 2002). Changes of higher magnitude are also likely to occur in the brain, but these may be restricted to small number of cells and "diluted" when a whole tissue is analysed (Barlow and Lockhart, 2002).

Use of an alternative technique for detection of hippocampal transcripts, serial analysis of gene expression (SAGE) in parallel with *Affymetrix* microarray analysis revealed the unreliability of the latter technique for the detection of low abundance transcripts (Evans et al., 2002), which largely outnumber the high abundance transcripts. A post reverse transcription amplification could probably improve the detection of low abundant transcripts at the expense of potentially saturating expression levels of more abundant genes.

The small number of biological replicates used (four per group) and within group heterogeneity in gene profiles (Pritchard et al., 2001) imply an increased likelihood of detecting transcriptional differences that are not related to the mutation. The use of larger sample sizes would permit the "dilution" of within-group variations.

In the present analysis, the above factors were considered by setting a rather low threshold (30%) of fold change in gene expression between the groups. The obvious disadvantage of setting such a low threshold is an increased false positivity rate.

The initial microarray screening aimed at the identification of transcriptional differences between WT and CaMKK$\beta$ mut mice after training in the MWM. The following experimental step addressed whether any of these transcriptional changes could be detected in the hippocampus of naïve male WT and CaMKK$\beta$ mutant mice. Because the follow up procedures focused on a group of mice under different conditions than the ones used for the initial microarray screening, the current experimental design did not allow the estimation of the positivity rate of the

microarray screening. For this reason it is not pertinent to compare the results of the current study with other published studies on microarray screenings of hippocampal tissue. Neverthless, the genes initially detected in the microarray screening and after confirmed to be CaMKK$\beta$-regulated in naïve animals (Arih1, SRp20, PSF; Table 3.3.), or after MWM training (Gaa1; Fig. 3.13), were all detected by GeneSpring and only one of these was detected by dCHIP. This suggests that Genespring yelded a higher positivity rate, at least within the group of genes tested. Discrepancies in the target genes detected by the two softwares can be interpreted on the bases of the normalilzation procedures used. While dCHIP starts by normalizing brightness across chips to the array with median overall brightness, GeneSpring starts by normalizing gene expression levels within a chip and only after normalizes levels of gene expression to the median brightness of each particular probe set. Under the prevailing conditions, GeneSpring represented a more reliable method of analysis as this was less biased by variations in hybridization procedures across chips. This also suggests that the hybridization step was critical for the final outcome of the analysis.

**Time points:**

The MWM is one of the most commonly used tasks to assess spatial memory formation in rodents. The parameter normally measured is selectivity towards the area where a hidden platform was located which implies a hippocampus dependent formation of a spatial representation of the environment (Morris et al., 1982). This is a complex learning task, with multiple behavioural and cognitive demands: swimming, learning that the platform is the only way to escape the water, and

learning the platform location (Gerlai, 2001a). Training paradigms comprise multiple trials generally given in the course of several days.

Experimental designs used in previous studies to detect changes in gene expression after training in the MWM differ in the number of training trials used, the number of days of training, the use of probe trials and the time points of sacrifice of the animals (Leil et al., 2003; Cavallaro et al., 2002; Leil et al., 2002). The complexity of the task and the time points chosen for the analysis of behavioural training induced changes affect strongly the final outputs from the transcriptional analysis, for this reason a comparison between the transcriptional outputs found in this study and found in other studies is not appropriate.

The present study used a three day training protocol, followed by a probe trial, 30 min after which the animals were sacrificed. The results of the transcriptional change should be strictly interpreted in the context of this experimental design: First of all, changes in transcription of particular target genes occur with a distinctive time course (Cavallaro et al., 2002), and the present experimental design missed transcriptional changes occurring earlier in the course of training; Second: the transcriptional changes associated with spatial training may be induced by the behavioural experience (handling, swimming, exposure to the room) without necessarily having a role in memory consolidation; Third: The training protocol encompassed a number of training trials over a number of days. Therefore, it is also possible that the transcriptional changes detected were elicited by memory reconsolidation, rather than consolidation (Morris et al., 2006). Fourth: in principle, the experience of swimming in the absence of the platform during the probe trial can induce extinction of the memory of the platform or platform location. Attempts to

distinguish between experience-related and learning-induced transcriptional changes are described in chapter IV.

### 3.5.4. QPCR FOLLOW UP ASSAY

QPCR was used as an independent follow-up method to identifiy CaMKK$\beta$ regulated genes. This technique is accurate enough to detect 20% changes in gene expression (Bustin, 2000). With this technique, variations in gene expression between CamKK$\beta$ null mutants and WT male mice were identified for four candidate genes. QPCR analysis for the remaining candidate genes failed to detect significant inter-group variations. This might be due to the fact that these are not CaMKK$\beta$ regulated genes, and their detection resulted from artefacts in the microarray procedure.

### 3.5.5. TRANSCRIPTIONAL CHANGES IDENTIFIED

Differences in transcriptional levels in the naïve hippocampus were identified for four genes (three of which at the baseline level, and one in induced by behavioural training in the MWM). Lack of CaMKK$\beta$ resulted in lower levels of expression of SRp20 and higher levels of expression of PSF in the hippocampus of male mice. However, lack of this kinase did not have an impact on levels of expression of these genes in the female hippocampus. For Arih1, loss of CaMKK$\beta$ resulted in opposite patterns of expression between the sexes, with a downregulation in male mutants and an upregulation in female mutants. These results point to a sex-specific role of this kinase in the regulation of gene expression in the naïve hippocampus. This finding was consistent with a sex specific role of this kinase in

hippocampal plasticity earlier described (Mizuno et al, 2006). Furthermore, these findings suggest sexual dimorphisms in the pattern of regulation of these genes in the naïve hippocampus, a hypothesis that was investigated and will be addressed in chapter IV.

Additionally, the identification of Gaa1, a gene not regulated by CaMKK$\beta$ in the naïve hippocampus but regulated by spatial training, points to differences in the spatial training transcriptional outputs between the genotypes. This was an expected finding considering differences in spatial training induced CREB activation and L-LTP described by Peters et al (2003).

It is important to emphasize, however, that investigation of most of the transcripts identified in the initial microarray screening was pursued no further. For example, the hypothesis that genes other than Gaa1 are regulated by CaMKK$\beta$ in an activity-dependent manner remains to be determined. As discussed in section 3.4.3.3, a role for the proteins encoded by some of these genes in synaptic plasticity and L&M has been previously described.

A more thorough investigation of the correlations between the transcriptional changes identified and L&M processes, the sex-specific patterns of gene expression, and the "behavioural training-induced" changes in gene expression are the focus of chapter IV. The potential significance of these transcriptional changes for memory consolidation will be addressed in chapter V.

## 3.5.6. A POSSIBLE ROLE FOR THE CANDIDATE GENES IN THE CONSOLIDATION OF HIPPOCAMPUS-DEPENDENT MEMORY

### 3.5.6.1. ARIADNE UBIQUITIN-3 LIGASE (Arih1)

#### 3.5.6.1.1. UBIQUITINATION AT THE SYNAPSE

Selective protein degradation plays an essential role in cell homeostasis, allowing clearance of harmful metabolites, cell cycle progression and regulation of transcription (Voet and Voet, 1995).

Proteins are targeted for degradation via covalent attachment of a small monomeric protein – ubiquitin. Ubiquitination occurs through three sequential processes catalyzed by ubiquitin activating (E1), conjugating (E2), and ligase (E3) enzymes. Multi-ubiquitinated targets are recognized by a large protein complex: the proteosome, which hydrolyses long polypeptide chains into small peptides (Voet and Voet, 1995).

Converging evidence establishes the importance of ubiquitination in normal synaptic function: In *Drosophila*, mutations in components of the ubiquitination pathway cause phenotypes including loss of synaptic growth control and axon guidance and defects in presynaptic neurotransmitter release (DiAntonio et al., 2001; Kitada et al., 1998; Muralidhar and Thomas, 1993). In *Aplysia*, proper function of the ubiquitination machinery is crucial for activity-dependent transcriptional activation (Yamamoto et al., 1999; Hegde et al., 1993). In addition, the gene encoding Ubiquitin C-terminal hydrolase (an enzyme that contributes to faster proteolysis by the proteosome) is an activity-dependent immediate early gene both in *Aplysia* and rodents (Foley et al., 2000; Hegde et al., 1997).

### 3.5.6.1.2. A ROLE FOR Arih1 IN MEMORY CONSOLIDATION?

Several classes of E3 ubiquitin ligase have been described. Each individual E3 ligase recognizes a restricted a set of proteins, which accounts for a tight regulation of ubiquitination of distinct substrates (reviewed by Pickart, 2004). Mutations in genes encoding E3 ubiquitin ligases are related to human diseases associated with cognitive impairments such as juvenile onset Parkinsonism and Angelman´s syndrome (Kitada et al., 1998; Jiang et al., 1998) .

Ariadne-1 is a E3 ubiquitin ligase gene that belongs to the Really Interesting New Gene (RING)-finger family of proteins (Aguilera et al., 2000). *Drosophila* mutants for the orthologue of Arih-1 exhibit severe developmental deficits affecting neuronal connectivity, and reduced rough endoplasmic reticulum suggesting compromised transmenbrane protein processing (Aguilera et al., 2000). This protein is highly conserved between *Drosophila,* mouse and humans and hence likely to display affinity for similar substrates (Tan et al., 2000). A known substrate of the human protein is the translation initiation factor eif4, suggesting that Arih1 can not only regulate protein degradation, but also protein translation (Tan et al., 2003).

As summarized in table 5.1, Arih1 levels display a sex specific pattern of expression being higher in the female than in the male adult naïve hippocampus. Curiously, in pre-pubertal animals the sex difference was observed in the opposite direction, suggesting that estrogen exposure may play a role in the transcriptional regulation in Arih1. In order to determine whether estrogens influence its expression level, Arih1 regulation could be studied in gonadectomized animals: First, an age effect would not be expected in gonadectomized females; Second, castration of males immediately after birth would prevent the organizational effects of estrogen and ablate the pre-pubertal sex difference.

Additionally, mice trained in the MWM and contextual fear conditioning express, on average, higher levels of the gene than the naïve counterparts, but this difference did not reach significance. In females, this lack of statistical significance can be due to hormonally driven fluctuations in levels of gene expression. This hypothesis could be tested by the monitoring levels of gene expression in different stages of the estrous cycle.

### 3.5.6.2. GPI-anchor attachment protein 1 (Gaa1)

#### 3.5.6.2.1. PROTEIN GLYCOSILATION

The attachment of glycosylphosphatidylinositol (GPI) moieties to proteins constitutes a post-translational modification mechanism that permits anchoring of proteins lacking transmembrane domains to the exterior surface of the plasma membrane (Fivaz and Meyer, 2003; Voet and Voet, 1995).

A role for glycosilation processes in memory consolidation has been established, as specific inhibitors of glycosilation exert potent amnesic effects if administered right before or shortly after training or 5-6 hours after training (Rose and Jork, 1987). GPI-anchored proteins are incorporated into lipid rafts- subregions of the plasma membrane that are rich in cholesterol. A number of cell adhesion molecules that support stable contacts between neurons are GPI anchored and cluster in this region. These are also thought to intervene in the transmission of intracellular signals presumably by interaction with transmembrane signalling receptors (Tsui-Pierchala et al., 2002).

GPI anchoring occurs in the rough endoplasmic reticulum and is catalysed by a transamidase complex. Gaa1 is an important component of this complex, as genetic deletion in yeast or disruption of protein function in murine cell lines prevent

expression of GPI anchored proteins on the cell surface membrane (Hiroi et al., 2000; Hamburger et al., 1995).

#### 3.5.6.2.2. A ROLE FOR Gaa1 IN MEMORY CONSOLIDATION?

Gaa1 mRNA levels are increased after training in the MWM and contextual fear conditioning in WT mice. In addition, the results described on chapter III indicate that Gaa1 expression is regulated by CaMKK$\beta$ in male mice, in response to behavioural training in the MWM.

Synaptic remodelling is believed to be one of the mechanisms supporting memory encoding in the hippocampus. In line with this view, a training induced upregulation of Gaa1, if mirrored at the protein level, could increase transamidase activity and levels of GPI anchored proteins incorporated into synapses; These GPI anchored proteins, for example, adhesion molecules, could contribute to the formation of new synaptic contacts and/or reinforcement of previously existing ones.

### 3.5.6.3. ALTERNATIVE SPLICING IN NEURONS

Maturation of pre-mRNA into translatable mRNA requires intron removal and exon ligation – a process known as splicing. This process is catalysed by the spliceosome- a complex composed of a large number of ribonucleoproteins and small nuclear RNAs (Roberts and Smith, 2002).

Alternative inclusion/exclusion of exons is influenced by cis-regulatory elements in exonic and intronic sequences. These sequences can recruit a number of trans acting splicing factors which can either promote or prevent spliceosome

assembly (Roberts and Smith, 2002; Will and Luhrmann, 1997). Cell-specific exon selection for a given pre-mRNA is determined by the stoichometry between splicing repressors and activators.

A number of studies describe regulation of alternative splicing in neuronal cells in response to membrane depolarization (Xie and Black, 2001), induction of LTP (O'Connor et al., 2004) or seizures (Bottai et al., 2002), exposure to stressful conditions (Nijholt et al., 2004), and physical activity (Beffert et al., 2005).

The present work studied the transcriptional profiles of two genes encoding two multifunctional nuclear proteins (PSF and SRp20), initially described as splicing regulators. These genes were identified as CaMKK$\beta$ regulated genes in the naïve male hippocampus and shown to be regulated by training in the MWM and contextual fear conditioning in males in the case of SRp20 and in both sexes in the case of PSF.

#### 3.5.6.3.1. SPLICING REGULATOR 20 (SRp20)

SR proteins constitute a multifunctional family of proteins primarily involved in regulation of alternative splicing. SR family members display overlapping specificities, which may account for redundant functions (reviewed in Graveley, 2000). However, targeted deletion of SRp20 is embryonically lethal at the blastocyst stage indicating that, at least at this stage of development lack of SRp20 can not be compensated by other proteins (Jumaa et al., 1999).

Cellular functions of SRp20 are fundamentally related to RNA metabolism and nuclear export. These encompass: (a) regulation of alternative splicing in a number of mRNAs (Galiana-Arnoux et al., 2003; Jumaa and Nielsen, 1997) including its own (Jumaa and Nielsen, 2000); (b) Regulation of alternative polyadenylation (Lou et al.,

1998) and (c) Nucleocytoplasmic export of intronless mRNA (Shav-Tal and Zipori, 2002).

### 3.5.6.3.2. A ROLE FOR SRp20 IN MEMORY CONSOLIDATION?

As described in chapter III, SRp20 is a CaMKK$\beta$ regulated gene in the male but not in the female naïve hippocampus. Furthermore, baseline expression in the WT naïve hippocampus is higher in males than in females; In addition, training in hippocampus-dependent tasks induces SRp20 transcription in males but not in females.

In addition to antisense, other possible approach towards establishing the importance of SRp20 in the cellular mechanisms underlying memory consolidation would be to inject the hippocampus with oligonucleotides corresponding to SRp20 binding consensus sequences to titrate the pool of SRp20 protein in the cell. Possible limitations of these approaches would be compensation by other SR protein family members.

The identification of the mRNAs that are regulated by SRp20 for example by means of a three hybrid system (Hook et al., 2005) or bioinformatics analysis, would be necessary to understand how SRp20 regulation could account for the cellular mechanisms underlying memory consolidation.

### 3.5.6.3.3. PTB- Splicing Factor associated protein (PSF)

PSF was initially identified in co-purification assays with the polypyrimidine tract binding protein PTB (Patton et al., 1993). Structurally, it comprises RNA recognition motifs and DNA binding domains. Apart from a well described function in splicing regulation both in association and independently of PTB (Will and Luhrmann,

1997), a number of other functions have been assigned to PSF: (a) nuclear retention of mis-edited mRNA through interaction with nuclear scaffolding proteins (Shav-Tal and Zipori, 2002); (b)Transcriptional repression of progesterone receptor and insulin growth factor mediated transcription (Urban and Bodenburg, 2002; Mathur et al., 2001); (c) Nuclear anchoring protein of the γisoform of protein kinase C which is usually cytoplasmic (Rosenberger et al., 2002); (d) DNA repair, by rejoining of DNA strand breaks (Bladen et al., 2005).

PSF expression is developmentally regulated being higher in post-mitotic differentiating neurons both in the last postgestational and early postnatal weeks. In adulthood PSF expression is restricted to areas maintaining a high level of synaptic plasticity like the hippocampus and the olfactory bulb (Chanas-Sacre et al., 1999).

Insights into the potential importance of this protein in the brain come from the observation that fetuses with Down syndrome have much lower levels of PSF in the brain than do healthy controls, accompanied by a downregulation of important components of the basal transcriptional machinery (Freidl et al., 2001).

### 3.5.6.3.4. A ROLE FOR PSF IN MEMORY CONSOLIDATION?

PSF mRNA levels are higher in CaMKKβ null mutant animals, and in WT animals trained in the MWM and contextual fear conditioning tasks. The observed upregulation of PSF after training in the MWM and its higher levels of expression in CaMKKβ null mutant naïve mice, which were initially described as being impaired in MWM performance, raise two alternative hypotheses: (a) PSF upregulation in naïve CaMKKβ null mutants is a compensatory mechanism triggered in response to lack of CaMKKβ; (b) CaMKKβ signalling represses PSF transcription in WT mice. In addition, if PSF does indeed play a role in memory consolidation, it is possible that

higher basal PSF expression in CaMKK$\beta$ null mutant males account for normal spatial memory in some of the cohorts tested, such as the ones described in this thesis.

Functional studies both in WT and CaMKK$\beta$ null mutants, such as the ones suggested for SRp20 would be essential to elucidate this hypothesis. In addition, identification of the pre-mRNA targets of PSF and of the target genes upon whose expression PSF exerts a repressor activity could provide deeper insights into the patterns of gene regulation elicited by behavioural training. For example, it is possible that some of these target genes encode products with an inhibitory function in memory consolidation. Therefore, transcriptional repression of these genes would represent a way of reducing inhibitory constraints in memory storage (Chen et al., 2003; Abel et al., 1998).

# CHAPTER IV:
# RESULTS II

## 4.1. INTRODUCTION

Peters and colleagues (2003) demonstrated the contribution of CaMKK$\beta$ to memory consolidation in some hippocampus-dependent tasks (STFP and spatial version of the MWM) in male mice. Furthermore, CaMKK$\beta$ null mutant male mice failed to activate the transcription factor CREB after spatial training and exhibited impairments in the transcription dependent phase of LTP. These results demonstrate the importance of the transcriptional events mediated by CaMKK$\beta$ in synaptic plasticity and in the formation spatial memories. Importantly, deletion of CaMKK$\beta$ did not produce any of these effects in females (Mizuno et al., 2006), suggesting a male-specific requirement of this kinase for the processes described above.

The previous chapter reports the identification of four CaMKK$\beta$ regulated genes encoding a GPI-anchor transamidase (Gaa1), an E3-ubiquitin ligase (Arih1) and two multifunctional nuclear proteins (SRp20 and PSF). Different levels of expression of these genes were initially detected in a microarray analysis of hippocampal tissue of male WT and CaMKK$\beta$ null mutant mice after training in the MWM. Confirmatory studies using an independent technique (QPCR) revealed that levels of expression of three of these genes (SRp20, PSF and Arih1) differed between genotypes in the naïve hippocampus, while for a fourth gene (Gaa1) this difference arised from a spatial training induced upregulation occurring only in WT animals.

A number of studies have established correlations between behavioural training and transcriptional regulation of target genes, some of which specifically induced after training in hippocampus-dependent tasks (von Hertzen and Giese,

2005; Levenson et al., 2004a; Leil et al., 2003; Cavallaro et al., 2002; Leil et al., 2002; Ressler et al., 2002; Hall et al., 2000). Functional studies in rats (Lee et al., 2004; Guzowski et al., 2000) and insights from mutant mouse studies (e.g. Giese et al., 2001; Tsien et al., 1996; Bourtchuladze et al., 1994) have revealed the direct contribution of some of these genes to memory consolidation. The work described in the present chapter investigated the transcriptional profile of the four CaMKK$\beta$ regulated genes after training in two hippocampus-dependent tasks: the MWM and contextual fear conditioning.

As described in the previous chapter, a between-genotype comparison of levels of gene expression in the female naïve hippocampus, revealed a sex-specific requirement of CaMKK$\beta$. In the present chapter, the characterization of the pattern of gene expression in the naïve hippocampus was further pursued: First, levels of expression were compared between the sexes in adult mice, revealing sex specific levels of expression; Second, in order to determine whether this sex-specificity is determined before or after the onset of the pubertal hormone surge, levels of expression were compared between adult and pre-pubertal mice; Third, *in situ* hybridizations were used to characterize the pattern of mRNA expression within hippocampal subfields, CA1, CA3 and DG, and to compare it between the sexes. In addition, whether the training induced upregulation detected in males was also triggered in females, was also investigated.

## 4.2. SEX DIFFERENCES IN GENE EXPRESSION IN THE NAÏVE HIPPOCAMPUS

The findings reported in chapter III indicate: a requirement of CaMKK$\beta$ for the basal expression of Arih1, PSF and SRp20 in the male hippocampus; a sex-specific requirement of CaMKK$\beta$ for Arih1 basal expression; and the requirement of CaMKK$\beta$ for the MWM spatial training induced upregulation of the gene Gaa1 in the male hippocampus. Based on these findings, the baseline levels of expression of the target genes were compared between naïve adult male and female WT mice (Fig. 4.1). Hippocampal tissue from eight WT males and seven WT females was used for the quantitative real-time PCR assay. As explained in chapter II, individual levels of expression that deviated from the Mean by more than two standard deviations were excluded from the analysis. The number of animals used for the gene expression analysis is indicated below each bar.

Levels of gene expression of Arih1, Gaa1, PSF and SRp20 differed significantly between the sexes in the hippocampus of naïve adult male mice and naïve randomly cycling adult female mice. Levels of expression of Arih1 were higher in females than in males [Fig. 4.1A, male: 100.0 ± 16%, female: 143.9 ± 8.8%, $F(1,11)=5.99$, $p<0.05$, one-way ANOVA with genotype as variable]. For the remaining genes the opposite pattern, higher levels of expression in males than in females, was found [Fig. 4.1B, Gaa1, male: 100.0 ± 5.8%, female: 70.3 ± 2.6%, $F(1,13)=21.33$, $p<0.001$; Fig. 4.1C, PSF, male: 100 ± 11%, female: 60 ± 14 %, $F(1,11)=16.08$, $p<0.001$; Fig. 4.1D, SRP20, male: 100.0 ± 7.6%, female: 78.9 ± 3.7%, $F(1,13)=5.93$, $p<0.05$, one-way ANOVA with genotype as variable].

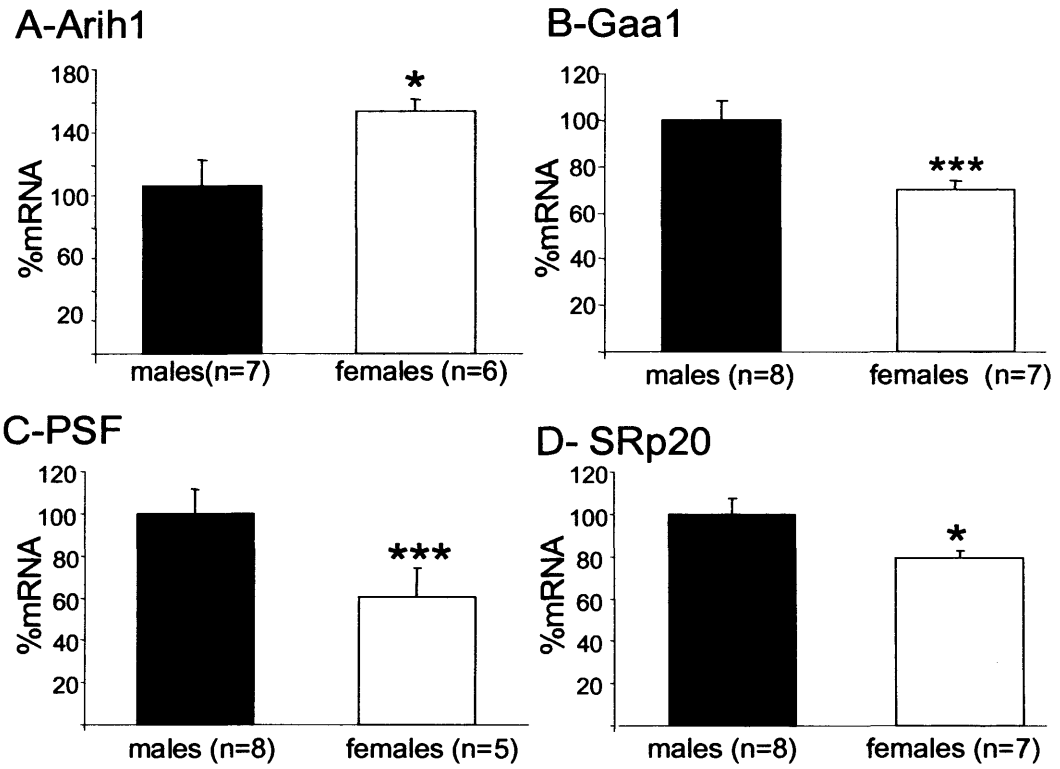


**Figure 4.1- Sex differences in levels of gene expression in the naïve hippocampus.** **(A)** Transcriptional levels of expression of Arih1 were higher in female than male mice. **(B, C, D)** Hippocampal transcriptional levels of Gaa1, PSF and SRp20 were higher in males than in females. (MEAN ± SEM; *p<0.05; ***p<0.001; % mRNA levels relative to WT males).

## 4.3. HIPPOCAMPAL SUBFIELD LOCALIZATION OF SEX DIFFERENCES IN GENE EXPRESSION

The previous section described sex differences in the levels of gene expression of four candidate genes in whole hippocampal tissue. The hippocampus is constituted by a set of subfields characterized by distinct morphology, connectivity and electrophysiological properties (Amaral and Witter, 1995). These hippocampal subfields are also molecularly distinct, as a number of studies report restricted or enriched expression of particular genes in discrete hippocampal subfields (Datson et al., 2004; Lein et al., 2004; Zhao et al., 2001). Characterization of the pattern of expression and relative quantification of mRNA levels of the candidate genes within hippocampal subfields in male and female

mice was performed by *in situ* hybridizations of coronal brain sections covering the central part of the hippocampus (bregma -1.46 mm to -2.46 mm, according to Paxinos and Franklin, 2002). This approach allowed also a comparison of levels of gene expression between the sexes in the three principal hippocampal subfields: CA1, CA3 and DG.

Two alternative methods were used to quantify levels of gene expression: densitometry and number of counts above threshold density, as described in chapter II. As explained in the methods section, these measurements were averaged for the number of sections within each group and subfields considered. The results were not analysed statistically, as the number of animals in each group was very small. For most of the comparisons performed there were no obvious differences in levels of gene expression between the conditions compared, contrarily to what was previously determined by QPCR. Technical and biological factors may contribute to these discrepancies. Possible technical causes include: (a) the small number of animals used in each *in situ* analysis (2-3 animals per condition), as opposed to an average of six per group in the QPCR analysis and (b) variations in overall densities between different slides arising from different hybridization efficiencies and contributing to a high within-group variation. Possible biological causes include intra-group variability in hippocampal mRNA levels of the target genes. In addition, a recent report describes heterogeneity in expression of a number of target genes along the dorsal-ventral axis of the hippocampus (Leonardo et al., 2005). If this is the case for Arih1, measurements of mRNA expression in coronal sections spanning a relatively broad longitudinal area may have eclipsed true differences restricted to discrete areas.

### 4.3.1. Arih1

Arih1 mRNA is expressed at similar levels in both sexes in all the hippocampal subfields considered (Fig. 4.2A). Neither of the measurements detected obvious sex differences in levels of gene expression in the distinct hippocampal subfields considered (Fig. 4.2A-D).

### 4.3.2. Gaa1

Densitometric analysis of Gaa1 mRNA expression revealed no differences in regional distribution and no overall sex differences between hippocampal subfields (Fig. 4.3A). The number of counts above threshold measurement suggested lower levels of Gaa1 mRNA in females in the CA1 (Fig. 4.3B) and DG (Fig. 4.3D) subfields.

### 4.3.3. PSF

Densitometric analysis of anatomical distribution of PSF mRNA revealed no apparent sex differences in expression in either of the hippocampal subfields compared. There were apparently higher levels of PSF mRNA in the DG compared to CA1 or CA3 in both sexes. Number of counts above threshold pointed to slightly lower levels of expression of PSF in females in all three hippocampal subfields considered (Fig. 4.4B-D).

### 4.3.4. SRP20

Analysis of the anatomical distribution of SRp20 mRNA revealed slightly higher levels of expression in the DG compared to areas CA1 and CA3 in both sexes (Fig. 4.5A). No apparent sex differences in densities were detected in any of the subfields considered. The number of counts above suggested sex differences in the expression of SRp20 in the naïve hippocampus particularly in areas CA1(Fig. 4.5B) and CA3 (Fig. 4.5C) but not in the DG (Fig. 4.5D).

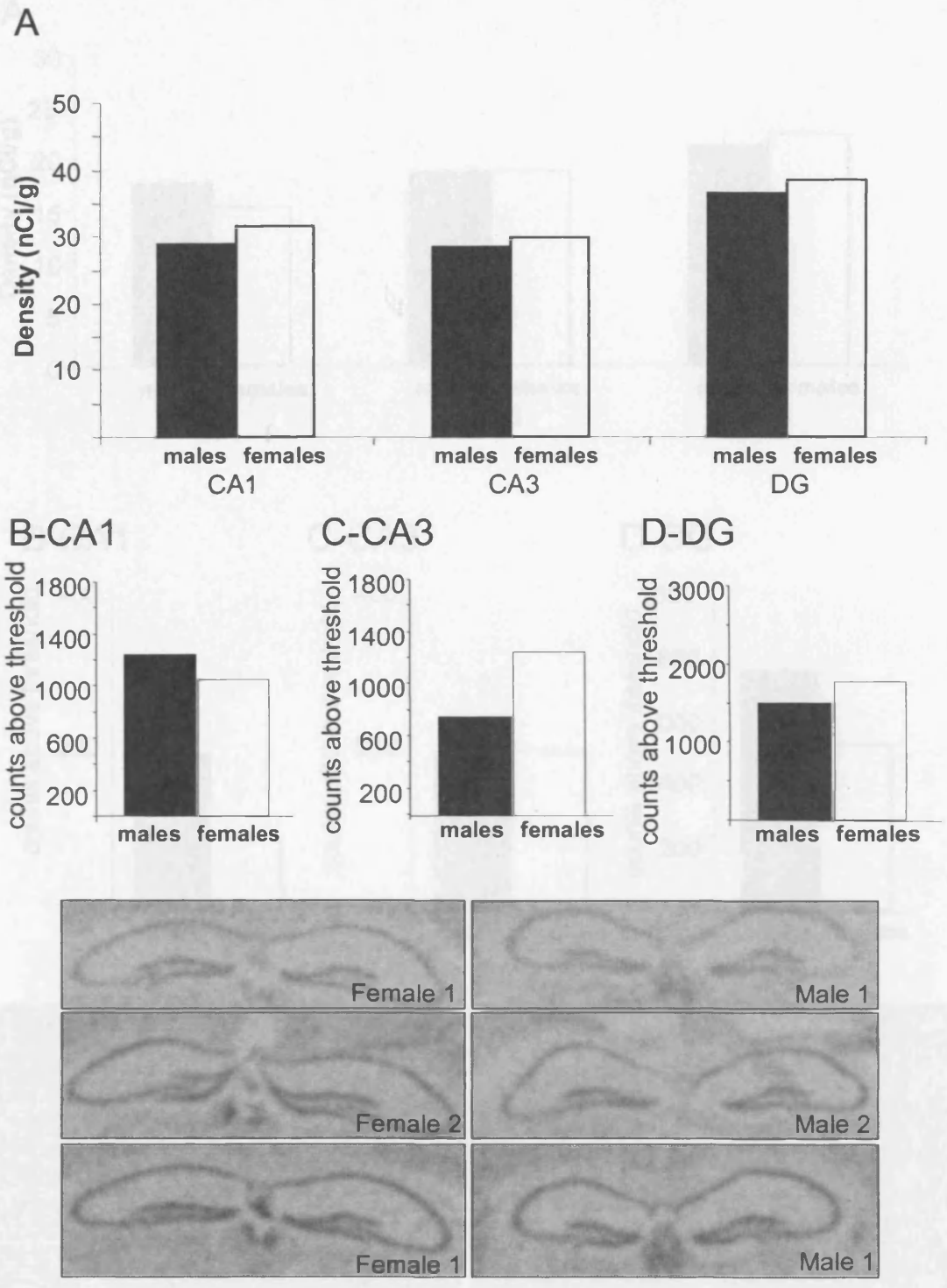


**Figure 4.2- Expression of Arih1 mRNA in the hippocampus of naïve male and female mice. (A)** Density levels of expression did not differ between the sexes in either of the hippocampal subfields considered; Number of counts above threshold density did not differ between the sexes for **(B)** CA1, **(C)** CA3 or **(D)** DG hippocampal subfields. **(E)** Representative *in situ* hybridizations of coronal brain sections of male and female naïve mice (males n=3 ; females, n=2; 8 sections/group).

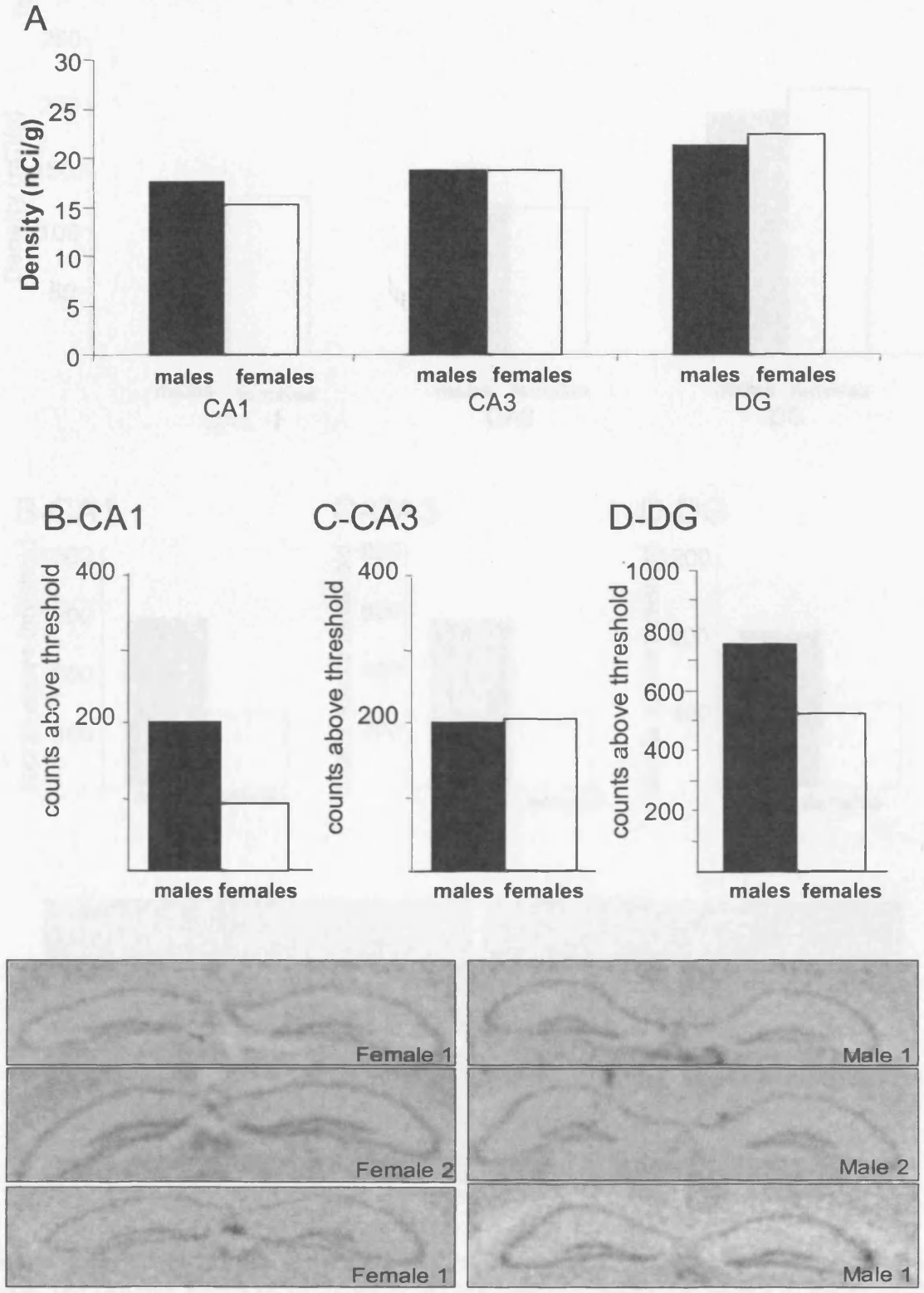


**Figure 4.3- Expression of Gaa1 mRNA in the hippocampus of naïve male and female mice. (A)** Density levels of expression did not differ between the sexes in either of the hippocampal subfields considered; Number of counts above threshold density was significantly higher in males in **(B)** CA1 but not in **(C)** CA3 or **(D)** DG hippocampal subfields. **(E)** Representative *in situ* hybridizations of coronal brain sections of male and female naïve mice (males n=3 ; females, n=2; 10 sections/group).

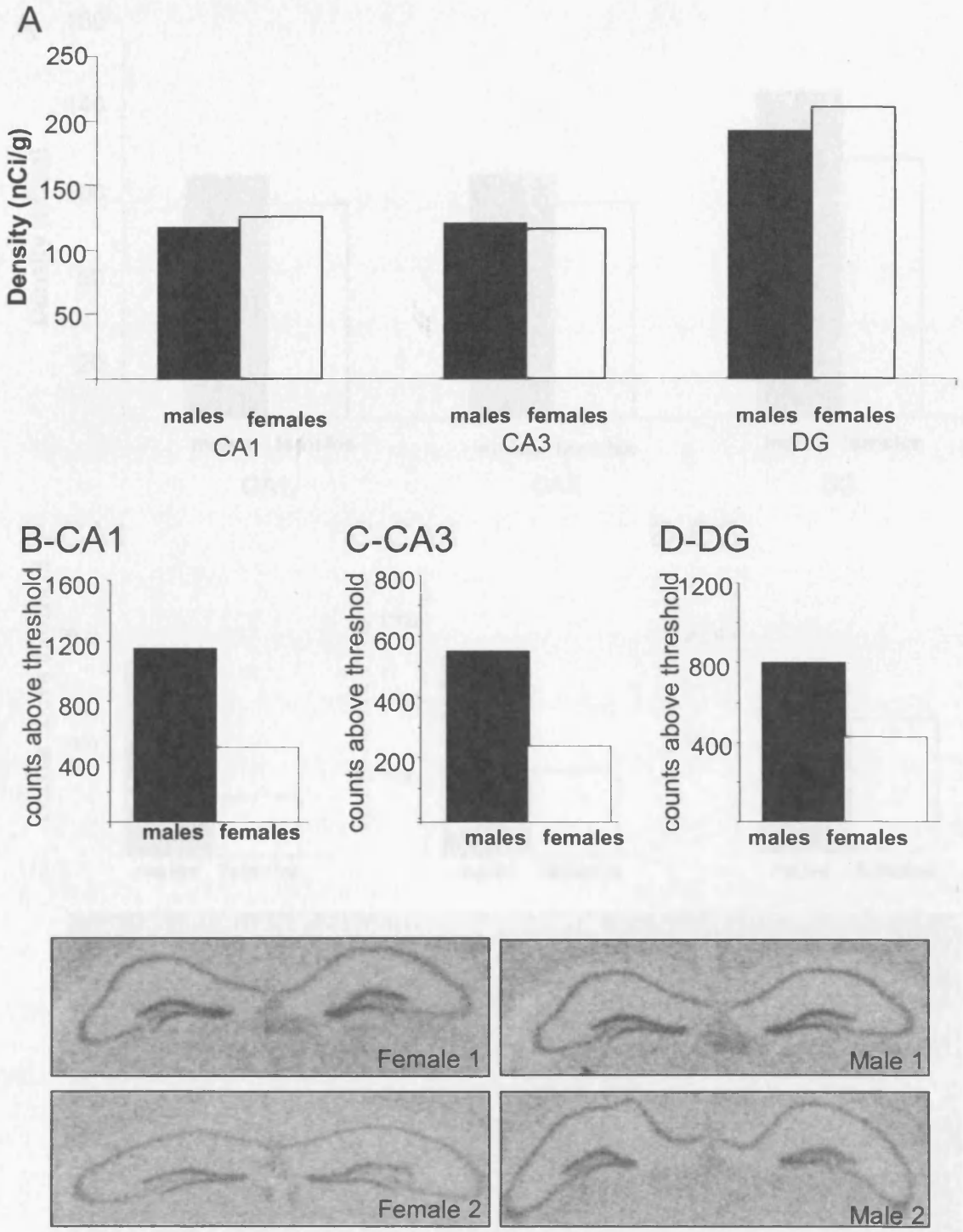


**Figure 4.4- Expression of PSF mRNA in the hippocampus of naïve male and female mice. (A)** Density levels of expression did not differ between the sexes in either of the hippocampal subfields considered; Number of counts above threshold density was significantly higher in males in subfield **(B)** CA1 and **(C)** CA3 but not in the **(D)** DG. **(E)** Representative *in situ* hybridizations of coronal brain sections of male and female naïve mice (males n=3 ; females, n=2; 10 sections/group).

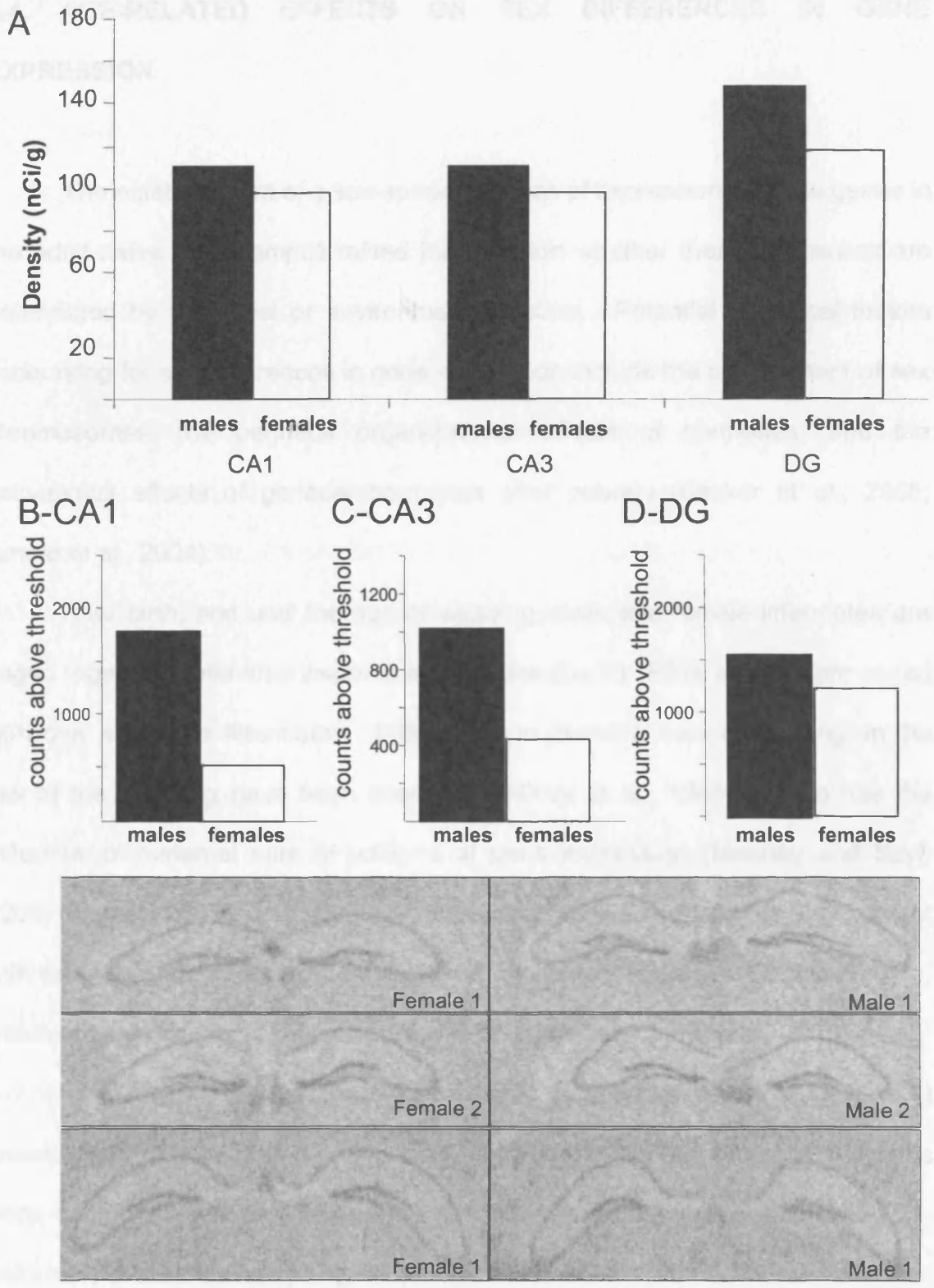


**Figure 4.5- Expression of Srp20 mRNA in the hippocampus of naïve male and female mice. (A)** Density levels of expression differed significantly between the sexes in all hippocampal subfields. Number of counts above threshold revealed significantly higher density in males in subfield **(B)** CA1 a trend towards higher expression in males in subfield **(C)** CA3 and no difference in the **(D)** DG. **(E)** Representative *in situ* hybridizations of coronal brain sections of male and female naïve mice (males n=3/12 sections; females, n=3/10 sections).

## 4.4. AGE-RELATED EFFECTS ON SEX DIFFERENCES IN GENE EXPRESSION

The establishment of a sex-specific pattern of expression of these genes in the adult naïve hippocampus raised the question whether these differences are determined by biological or environmental factors. Potential biological factors accounting for sex differences in gene expression include the complement of sex chromosomes, the perinatal organizational effects of hormones, and the activational effects of gonadal hormones after puberty (Becker et al., 2005; Arnold et al., 2004).

After birth, and until the age of weaning, male and female littermates are caged together, while after weaning at postnatal day 21 (P21), animals are caged with only same sex littermates. Differences in maternal care depending on the sex of the offspring have been described (Alleva et al., 1989) and so has the influence of maternal care in patterns of gene expression (Meaney and Szyf, 2005; Meaney, 2001). In addition, after weaning, males and females only interact with same sex littermates and are exposed to different pheromone environments, which are also known to influence gene expression (e.g. Gore et al., 2000)

To address whether the transcriptional differences described (Fig. 4.1) arose before or after puberty, hippocampal mRNA levels of the candidate genes were compared between 2-3 month old animals (adult) and animals at P21, before the onset of puberty (Fig. 4.6).

### 4.4.1. Arih1

Levels of Arih1 mRNA expression are affected by sex and age [Fig. 4.6A, effect of sex: $F(1,1)=1.63$; $p=0.32$; effect of age: $F(2,20)=29.52$, $p<0.001$; sex x age interaction: $F(2,20)=26.32$, $p<0.001$, two-way ANOVA with sex and age as variables]. Student-Newman-Keuls *post-hoc* analysis revealed significantly higher levels of gene expression in pre-pubertal males when compared to pre-pubertal females ($p<0.001$). The direction of this difference is reversed in adult animals, as males express significantly lower levels of the transcript than females. A possible cause for this effect is pubertal secretion of gonadal hormones in females, as levels of expression are significantly higher in adult than in P21 females, ($p<0.001$), but do not differ with age in males ($p=0.83$).

### 4.4.2. Gaa1

Levels of expression of Gaa1 are significantly influenced by sex but not by age [Fig. 4.6B, effect of sex: $F(1,1)=7.25$; $p<0.05$; effect of age: $F(2,20)=3.21$, $p=0.089$; sex x age interaction: $F(2,20)=0.017$, $p=0.90$, two-way ANOVA with sex and region as variables]. Student-Newman-Keuls *post-hoc* analysis confirmed the sex difference in adults previously described (Fig. 4.1). Furthermore, a trend towards higher levels of gene expression in males was detected in P21 animals. Increasing the number of animals in the P21 group would be necessary to confirm whether the sex difference in SRp20 expression is indeed present before puberty. No age effect was detected in either sex (males: $p=0.26$; females: $p=0.80$).

### 4.4.3. PSF

Levels of expression of PSF are significantly influenced by age [Fig. 4.6C, effect of sex: $F(1,1)=0.84$; $p=0.37$; effect of age: $F(2,20)=24.28$, $p<0.001$; sex x age interaction: $F(2,20)=2.92$, $p=0.10$, two-way ANOVA with sex and age as variables]. Sex differences in levels of expression of PSF only arise in adulthood (P21 males vs females: $p=0.60$, Student-Newman-Keuls *post-hoc* test). Furthermore, levels of expression are reduced with age in both sexes (males: $p<0.05$; females: $p<0.001$).

### 4.4.4. SRp20

Sex differences in expression of SRp20 are detectable before the onset of puberty with females expressing lower levels of the transcript [Fig. 4.6D, effect of sex: $F(1,1)=7.75$; $p<0.05$; effect of age: $F(2,20)=2.65$, $p=0.12$; sex x age interaction: $F(2,20)=0.53$, $p=0.48$, two-way ANOVA with sex and age as variables; Student-Newman-Keuls *post-hoc* analysis: P21 males vs P21 females: $p<0.05$; P21 vs adult males: $p=0.12$; P21 vs adult females, $p=0.52$].

In summary, the results described in this section indicate that sex differences in Arih1 and SRp20 expression levels can be detected before the onset of puberty. A trend for sex differences in Gaa1 expression was detected in P21 animals, but an increased number of animals would be necessary to draw conclusions on age effects in the regulation of this gene. Sex differences in PSF expression only arise after puberty.

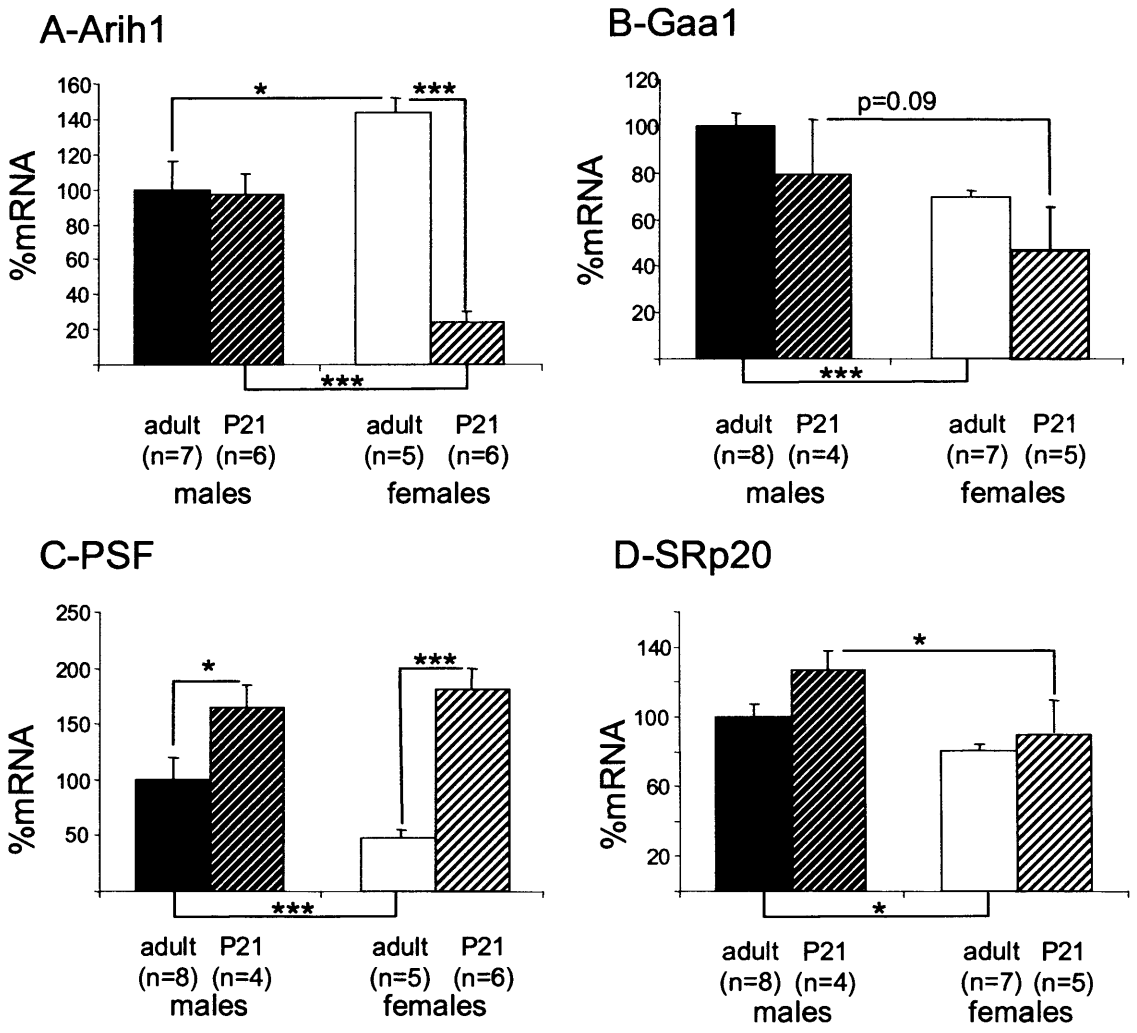


**Figure 4.6- Comparison of hippocampal levels of expression of the candidate genes between pre-pubertal (P21) and adult mice. (A)** Arih1 mRNA levels differed between the sexes before the onset of puberty, and between P21 and adults in females only; **(B)** A trend towards a sex difference in Gaa1 mRNA was detected before the onset of puberty; **(C)** PSF mRNA levels did not differ between the sexes in pre-pubertal, and were lower in adults of both sexes; **(D)** Sex differences in SRp20 mRNA levels were detected before the onset of puberty (Mean ± SEM; *p<0.05; ***p<0.001; % mRNA levels relative to WT adult males).

## 4.5. TRAINING INDUCED CHANGES IN GENE EXPRESSION – MORRIS WATER MAZE

### 4.5.1. TRAINING OF MALE AND FEMALE MICE IN THE MORRIS WATER MAZE

Adult WT male (n=11) and female (n=11) littermates were trained in the hidden platform version of the MWM in a 4 trials per day protocol, and tested in a probe trial at the end of the sixth day of training. There was a significant effect of training in decreased latency time to find the platform and a significant sex x training interaction [Fig. 4.7A, effect of sex: $F(1,20)=2.579$, $p=0.12$; effect of training: $F(5,100)=38.68$ $p<0.001$; sex x training interaction: $F(5,100)=2.85$, $p<0.05$, two-way repeated measures ANOVA]. Student-Newman-Keuls *post-hoc* analysis revealed a significantly higher escape latency in the female group on the third day of training ($p<0.01$).

During probe trials there was no difference in average swim speed [Fig. 4.7B, males: 26.3 ± 1.7 cm/s; females: 28.2 ± 1.7 cm/s; $F(1,20)=0.63$; $p=0.44$, one-way ANOVA with sex as variable]. A trend towards higher percentage of time spent in the thigmotaxis zone was detected in the female group [Fig. 4.7C, males 8.8 ± 2.0 %; females: 19.1 ± 5.0 %; $F(1,20)=3.75$; $p=0.060$, one-way ANOVA with sex as variable]. The percentage of time spent swimming at a speed lower than 5cm/s, considered as floating, was similar between the sexes [Fig. 4.7D, males: 14.4 ± 1.8%; females: 16.2 ± 2.4%; $F(1,20)=0.36$; $p=0.55$; one-way ANOVA with sex as variable).

A probe trial at the end of the sixth day of training revealed the same degree of selectivity in males and females. One-way ANOVA with pool quadrant as variable revealed a global preference for the previous platform location within each sex group, measured by percentage of time spent in the quadrant [Fig. 4.8A, males: $F(3,40)=4.82$, $p<0.01$, *post-hoc* Student-Newman-Keuls comparisons: TQ vs AL and TQ vs OP: $p<0.01$; TQ vs AR: $p<0.05$; females: $F(3,40)=4.32$; $p<0.01$, *post-hoc* Student-Newman-Keuls comparisons: TQ vs AR: $p<0.01$; TQ vs AL and TQ vs OP: $p<0.10$], number of platform crossings [Fig. 4.8B, males: $F(3,40)=4.55$; $p<0.01$; *post-hoc* Student-Newman-Keuls comparisons: TQ vs OP: $p<0.01$; TQ vs AR: $p<0.05$; females: $F(3,40)=3.56$; $p<0.05$, *post-hoc* Student-Newman-Keuls comparisons: TQ vs AR and TQ vs OP, $p<0.05$: TQ vs AL, $p<0.10$] or cumulative proximity to platform in TQ [Fig. 4.8C, males: $F(3,40)=5.23$; $p<0.01$, *post-hoc* Student-Newman-Keuls: TQ vs OP: $p<0.01$; TQ vs AR and TQ vs AL: $p<0.05$; females: $F(3,40)=2.81$; $p=0.05$, *post-hoc* Student-Newman-Keuls: TQ vs AR: $p<0.05$; TQ vs OP: $p=0.10$]. Furthermore, one-way ANOVA with sex as variable revealed similar percentages of time spent in TQ [Fig. 4.8A, males: 34.8 ± 4.0%, females: 32.6 ± 3.4%; $F(1,20)=0.17$; $p=0.69$], number of platform crossings [Fig. 4.8B, males: 5.2 ± 1.0, females: 4.8 ± 0.9; $F(1,20)=0.071$; $p=0.79$) and cumulative proximity to platform [Fig. 4.8C, males: 46.4 ± 3.0m, females: 54.8 ± 3.5m; $F(1,20)=3.32$; $p=0.083$].

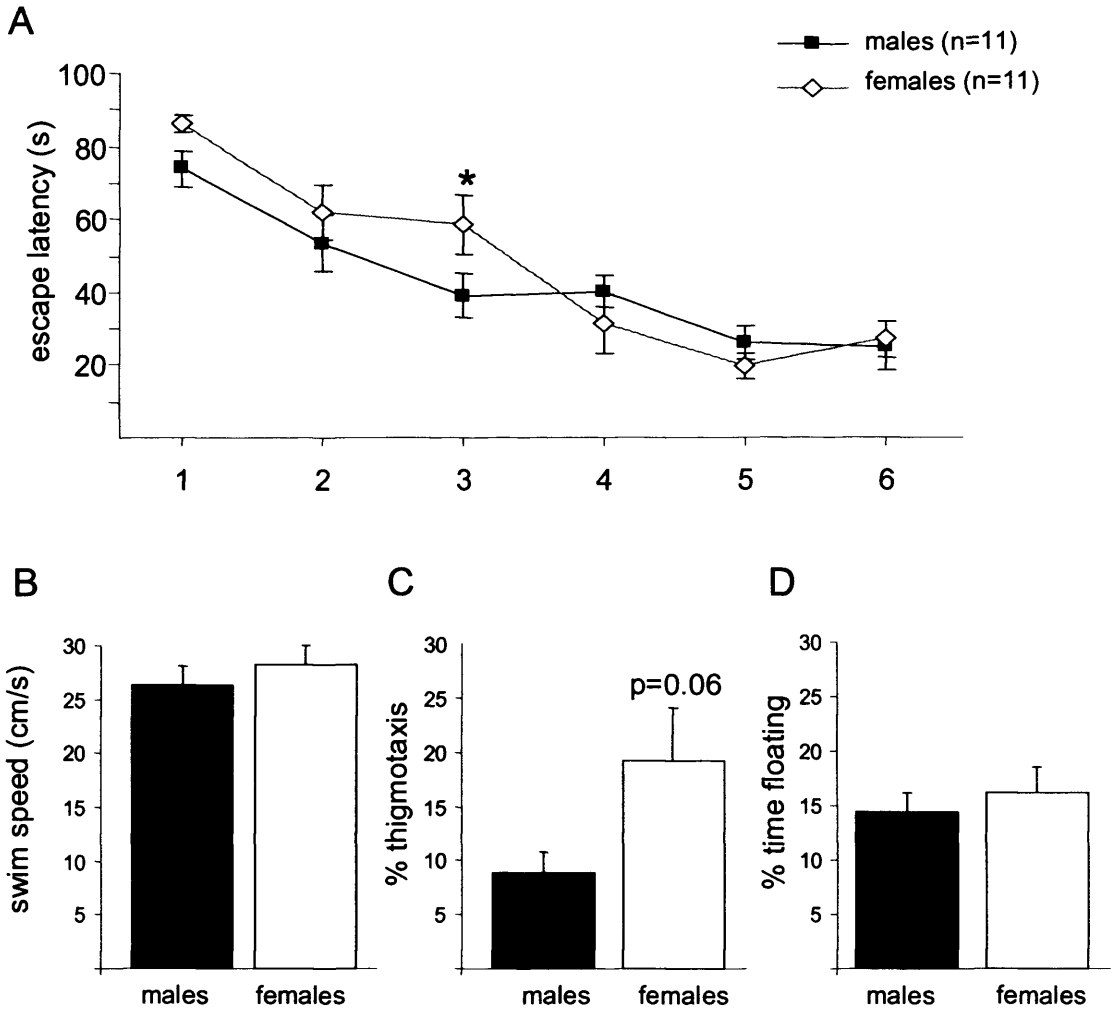


**Figure 4.7- Acquisition and swimming abilities of male and female WT mice trained in the Morris Water Maze.**
**(A)** Both sexes decreased their latency times to find the platform with the number of training trials; **(B)** During the probe trial, swim speeds did not differ between genotypes; **(C)** A trend towards a higher percentage of time swimming in the thigmotaxis area was detected for the female group. **(D)** The percentage of time spent floating did not differ between the sexes. (Mean ± SEM, *p<0.05)

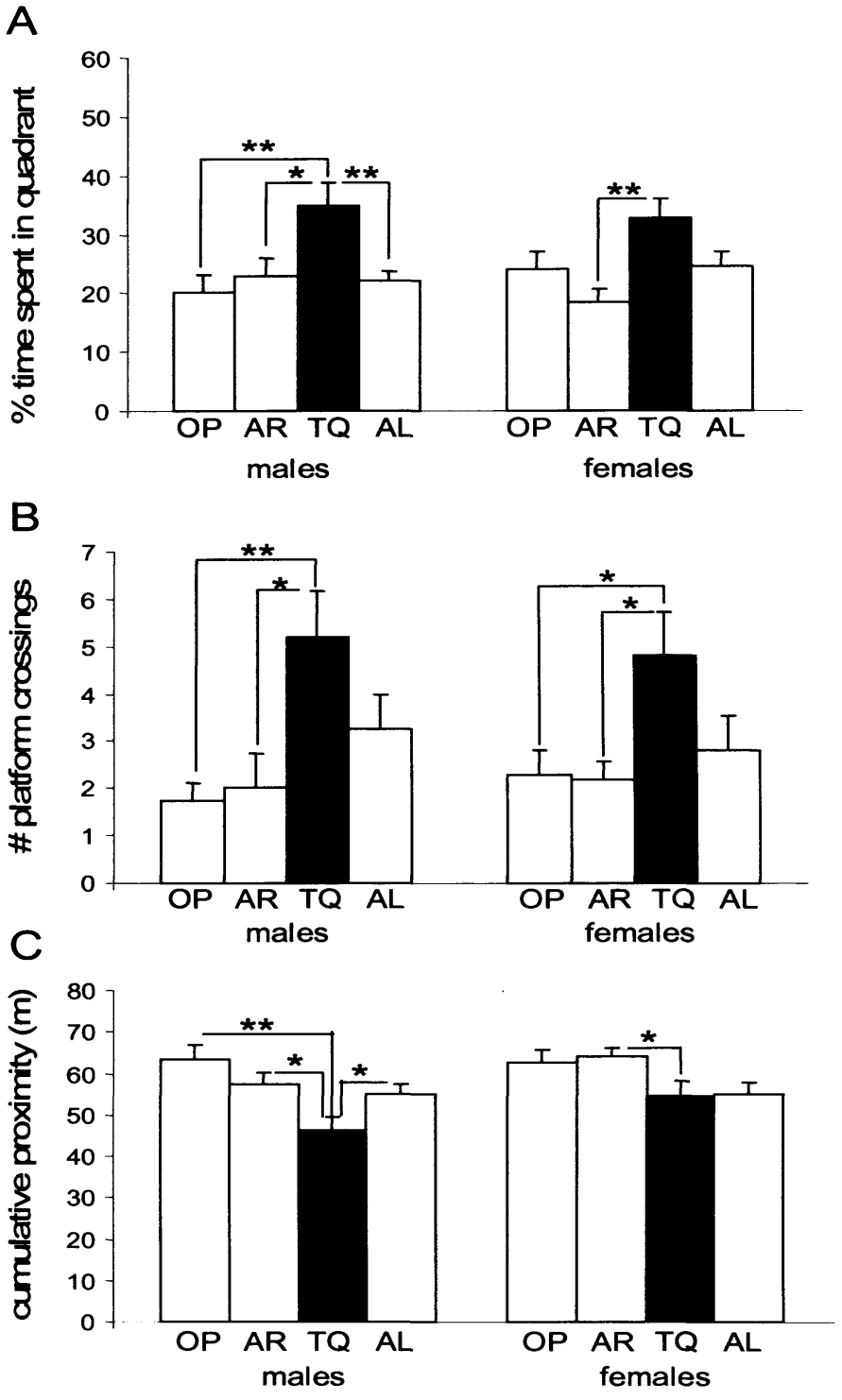


**Figure 4.8- Probe trial performance of male and female WT mice trained in the Morris Water Maze. (A)** Males and females spent a significantly higher percentage of their search time in TQ, and this selectivity did not differ between the sexes; **(B)** Both sexes crossed the platform position in TQ more often than any other quadrant, and number of platform crossings in TQ did not differ between the sexes; **(C)** Cumulative distance to platform position in TQ did not differ between the sexes, but, in the female group, cumulative distance to TQ was only different from cumulative distance to AR. (Mean ± SEM, *p<0.05, **p<0.01, TQ- target quadrant; AR- adjacent right; AL- adjacent left; OP- opposite)

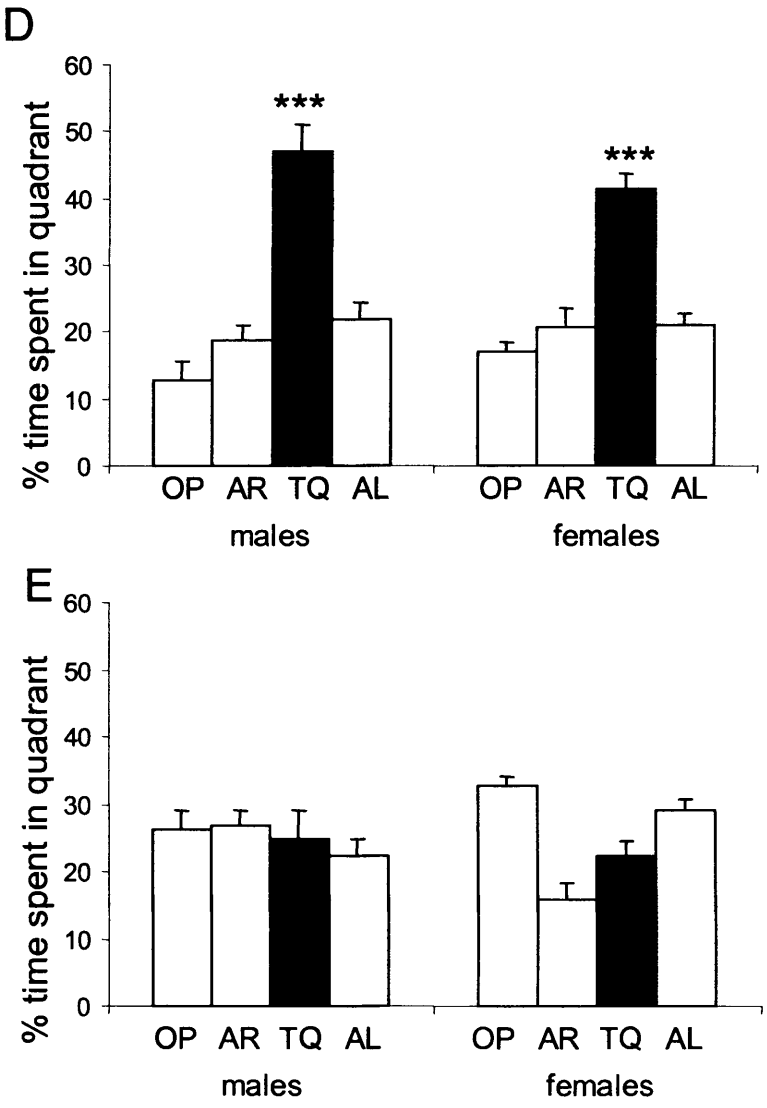


**Figure 4.8 (cont)- Probe trial performance of male and female WT mice trained in the Morris Water Maze.** Percentage of time spent in quadrant for selective (D) and non selective (E) animals (Mean ± SEM, ***p<0.001; TQ- target quadrant; AR- adjacent right; AL- adjacent left; OP- opposite).

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### 4.5.2. EXPERIMENTAL GROUPS

Preliminary data pointed to an upregulation of mRNA levels of Gaa1 (Fig. 3.12), Srp20 and PSF (data not shown) after spatial training in the MWM in a four trials per day training protocol, followed by a probe trial at the end of the sixth day

of training. In order to address whether these transcriptional changes were specifically elicited by learning of the platform location or by other factors such as handling or motor activity, four experimental groups were used in the gene expression analysis: (a) Naïve: Animals were sacrificed directly from their home cages; Animals trained in the hidden platform version of the MWM with a 4 trials per day protocol for 6 days and tested in a probe trial at the end of the sixth day of training. According to the percentage of time spent in the TQ during the probe trial animals were divided in a subgroup of (b) selective animals (that spent significantly more time in TQ than in any other quadrant; Fig. 4.8D) and (c) non-selective animals (that did not display preference towards TQ; Fig. 4.8E). An additional group: (d) swim control group swam in the pool without a platform, for an equal number of sessions and trials and for the same period of time as the average duration of a training trial on the respective day of training.

### 4.5.3. REGULATION OF HIPPOCAMPAL GENE EXPRESSION AFTER TRAINING IN THE MWM IN MALE MICE

Arih1 expression was not regulated after MWM training under the current training conditions [Fig. 4.9A; naïve: 100 ± 16%; selective: 130 ± 19%; $F(1,10)=1,67$; $p=0.22$, one-way ANOVA with training as variable].

In contrast, levels of expression of the remaining genes differed significantly between the naïve and selective groups. Therefore, a transcriptional analysis was performed in two additional experimental groups: animals trained in the MWM that failed to display selectivity towards TQ in the probe trial (non selective animals), and swim control groups (Fig. 4.9 B, C, D).

For Gaa1, a significant difference in the levels of expression between the groups was found [Fig. 4.9B, naïve: 100.0% ± 5.9%, swim controls: 113± 13 %, non selective: 141 ± 11 %; selective: 149 ± 12 %; $F(3,22)=5.06$; $p<0.01$ one-way ANOVA with training as variable]. Student-Newman-Keuls *post-hoc* comparisons revealed a significant difference between naïve vs selective and naïve vs non selective animals ($p<0.05$), a trend towards significance between selective and non selective animals when compared to swim controls ($p<0.10$), and no significant difference between the groups that had been trained in the MWM. These findings suggest that swimming and exposure to the room are not sufficient to trigger Gaa1 upregulation.

PSF mRNA levels were significantly different between the four groups [Fig. 4.9C, naïve: 100.0% ± 4.1%; swim controls 144.2 ± 9.2%; non selective, 161.4 ± 8.1%; selective 139 ± 15 %; $F(3,23)=9.48$; $p<0.001$, one-way ANOVA with training as variable]. Training in the absence of a platform was sufficient to trigger PSF upregulation as levels of expression differed significantly between naïve and all the remaining groups (naïve vs non selective and naïve vs swim controls $p<0.001$; naïve vs selective $p<0.05$, *post-hoc* Student-Newman-Keuls comparisons), but not between swim controls and animals trained in the MWM ($p>0.05$).

Levels of expression of SRp20 differed significantly between the groups (Fig. 4.9D, naïve 100 ± 10 %; swim controls 130.5 ± 7.1%; non selective, 152 ± 16 %; selective 177.0 ± 11%; $F(3,20)=9.47$; $p<0.001$, one-way ANOVA with training as variable). *Post-hoc* Student-Newman-Keuls analysis revealed significant differences between naïve and all the remaining groups ($p<0.001$ vs selective group; $p<0.01$ vs non selective and $p<0.05$ vs swim controls), and

higher levels in selective animals than in swim controls ($p<0.05$). This suggests that the conditions to which the swim controls were exposed can only partially account for the upregulation of SRp20 in the selective animals.

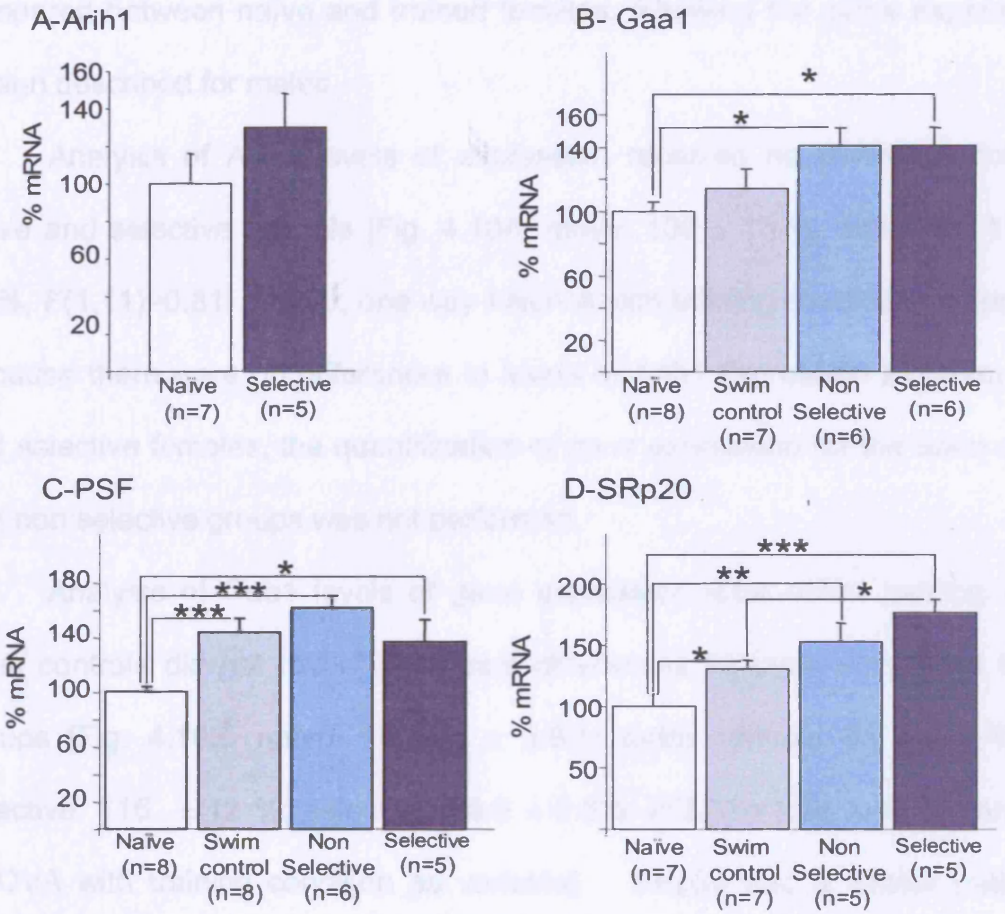



**Figure 4.9- Comparison of hippocampal levels of gene expression between male naïve and MWM trained mice. (A)** Arih1 mRNA levels did not differ significantly between naïve and selective animals; **(B)** Gaa1 mRNA levels did not differ between naïve and swim controls, but differed between naïve and trained animals; **(C, D)** PSF and SRp20 mRNA levels differed between naïve and all the remaining groups. (Mean ± SEM, *$p<0.05$, **$p<0.01$, ***$p<0.001$, % mRNA levels relative to naïve).

### 4.5.4. REGULATION OF HIPPOCAMPAL GENE EXPRESSION AFTER TRAINING IN THE MWM IN FEMALE MICE

The genes shown to be regulated after MWM training in male mice (Fig. 4.9), display sex-specific levels of expression in the naïve hippocampus (Fig. 4.1)

and a requirement of CaMKK$\beta$ for basal expression in males but not females (Figs. 3.5 and 3.6). In order to determine whether behavioural training-induced expression of these genes was sexually dimorphic, levels of expression were compared between naïve and trained females, following the same experimental design described for males.

Analysis of Arih1 levels of expression revealed no difference between naïve and selective animals [Fig. 4.10A, naïve: 100 ± 13 %, selective: 112.0 ± 9.0%, $F(1,11)=0.81$; $p=0.39$, one way ANOVA with training condition as variable]. Because there were no differences in levels of Arih1 expression between naïve and selective females, the quantification of gene expression for the swim control and non selective groups was not performed.

Analysis of Gaa1 levels of gene expression after MWM training and in swim controls did not reveal significant differences between naïve and trained groups [Fig. 4.10B, naïve: 100.0% ± 3.8%; swim controls 98 ± 10 %; non selective: 116 ± 12 %; selective: 96.0 ± 5.5%; $F(3,23)=1.16$, $p=0.35$, one-way ANOVA with training condition as variable]. SRp20 had a similar pattern of expression [Fig. 4.10D, naïve: 100.0% ± 4.1%; swim controls: 98.6 ± 6.6%; non selective: 115.6 ± 9.4%; selective: 106.4 ± 2.9; $F(3,22)=1.58$; $p=0.22$, one-way ANOVA with training condition as variable]. Training in the MWM induced upregulation of expression of PSF in females [Fig. 4.10C, naïve: 100.0% ± 9.5%; swim controls: 125.8 ± 9.4%; selective: 145 ± 11 %; $F(2,17)=7.20$; $p<0.01$, one-way ANOVA with training condition as variable]. Student-Newman-Keuls *post-hoc* analysis revealed a significant difference in PSF mRNA levels between the naïve and swim control groups ($p<0.05$) and naïve and selective animals ($p<0.01$), but not between swim controls and selective animals ($p=0.16$).

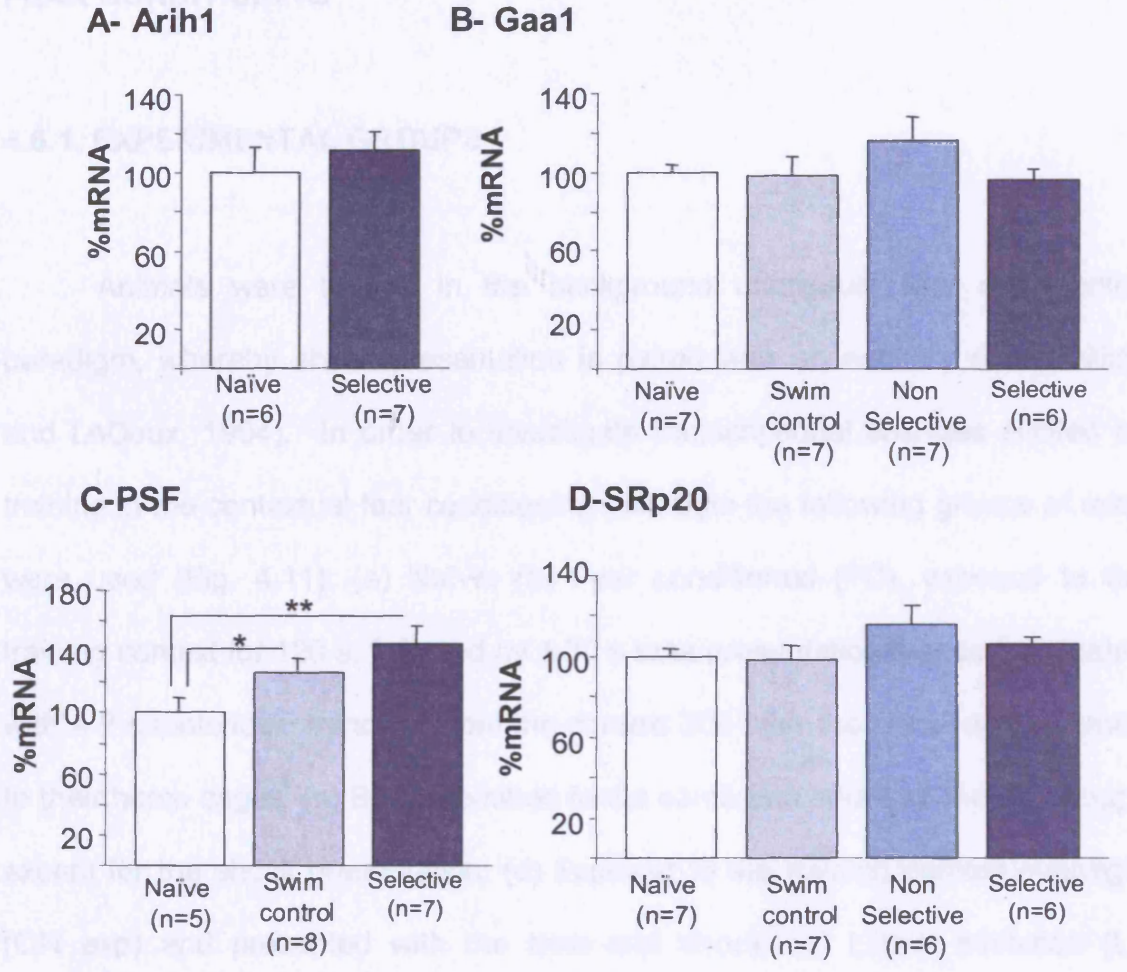



**Figure 4.10- Comparison of hippocampal levels of gene expression between naïve and trained female mice (MWM). (A)** Arih1 mRNA levels did not differ significantly between naïve and selective animals; **(B)** Gaa1 mRNA levels did not differ between the four groups; **(C)** PSF mRNA levels differed between naïve and the remaining groups; **(D)** SRp20 mRNA levels did not differ between the four groups. (Mean±SEM, *p<0.05, **p<0.01; % mRNA levels relative to naïve).

## 4.6. TRAINING INDUCED CHANGES IN GENE EXPRESSION- CONTEXTUAL FEAR CONDITIONING

### 4.6.1. EXPERIMENTAL GROUPS

Animals were trained in the background contextual fear conditioning paradigm, whereby shock presentation is paired with an auditory cue (Phillips and LeDoux, 1994). In order to investigate transcriptional changes elicited by training in the contextual fear conditioning paradigm the following groups of mice were used (Fig. 4.11): (a) Naïve; (b) Fear conditioned (FC), exposed to the training context for 120 s, followed by a 30 s tone presentation that co-terminated with a 2 s footshock, removed from the context 30s after the shock and returned to their home cages; (c) Box, submitted to the same procedure as the FC groups except for the shock presentation; (d) Exposed to the training context overnight (ON exp) and presented with the tone and shock; (e) Latent inhibition (LI) exposed to the training context overnight and presented with the shock without the tone.

For the expression studies, mice were sacrificed 30 min after the end of the tone, and the naïve group was sacrificed at the same time. Experimental control groups were tested 24 hours after conditioning.

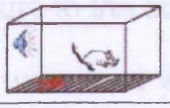
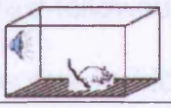
| | FC | Box | ON exp | LI |
|---|---|---|---|---|
| |  |  |  |  |
| Total exposure time | 3 min | 3 min | ON | ON |
| Tone | + | + | + | - |
| Shock | + | - | + | + |

**Figure 4.11- Experimental groups for the contextual fear conditioning task.**
FC- trained in a background contextual fear conditioning paradigm; Box- Exposed to the tone and context in the absence of the shock; ON exp- Exposed overnight to the context followed by tone and shock; LI- latent inhibition, Exposed overnight to the context followed by the shock in the absence of the tone.

### 4.6.2. BEHAVIOURAL TESTING OF THE EXPERIMENTAL GROUPS

The transcriptional profiles of the candidate genes were investigated in a range of experimental groups. Control groups were used in order to investigate whether the transcriptional changes detected after contextual fear conditioning were specifically caused by association of the foot shock with the context or the result of non specific factors. Animals trained in the contextual fear conditioning paradigm exhibited significant freezing responses when tested 24 h after conditioning. In contrast, exposure to the context and tone in the absence of foot shock (Box) did not evoke a freezing response [Fig. 4.12.A, Box: 3.8 ± 2.2%; FC: 47.8 ± 7.5%; $F(1,5)=42.30$; $p<0.01$, one-way ANOVA with training as variable].

A prolonged exposure to the context followed by tone and shock presentation elicited significantly lower freezing when compared to the contextual fear conditioning paradigm [Fig. 4.12.B, ON exp: 27.1 ± 5.6%; FC: 62 ± 13 %;

F(1,5)=7.54; p=0.040, one-way ANOVA with training as variable]. However, prolonged exposure to the context followed by shock presentation in the absence of the tone (LI) was more effective in blocking the conditioned response [Fig. 4.12.C, LI: 7.5 ± 4.8%; FC: 89.4 ± 8.9%; F(1,5)=76.27; p<0.001, one-way ANOVA with training as variable], indicating that, in this group, the animals failed to form the context-shock association. Because the LI group failed to form the context-shock association and was subject to the same degree of electrical stimulation as the FC group, this group was suitable for the study of shock induced transcriptional changes.

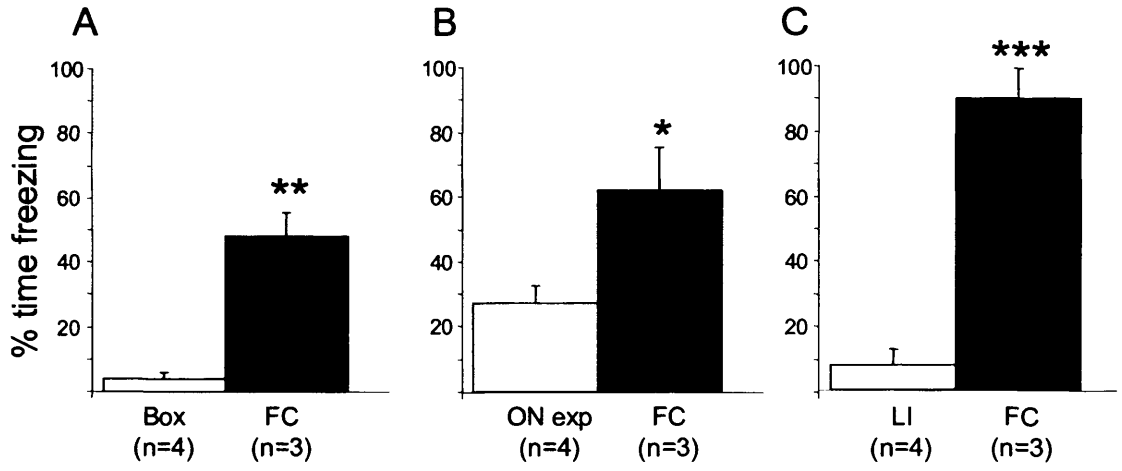



**Figure 4.12- Freezing scores of the control groups for the fear conditioning task.** **(A)** Animals exposed to the context and tone for 3 minutes failed to display a freezing response **(B)** Animals exposed to the context overnight followed by tone and shock displayed some degree of freezing, albeit less than the group tested in the contextual fear conditioning paradigm; **(C)** Animals exposed to the context overnight followed by the shock without the tone failed to display a freezing response. (Mean±SEM, *p<0.05, **p<0.01, ***p<0.001).

### 4.6.3. REGULATION OF HIPPOCAMPAL GENE EXPRESSION AFTER CONTEXTUAL FEAR CONDITIONING IN MALE MICE

Analysis of Arih1 mRNA levels after fear conditioning revealed an increase relative to the naïve group (Fig 4.13A, naïve: 100 ± 14 %; FC: 132 ± 10 %). However, presumably because of the high within group variability in Arih1 mRNA levels, this difference did not reach significance [$F(1,11)=3,70$; $p=0.08$, one-way ANOVA with training as variable], and characterization of the transcriptional profile of this gene was pursued no further.

A pilot experiment (data not shown) indicated that hippocampal mRNA levels of Gaa1, PSF and SRp20 were significantly higher in FC animals compared to naïve controls. The transcriptional profiles of these genes were studied in the control groups. This allowed investigating whether the up regulation was specifically related to the formation of a context-shock association or elicited by non specific factors such as handling, exposure to the context and/or tone or the electrical stimulation provided by the shock.

Analysis of gene expression levels after training in a fear conditioning paradigm revealed a significant effect of training for Gaa1 [Fig. 4.13B, naïve: 101.1% ± 4.5%; box: 99.1 ± 6.2%; LI: 94.3 ± 3.3%; ON exp: 123.4 ± 4.7%; FC: 120.6 ± 4.6%; $F(4,30)=5.97$; $p<0.01$, one-way ANOVA with training as variable]. *Post-hoc* Student-Newman-Keuls comparisons revealed that Gaa1 mRNA levels differed significantly between in the following comparisons: naïve vs FC ($p<0.01$), naïve vs ON ($p<0.01$), box vs FC ($p<0.05$), box vs ON ($p<0.05$); LI vs FC ($p<0.01$) and LI vs ON groups ($p<0.05$) and did not differ between the remaining groups. Hence, these results rule out the possibility that Gaa1

upregulation after contextual fear conditioning was elicited by handling, exposure to the context or to the tone.

As shown in Fig. 4.13B, a prolonged exposure to the context followed by paired shock-tone presentation, elicited a significantly lower conditioned response than training in the contextual fear conditioning paradigm. Gaa1 mRNA levels differed significantly between the ON exp and naïve and box groups ($p<0.05$ for both comparisons), and not between the ON exp and FC groups ($p>0.05$). Because these animals form some degree of context-shock association, albeit less than the animals trained in the contextual fear conditioning paradigm, this group is not suitable as a control for transcriptional events elicited by the shock alone. In the latent inhibition group, the freezing response was blocked suggesting a failure to associate the shock and the context. Levels of Gaa1 mRNA after training in the LI paradigm did not differ when compared to naïve control animals indicating that the shock alone did not elicit upregulation of Gaa1 mRNA.

Taken together, these findings suggest that the upregulation of Gaa1 expression in the hippocampus is the consequence of a context-shock specific association formed in the course of background contextual fear conditioning.

PSF gene expression levels differed between experimental groups [Fig. 4.13C, naïve: 102.0 ± 3.7 %; box: 107.7 ± 3.2%; LI: 97.1 ± 6.2%; ON exp: 136.2 ± 7.9%; FC: 132.0 ± 8.2 %; $F(4,32)=8.93$; $p<0.001$; one-way ANOVA with genotype as variable]. Post-hoc analysis revealed significant differences in the following comparisons: naïve vs FC ($p<0.01$), naïve vs ON ($p<0.001$), box vs FC ($p<0.05$), box vs ON ($p<0.01$); LI vs FC ($p<0.01$) and LI vs ON groups ($p<0.01$).

Upregulation of PSF expression was not elicited by the context alone (naïve vs box: $p>0.05$), or the shock alone (naïve vs LI: $p>0.05$).

Finally, SRp20 was specifically regulated in the FC and ON exp groups that received the tone paired with the shock [Fig. 4.13D, Naïve: 97.4 ± 7.2%; Box: 96 ± 12%; LI: 79.3±7.5%; ON exp: 116.6 ± 6.7%; FC: 132.0 ± 4.8%; $F(4,31)=6.29$; $p<0.001$, one-way ANOVA with training as variable]. *Post hoc* Student-Newman-Keuls analysis revealed significant differences in the FC vs Naïve ($p<0.01$); FC vs Box ($p<0.05$); FC vs LI ($p<0.001$); and in the ON exp vs LI comparison ($p<0.01$) but no significant differences between the remaining groups.

### 4.6.4. REGULATION OF HIPPOCAMPAL GENE EXPRESSION AFTER CONTEXTUAL FEAR CONDITIONING IN FEMALE MICE

Under the prevailing training conditions, both males and females were able to form and consolidate memories for context-shock associations as assessed by freezing responses upon re-exposure to the context 24 h after training. In both sexes the groups trained in the contextual conditioning paradigm displayed significantly higher freezing responses than their respective box controls [Fig. 4.14, males: Box: 4.0 ± 1.3%; FC: 54.1 ± 6.8%; $F(1,13)=60.58$, $p<0.001$; females: Box: 1.1 ± 0.1%; FC: 71.7 ± 9.7%; $F(1,5)=37.85$, $p<0.01$; one-way ANOVA with training condition as variable].

Freezing scores for the contextual fear conditioning paradigm did not differ between the sexes [$F(1,9)=2.34$; $p=0.16$; one-way ANOVA with sex as variable] .

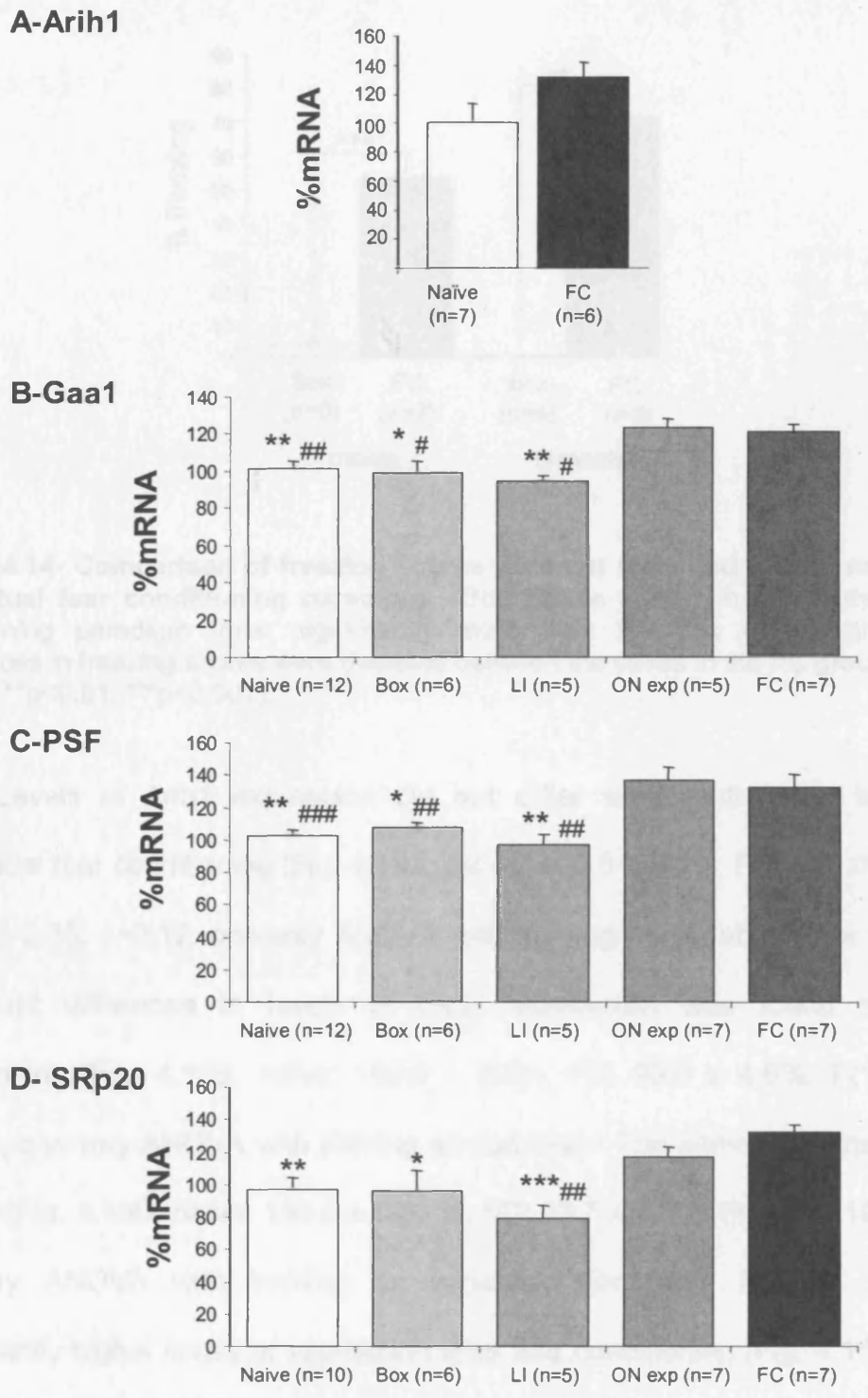



**Figure 4.13- Comparison of hippocampal levels of gene expression between male naïve and trained mice (contextual fear conditioning). (A)** Arih1 mRNA levels did not differ significantly between the naïve and FC groups; **(B)** Gaa1 mRNA levels differed significantly between the shocked and non-shocked groups **(C)** PSF mRNA levels differed significantly between the shocked and non-shocked groups; **(D)** SRp20 mRNA levels differed significantly between naïve and FC groups and between box and FC groups (Mean ± SEM, *p<0.05, **p<0.01, relative to FC, #p<0.05, ##p<0.01 relative to ON exp; % mRNA levels relative to naïve).

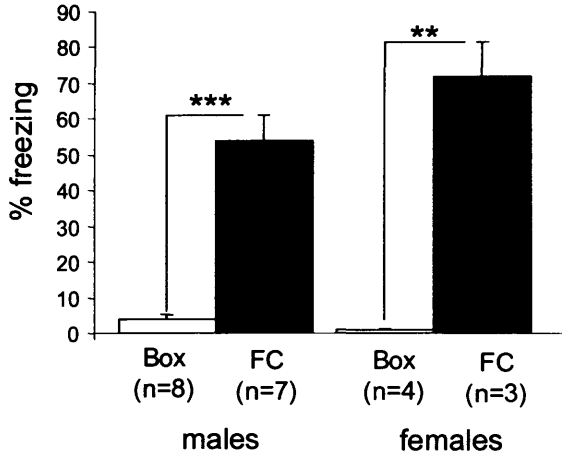



**Figure 4.14- Comparison of freezing scores between male and female mice in the contextual fear conditioning paradigm.** Both sexes trained in the contextual fear conditioning paradigm froze significantly more than the box control groups. No differences in freezing scores were detected between the sexes in the FC groups. (Mean ± SEM, **p<0.01, ***p<0.001).

Levels of Arih1 expression did not differ significantly after training in contextual fear conditioning [Fig. 4.15A, naïve: 100.0 ± 9.1%; FC: 150.0 ± 28.0%, F(1,10)=2.88; p=0.12, one-way ANOVA with training as variable]. For Gaa1, no significant difference in levels of gene expression was found after fear conditioning [Fig. 4.15B, naïve: 100.0 ± 3.5%; FC: 93.0 ± 4.6%, F(1,9)=1.54; p=0.25, one-way ANOVA with training as variable]. The same was observed for SRp20 [Fig. 4.15D, naïve: 100.0 ± 12.0 %; FC: 93.7 ± 7.5%, F(1,9)=0.19; p=0.67, one-way ANOVA with training as variable]. For PSF, females displayed significantly higher levels of expression after fear conditioning [Fig. 4.15C, naïve: 100.0 ± 9.5%; FC: 127.4 ± 9.3%, F(1,9)=6.87; p<0.05, one-way ANOVA with training as variable].

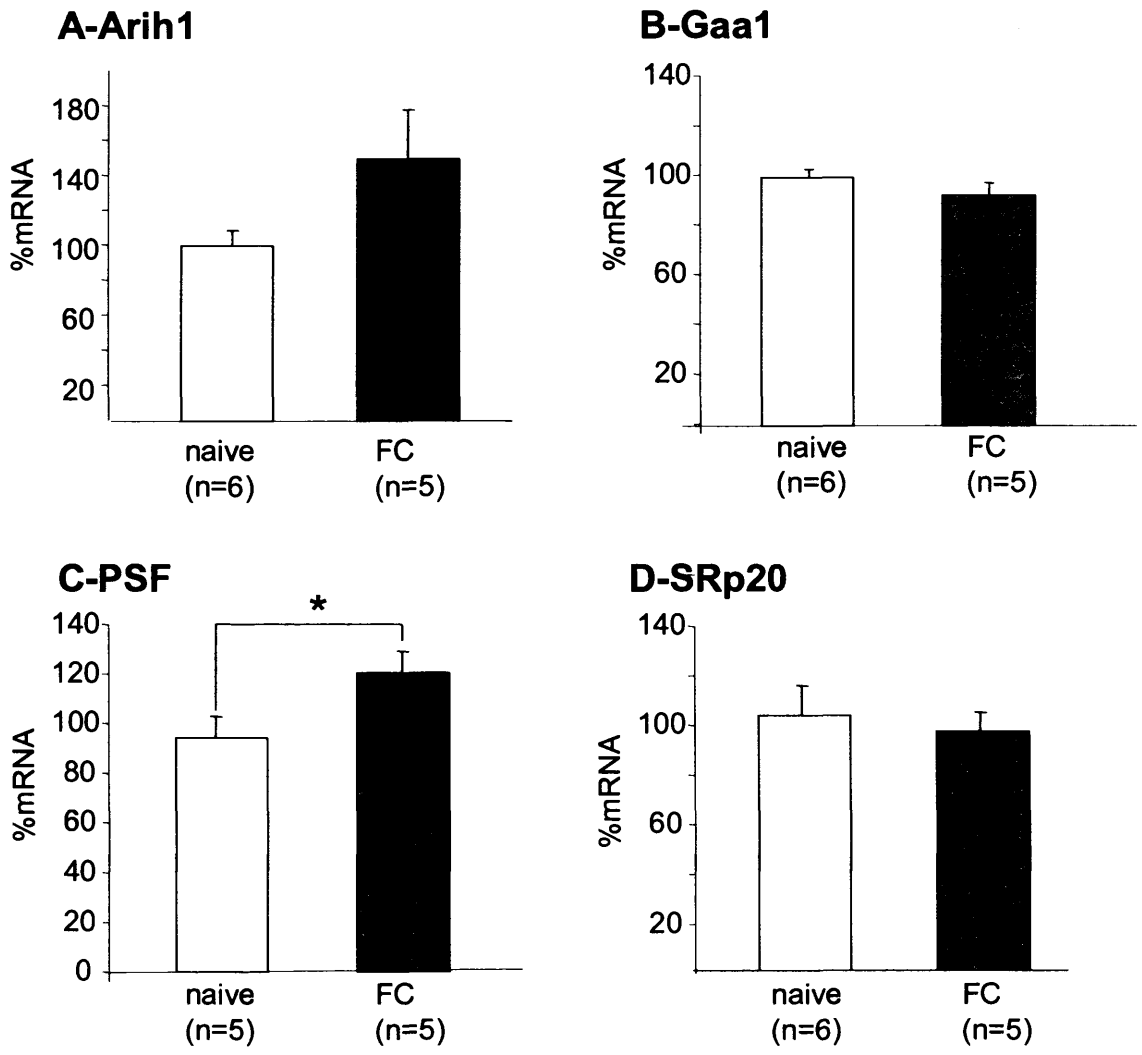



**Figure 4.15- Comparison of hippocampal levels of gene expression between female naïve and trained mice (contextual fear conditioning).** mRNA levels of **(A)** Arih1, **(B)** Gaa1 and **(D)** SRp20 did not differ significantly between the naïve and conditioned groups.**(C)** An upregulation of PSF expression was observed in the conditioned group relative to naïve. (Mean±SEM, *$p<0.05$, %mRNA relative to naïve).

### 4.6.5. SUMMARY

The results described in the previous section point to a sex-specific regulation of expression of three genes (Gaa1, PSF and SRp20) in the naive hippocampus, with males expressing higher levels than females. In addition, training in two hippocampus-dependent tasks: the MWM and contextual fear conditioning induced an upregulation of PSF in both sexes. However,

transcriptional upregulation of Gaa1 and SRp20 after training in these tasks was only triggered in males. Table 4.1 summarizes the data.

**Table 4.1- Sex and behavioural training specific regulation of Gaa1, PSF and SRp20 gene expression in the hippocampus.**

| Gene | Naïve hippocampus | Training in the MWM (trained selective animals compared to naive animals) | | Training in contextual fear conditioning (trained animals compared to naive) | |
|---|---|---|---|---|---|
| | | Males | Females | Males | Females |
| Gaa1 | Male > Female | Upregulated | Unchanged | Upregulated | Unchanged |
| PSF | Male > Female | Upregulated | Upregulated | Upregulated | Upregulated |
| SRp20 | Male > Female | Upregulated | Unchanged | Upregulated | Unchanged |

## 4.7. HIPPOCAMPAL SUBFIELD LOCALIZATION OF CONTEXTUAL FEAR CONDITIONING INDUCED TRANSCRIPTIONAL CHANGES

In order to determine whether the upregulation of Gaa1, Srp20 and PSF expression could be mapped to discrete hippocampal subfields, *in situ* hybridizations were performed in brain coronal sections of male naïve animals (n=2) and animals trained in contextual fear conditioning and sacrificed 30 min after delivery of the shock (n=2). PSF regulation after training in contextual fear conditioning was also investigated in female mice.

### 4.7.1. Gaa1 EXPRESSION IN THE MALE HIPPOCAMPUS

Gaa1 mRNA levels measured by densitometry or estimated counts above threshold did not differ between the naïve and FC groups in any of the subfields considered (Fig. 4.16A-D).

### 4.7.2. PSF EXPRESSION IN THE MALE HIPPOCAMPUS

Densitometric measurements revealed slightly higher levels of PSF mRNA density in the group trained in the fear conditioning paradigm (Fig. 4.17A). Estimation of the number of counts above threshold suggested higher levels of expression in the FC group in subfields CA1 (Fig, 4.17B) and DG (Fig, 4.17D), and similar levels in CA3 (Fig. 4.17C).

### 4.7.3. PSF EXPRESSION IN THE FEMALE HIPPOCAMPUS

No differences in PSF mRNA levels were apparent between the hippocampal subfields as assessed by the densitometric measurement (Fig. 4.18A). The number of counts above threshold density suggested that levels of expression are higher in areas CA1 (Fig, 4.18B) and CA3 (Fig, 4.18C) in the trained group.

### 4.7.4. SRp20 EXPRESSION IN THE MALE HIPPOCAMPUS

Densitometric measurements did not reveal differences in SRp20 mRNA expression between the naïve and FC groups in either hippocampal subfield considered (Fig. 4.19A). Estimated counts above threshold suggest higher levels of expression in areas all three hippocampal subfields considered (Fig. 4.19B-D).

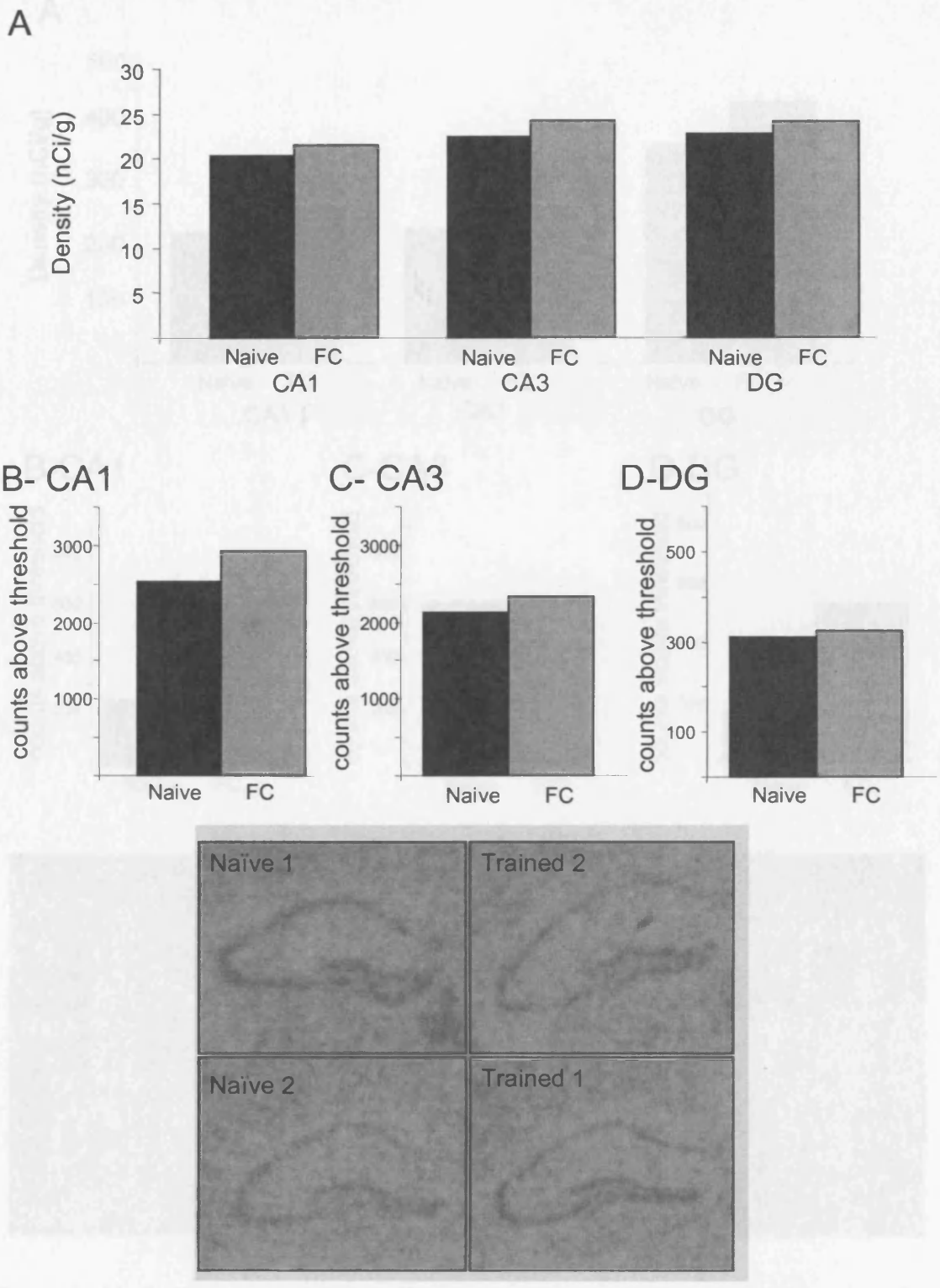



**Figure 4.16- Expression of Gaa1 mRNA in the hippocampus of naïve and trained male mice (contextual fear conditioning). (A)** Density levels of expression did not differ between the conditions in either of the hippocampal subfields considered; Number of counts above threshold density did not differ in subfields **(B)** CA1, **(C)** CA3 or **(D)** DG. (naive n=3 ; fc n=2; 12 sections/group).

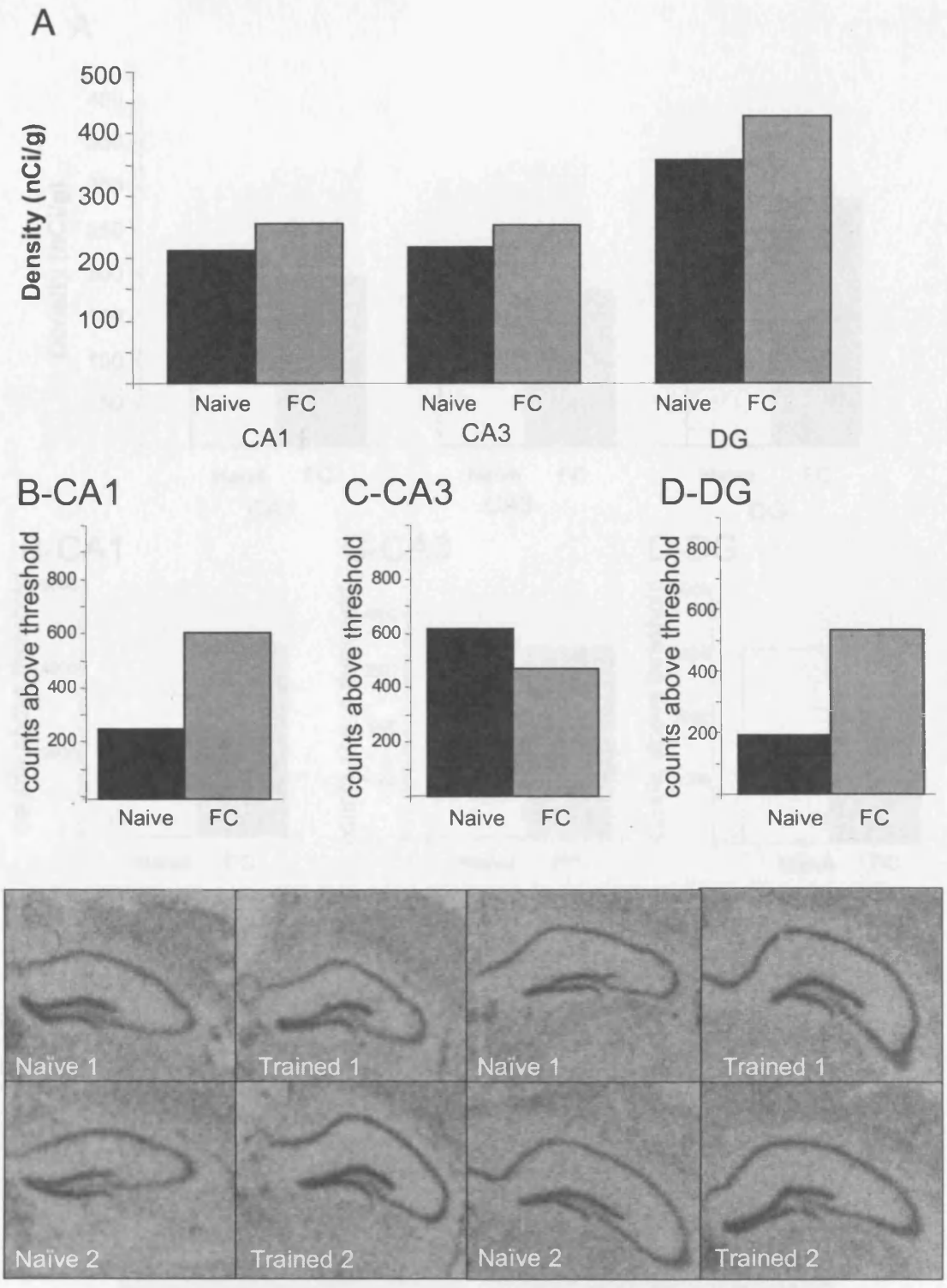



**Figure 4.17- Expression of PSF mRNA in the hippocampus of naïve and trained male mice (contextual fear conditioning). (A)** A trend towards higher density of PSF mRNA was detected in subfields CA1 and DG. Number of counts above threshold density did not differ in subfields **(B)** CA1, **(C)** CA3 or **(D)** DG. (9 sections/group)

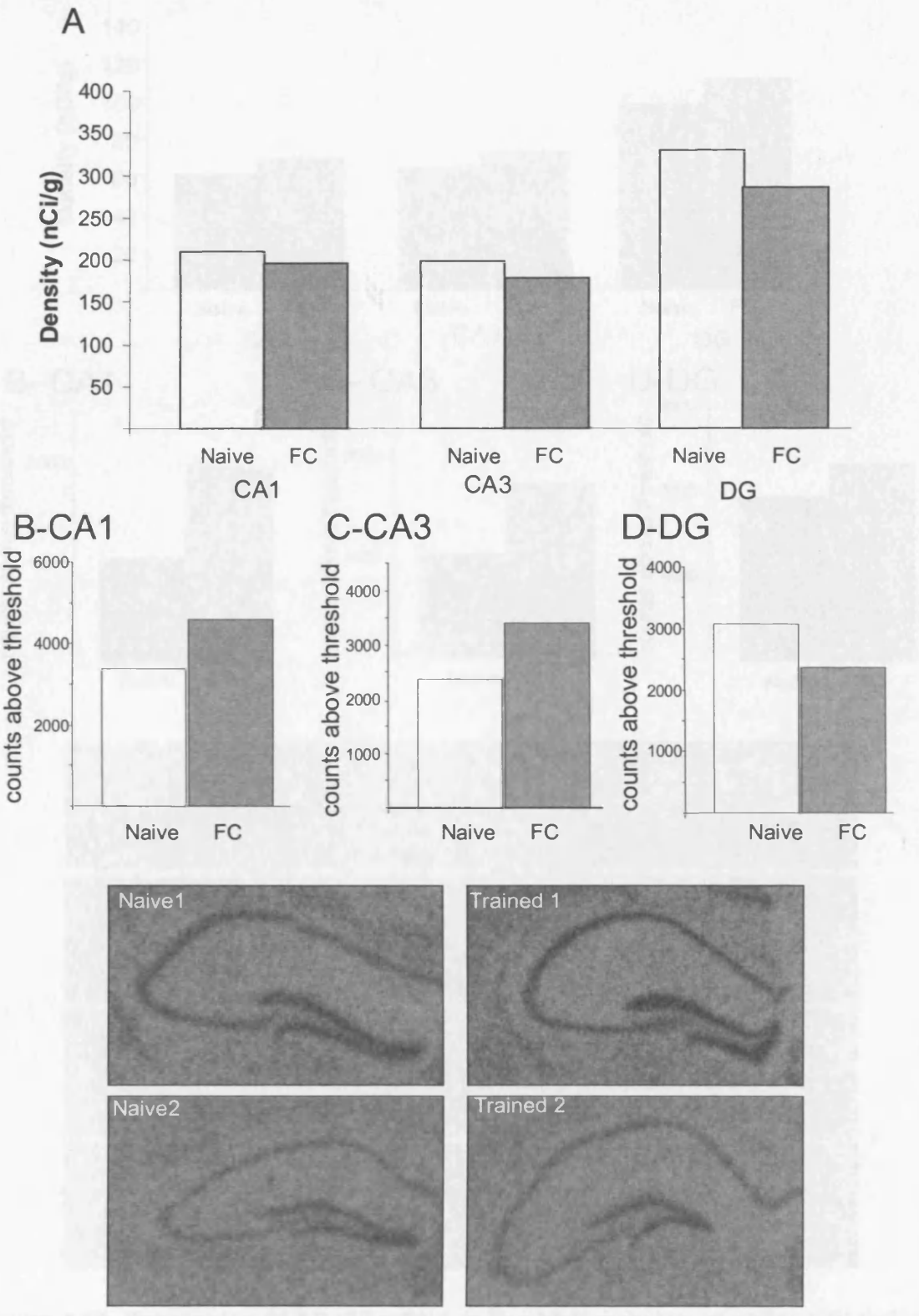



**Figure 4.18- Expression of PSF mRNA in the hippocampus of naïve and trained female mice (contextual fear conditioning). (A)** Density levels of expression did not differ between the conditions in either of the hippocampal subfields considered; Number of counts above threshold density revealed a trend towards higher levels of expression in **(B)** CA1 but not **(C)**CA3 or **(D)** DG. (12 sections/group).

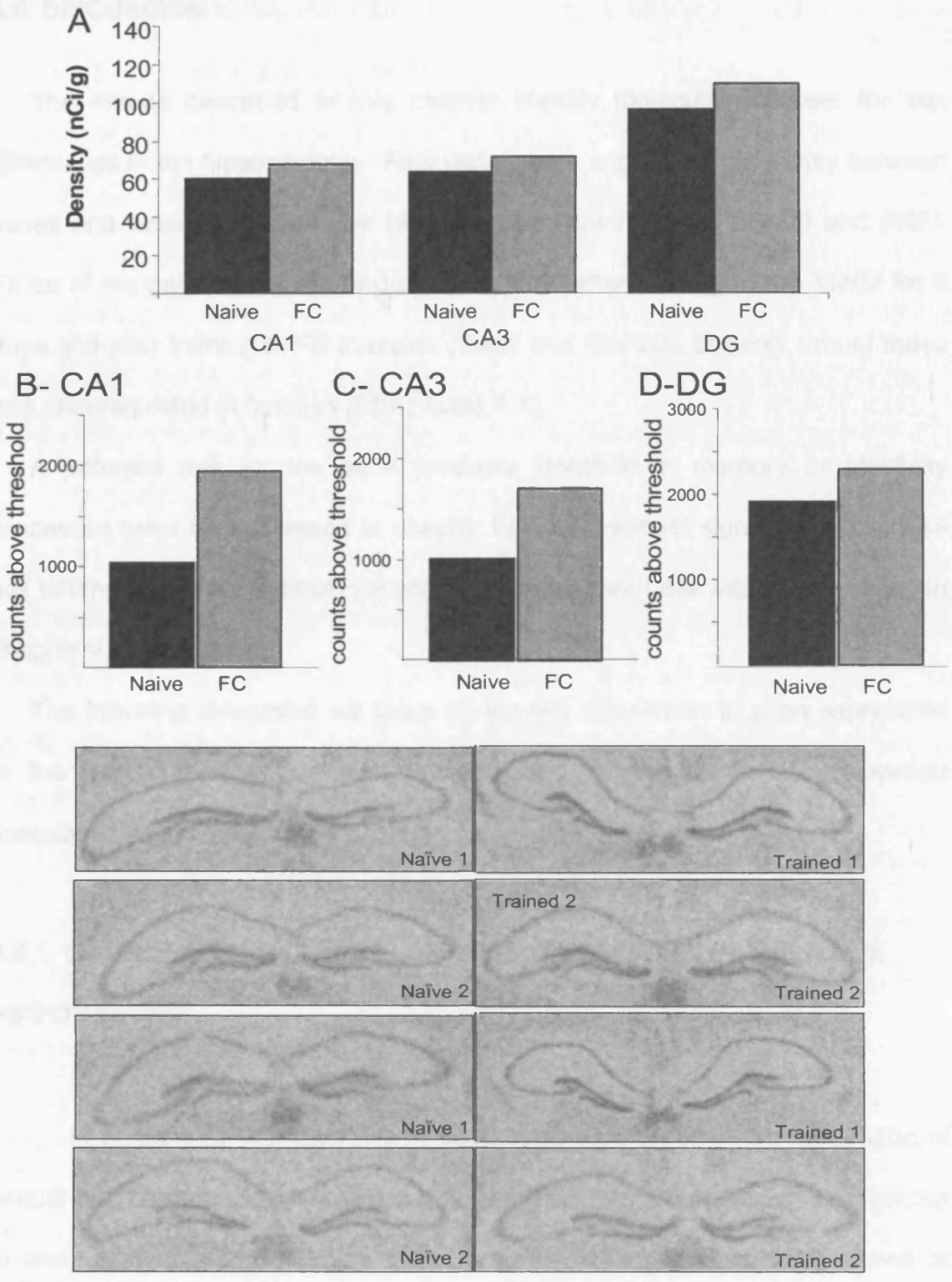



**Figure 4.19- Expression of SRp20 mRNA in the hippocampus of naïve and trained male mice (contextual fear conditioning). (A)** Density levels of expression do not differ between the conditions in either of the hippocampal subfields considered; Number of counts above threshold density revealed a trend towards higher levels of expression in **(B)** CA1 and **(C)** CA3 but not in the **(D)** DG. **(E)** Representative *in situ* hybridizations of coronal brain sections of naïve and trained mice. (naïves: 15 sections; FC: 13 sections).

## 4.8. DISCUSSION

The results described in this chapter identify molecular markers for sex differences in the hippocampus. Four genes were expressed differently between males and females in the naïve hippocampus (Arih1, Gaa1, SRp20 and PSF). Three of the genes were shown to be regulated after training in the MWM for 6 days and after training in FC in males (Gaa1 and SRP20), but only one of these was also regulated in females (PSF; Table 4.1).

A potential role for the gene products identified in memory or plasticity processes were be addressed in chapter III. The potential significance of these sex differences in the light of hippocampus-dependent L&M will be addressed in chapter V.

The following discussion will focus on the sex differences in gene expression in the naïve hippocampus, and the specificity of the transcriptional events described in male mice.

### 4.8.1. SEX-SPECIFIC PATTERNS OF GENE EXPRESSION IN THE NAÏVE HIPPOCAMPUS

In agreement with the roles of some dimorphic areas in the maturation of sexual and parental behaviours it is not surprising that morphological differences in sexually dimorphic nuclei are mirrored at the molecular level by enriched or decreased expression of certain genes (Devidze et al., 2005; Coelho et al., 2005). In addition, a number of other studies have also reported sex-specific

patterns of gene expression in the naïve hippocampus and amygdala (Koshibu and Levitt, 2005; Zhang et al., 1999).

The present study provides further evidence for sexually dimorphic gene expression in the hippocampus of adult naïve mice. QPCR analysis of whole hippocampal tissue revealed that levels of Arih1 were significantly higher in randomly cycling female mice as compared to male mice. For the remaining three genes (Gaa1, SRp20 and PSF), the opposite pattern was obtained with male expression being significantly higher than female expression.

The subsequent step was to investigate whether sex-differences in levels of gene expression were already present before the onset of puberty. For this purpose, the transcriptional profiles of the candidate genes were compared between P21 mice (before the onset of puberty) and 2-3 month old mice. A combination of biological and environmental factors may contribute to changes in levels of gene expression between these two ages.

Sex differences in gene expression in young animals can be determined before birth by a direct or indirect effect of the sex chromosome complement (Dewing et al., 2003), or perinatally by the organizational effects of gonadal hormones (Carruth et al., 2002). The onset of puberty in mice occurs between post-natal days 28 and 30. Comparison of levels of expression of a number of genes across different age groups revealed that sex-specific effects of puberty can be transient and restricted to the phase of onset, or remain in adulthood (Koshibu and Levitt, 2005).

A number of epigenetic mechanisms modulated by environmental factors can also contribute to sex-specific patterns of gene expression. For example, maternal behaviour strongly influences the regulation of certain genes in the

offspring both as infants and during adulthood (Meaney and Szyf, 2005; Meaney, 2001). And some studies report that male and female mouse pups are exposed to different patterns of maternal behaviour (Alleva et al., 1989) suggesting that sex-differences in gene expression in early life can be a consequence of maternal care. After weaning at postnatal day 21, animals are caged only with littermates of the same sex and are exposed to different pheromone environments, which are also known to influence gene expression (Gore et al., 2000).

Sex differences in levels of expression of Arih1 were present before puberty, with males expressing significantly higher levels of the gene. Surprisingly, the direction of this sex difference was reversed in adulthood. Furthermore, puberty correlated with significant upregulation of Arih1 in the female but not in the male hippocampus. A possible interpretation of these findings is that Arih1 expression was regulated by exposure to estrogen. In this interpretation: perinatal testosterone secretion and aromatization to estrogen contributes to the activation of the molecular pathways engaged in the regulation of transcription of Arih1; Because females are not exposed to high levels of estrogen in early life, Arih1 expression is kept to minimal levels, and the pubertal estrogen burst activates expression of this gene.

Sex differences in levels of expression of SRp20 were present before puberty and remain in adulthood, suggesting that the pattern of transcriptional regulation of this gene resulted from indirect effects from the sex chromosome complement, organizational effects of gonadal hormones, or different experiences in early life (for example, maternal care).

A trend towards sex differences in Gaa1 mRNA expression was detected in P21 animals. This comparison did not reach statistical significance presumably because the number of animals per group was relatively small (4 males, 5 females). Increasing the number of animals would be necessary to clarify whether sex differences in Gaa1 mRNA expression are present before puberty.

A significant age effect was detected for PSF mRNA levels: P21 animals expressed higher levels of the transcript in both sexes. A study by (Chanas-Sacre et al. (1999) describe an age-dependent regulation of PSF expression which is widespread throughout the brain in early life and restricted to the olfactory bulb and the hippocampus in adulthood. The present study complements these results by reporting an age-dependent decrease in levels of PSF expression in the hippocampus.

This finding is consistent with previous reports of high levels of PSF expression in brain during early life, and reduced levels of expression in adulthood

In this study, all the genes displaying sex specific patterns of gene expression are encoded by autosomes [Arih1: chromosome 9; Gaa1: chromosome 15; SRp20: chromosome 3; PSF: chromosome 1; (www.ensembl.org)], which rules out a direct contribution of sex chromosome complement for differences in transcriptional level. Distinct transcriptional levels of these genes can be justified by epigenetic mechanisms (as mentioned above), and/or differences in the regulation of gene expression at the level of transcription factors, and the signalling cascades that modulate their activity. This latter explanation is supported by the establishment of a sex specific requirement of

CaMKK$\beta$ in the regulation of expression of the target genes in the naïve hippocampus (a topic that will be addressed in Chapter V).

### 4.8.2. THE SEXES DISPLAYED SIMILAR LEVELS OF PERFORMANCE IN THE MWM AND CONTEXTUAL FEAR CONDITONING

Some studies describe sex differences in performance in the MWM. Growing evidence suggests that these sex differences arise from a slower rate of acquisition in females and tend to be eclipsed by increased training. Causes for this lower rate of acquisition can be related to stress factors (which tend to be more detrimental to females than to males), but also to the use of alternative learning strategies (with females relying predominantly on distal cues; Roof and Stein, 1999; Williams and Meck, 1991).

In the MWM experiment described in section 4.5.1., both sexes improved their escape latencies with the number of training trials. In addition, during probe trials, both groups were, on average, equally selective towards TQ. Only slightly higher escape latencies were detected for females at the third day of training, suggesting that, probably, a difference in probe trial performance could have been detected at this stage. In addition, on average females presented a trend towards a higher percentage of time swimming in the thigmotaxis zone during the probe trial. Thigmotaxic behaviour is difficult to interpret in the light of learning abilities as there is no consensus on whether this is a cause or consequence of delayed learning. Swimming close to the rim of the pool can be interpreted as a measure of fearfulness towards the open space (Gerlai and Clayton, 1999) and a thigmotaxic behaviour could prevent the animals from finding the platform in initial

stages of learning, which would then prevent or delay learning of the task. In contrast, thigmotaxis can also be a consequence of failure to learn the task and a choice of the animals towards a safer area of the pool.

A number of studies describe sex differences in contextual memory formation assessed by scoring freezing behaviours. These are dependent on task parameters such as exposure to shock interval, and conditioning apparatus (eg. Wiltgen et al., 2001). In the present conditions, the conditioning protocol triggered similar conditioned responses in both sexes (Fig. 4. 14).

In summary, for the two hippocampus-dependent tasks used in this study, both males and females exhibited robust learning. This was an important factor in the experimental design. Training-induced transcriptional changes could not be compared between the sexes if these had displayed differences at the behavioural level.

### 4.8.3. SPECIFICITY OF THE TRANSCRIPTIONAL CHANGES ELICITED BY TRAINING IN MALE MICE

Activation of transcription upon induction of neural activity has been described for a number of genes. Transcriptional events triggered by electrical or chemical synapse stimulation such as protocols used for the induction of LTP or seizures, can be useful in the understanding of cellular mechanisms underlying synaptic plasticity (Ressler et al., 2002; Bottai et al., 2002; French et al., 2001; Cole et al., 1989). A number of studies in behaving animals report induction of expression of a number of genes in response to behavioural training. Functional studies have identified some of these genes as "memory effector genes" due to

their importance in memory consolidation processes (Lee et al., 2004; Guzowski, 2002; Guzowski et al., 2001; Hall et al., 2000). The present study investigated the regulation of hippocampal expression of Arih1, Gaa1, PSF and SRp20 after training in hippocampus-dependent tasks.

### 4.8.3.1. TRANSCRIPTIONAL CHANGES ELICITED BY SPATIAL TRAINING IN THE MORRIS WATER MAZE

The MWM is one of the most commonly used tasks to assess spatial memory formation in rodents. In this task, an animal learns to locate a hidden escape platform placed in a pool located in a room where several landmarks are distributed on the walls. Successful learning of the task is assessed in a probe trial. The parameter normally measured is selectivity towards the area where the platform was located previously. A preference towards this area is considered to indicate a hippocampus-dependent representation of the environment, which the animal uses to find an object which can not be perceived by the senses (Morris et al., 1982).

Successful acquisition of the MWM task requires learning a series of aspects of the task: learning that there is a platform, learning that this is the only way to escape the water and learning the platform location (Gerlai, 2001a; Gerlai and Clayton, 1999). For this reason, transcriptional changes detected after spatial training and successful learning of the task may not be directly associated to learning of the platform location alone. In addition to complex cognitive demands, other factors intrinsic to the task can modulate gene expression. First, a number of studies reported that exposure to novel environments is sufficient to

trigger transcriptional activation of immediate early genes such as the ones encoding the transcription factor zif268 (Hall et al., 2000) and the cytoskeletal protein Arc (Guzowski et al., 1999). Second, motor activity elicits gene expression (Vaynman et al., 2004); Third, swimming and interaction with the human experimenter can also represent a potent stressor (Treit and Fundytus, 1988).

Different experimental designs have been used to control for the impact of the factors mentioned above in gene expression induced by spatial training. These are generally termed "swim controls" and include mice that swam in the absence of a platform (Leil et al., 2003; Oh et al., 2003; Cavallaro et al., 2002; Shors et al., 2000) or in a cued platform version of the task (Leil et al., 2002). In the present experiment, swim controls corresponded to animals that swam in the absence of a platform, for the same number of trials and for the same amount of time per trial as trained animals. Despite being trained in the absence of the platform, mice in the swim control group were exposed to the training environment and may have formed a spatial representation of it. For this reason, these animals can not be purely regarded as controls for physical activity.

A number of studies have established direct correlations between transcriptional levels of certain genes in the hippocampus and performance in the MWM (Brightwell et al., 2004; Balschun et al., 2003; Matsuyama et al., 1997). In order to address whether mRNA levels of any of the candidate genes could be related to the levels of performance in the MWM, trained animals were separated into "selective" and "non selective" groups according to probe trial performance.

In the current expression studies, mRNA levels of Gaa1 did not differ between naïve and swim control mice, but differed between these two groups

and animals that were trained in the MWM, regardless of probe trial performance. These findings can have the following interpretations: (a) Animal handling and swimming or the stress inflicted by these factors, are not sufficient to regulate Gaa1 expression; (b) Gaa1 regulation is not related to the encoding of spatial information induced by exposure to the training environment; (c) Gaa1 is specifically regulated when animals are trained to find the platform, suggesting that the regulation of the gene is related to learning that *there is* a platform and/or *where it is located.*

Levels of PSF and SRp20 mRNA gene expression were comparable between the swim control groups, selective and non selective animals. These findings can be interpreted as follows: (a) physical activity and handling of the animals are sufficient to trigger PSF and SRp20 upregulation; or alternatively (b) PSF and SRp20 participate in the encoding or consolidation of spatial information, independently of the need to find a hidden platform.

The fact that training in this set up triggers similar transcriptional events independently of the cognitive demands of the task or the degree of selectivity in the probe trial does not rule out a potential contribution of these genes to spatial memory consolidation. For example, the magnitude of induction of the immediate-early gene Arc does not differ between animals trained in a spatial and non spatial versions of the MWM (Guzowski et al., 2001), nevertheless disruption of Arc protein expression causes impairments in the Morris Water Maze task (Guzowski et al., 2000). These results suggest that some transcriptional events in the hippocampus can be triggered by exposure to the novel environment alone and yet be relevant for the encoding of spatial information.

Because both the swim controls and trained animals were subjected to four training trials during the course of six days, it is unlikely that the transcriptional events purely related to the initial learning of the task, were detected at the end of the sixth training day. In addition, acquisition of the task has, at this stage, already reached asymptotic levels. This suggests that the memory of the platform position has already been consolidated. In addition, the probe trial is thought to elicit memory extinction. For these reasons, it is possible that memory reconsolidation or extinction, rather than consolidation is being probed at this stage [for example as suggested by Morris et al. (2006)]. Under the prevailing conditions, memory consolidation, reconsolidation and extinction can not be dissociated at the molecular level. In order to attempt to establish the correlation between transcriptional events and memory consolidation, gene expression studies in the present report focused on a single trial hippocampus-dependent learning task: contextual fear conditioning.

### 4.8.3.2. TRANSCRIPTIONAL CHANGES ELICITED BY CONTEXTUAL FEAR CONDITIONING

In the contextual fear conditioning task, the animal is required to form an association between a context and a noxious event, and to display it through behaviour upon re-exposure to the context. A number of lesion and pharmacological studies established that the hippocampus is engaged in mediating context representations (Rudy and Matus-Amat, 2005; Rudy et al., 2002; Rudy and O'Reilly, 2001; Rudy and O'Reilly, 1999). In addition, a number of gene expression and functional studies have provided evidence for an

engagement of the hippocampus in the consolidation of memories not only for context but also for context-shock associations (von Hertzen and Giese, 2005; Lee et al., 2004; Ressler et al., 2002).

The transcriptional analysis reported in this chapter provides further evidence for the activation of transcription in the hippocampus as a consequence of context-shock association. This is because no upregulations in gene expression in males and PSF in females after contextual fear conditioning were elicited by the context alone or non-specific factors of the training procedure (Fig. 4.13).

First, exposure of the animals to the context and tone in the absence of the noxious event, did not trigger upregulation of either of the genes studied, ruling out the possibility that transcription of these genes correlates purely with hippocampal representations of the context.

Using an immediate shock freezing paradigm, Frankland et al. (2004) provided evidence for a requirement of protein synthesis for the formation of a representation of the shock, although the contribution of the hippocampus has not been established. In addition, the electrical activity to which the animal is exposed can, *per se,* trigger changes in gene expression. Other studies have also used the immediate shock freezing paradigm to control for transcriptional events induced by the shock (eg. Atkins et al., 1998). However, in our experimental setup, immediate shock does not prevent the formation of context-shock associations (as assessed by freezing on a 24h test) and is not a suitable control (L.S.J. von Hertzen and K.P. Giese, unpublished). For this reason, an alternative control for shock induced transcriptional changes, latent inhibition (LI), was used in this study.

In LI paradigms, animals are exposed to the context for a long period of time before receiving the shock. LI learning reflects the ability to ignore stimuli that historically predict no significant consequences, therefore preventing the association between the novel environment and the noxious stimulus (von Hertzen and Giese, 2005; reviewed in Meyer et al., 2005; Levenson et al., 2004b; Impey et al., 1998). In the present study, two attempts were made to establish a latent inhibition protocol: these groups were termed overnight exposed (ON exp) and latent inhibition (LI).

The ON exp group was exposed to the training context overnight, for approximately 14 hours. In the morning, the animals received a tone that co-terminated with a foot shock. The group trained under these conditions displayed a lower freezing response compared to that elicited by the fear conditioning protocol. Nevertheless, the percentage of time spent freezing (30%) was higher than that elicited by exposure to the context alone. This finding can be interpreted in two alternative ways: (a) A context-shock association was formed to some extent in this group; (b) the animals were conditioning to a discrete cue (the tone), and the freezing response observed is a response to tone-shock association, a phenomenon independent of the hippocampus (reviewed in McGaugh, 2004). Higher levels of gene expression in this group when compared to naïve and box animals can not be solely triggered by the delivery of the shock.

An additional group of animals was trained under the same conditions as the ON exp group except for the exposure to the auditory cue (LI group). In this case, the freezing response was at the same level as that elicited in the box group. These results suggest that exposure to the tone before the shock contributed to conditioning to the context presumably by resetting attention and

increasing awareness to the context (Restivo et al., 2002; Honey and Hall, 1989). This LI group represented a suitable control to test for shock-induced genes in the hippocampus. None of the genes tested was upregulated by training in LI protocol, suggesting that the shock alone does not account for the transcriptional events described.

Taken together, the controls used in this study allow to discriminate between the molecular events triggered in an association-specific manner and those triggered by novelty, tone or shock alone.

### 4.8.4. TIME POINT OF DETECTION OF CHANGES IN GENE EXPRESSION INDUCED BY BEHAVIOURAL TRAINING

In the initial transcriptional screen for MWM regulated genes, described in chapter III, the thirty minutes after probe trial time point was chosen. This choice was based on the observation that impairments in probe trial performance and the establishment of L-LTP in CaMKK$\beta$ were correlated with a failure to activate CREB immediately after spatial training (Peters et al., 2003). This time point has also been used in other MWM transcriptional studies and corresponds to peak levels of, for example the protein Arc, which has a functional importance in spatial memory consolidation (Guzowski et al., 2001).

The use of protein synthesis and transcription inhibitors has allowed the definition of time windows during which consolidation of long-term contextual fear memories is susceptible to disruption (Igaz et al., 2002; Bourtchouladze et al., 1998). These studies have identified two waves of transcription, the first one

occuring within the first hour and the second between 4-6 hours following conditioning.

The thirty minutes after conditioning in a single trial learning task was also used for the analysis of contextual fear conditioning regulated genes as CREB activation is detected 30 min after training in contextual fear conditioning (Kida et al., 2002), and the transcription levels of immediate early genes with well established functions in memory consolidation peak at this time point (Ressler et al., 2002).

It is important to mention at this stage, that a bioinformatics analysis of the regions in the upstream regulatory regions of the candidate genes, that are conserved between species, only revealed the presence of a CRE-element upstream of Arih1, and did not detect any CRE-elements upstream of Gaa1, PSF or SRp20 (data not shown). These results are not surprising as other screens for memory related genes have failed to find a strong enrichment in CREB dependent genes (e.g. Levenson et al., 2004a). Possible reasons for the lack of CRE-elements in the candidate genes include; (a) these genes were identified as CaMKK$\beta$ regulated and the CaM Kinase cascade is known to mediate activation of a number of transcription factors other than CREB (e.g. Kane and Means, 2000); (b) these genes are regulated by transcription factors which are, themselves, IEG regulated by CREB such as zif268 (West et al., 2001; Finkbeiner et al., 1997; Bito et al., 1996)

### 4.8.5. HIPPOCAMPAL SUBFIELD LOCALIZATION OF THE TRANSCRIPTIONAL CHANGES BY *IN SITU* HYBRIDIZATIONS

Sex-specific and behavioural-training induced transcriptional differences in whole hippocampal tissue were detected by QPCR. *In situ* hybridizations of brain coronal slices was performed in order to study whether the transcriptional differences detected by QPCR were occurring preferentially in any of the three major hippocampal subfields: CA1, CA3 or DG.

Gene expression studies in the rodent hippocampus after contextual fear conditioning suggest that the transcriptional events triggered by the behavioural experience occur predominantly in area CA1. First CRE-dependent transcription was activated in an association specific manner in area CA1 only (Kida et al., 2002). Second, upregulation of a number of learning induced genes in the hippocampus was restricted to area CA1 (von Hertzen and Giese, 2005; Hall et al., 2000); Third, targeted disruption of gene expression in dorsal areas of the hippocampus was shown to specifically impair the consolidation of contextual fear memories (Lee et al., 2004).

As explained in section 4.3, the current *in situ* hybridizations data was not analysed statistically due to the low number of animals used in each group. Therefore, it would be necessary to increase the number of animals per experimental group considered to map sex differences and behaviourally induced transcriptional events to distinct hippocampal subfields.

Nevertheless, comparison of average densities or estimated counts above threshold suggests that some of the transcriptional differences detected by QPCR can be mapped to specific hippocampal subfieds.

An interesting additional experiment would be to localize the transcriptional changes induced after training in the MWM, in order to determine whether the transcriptional changes would coincide in terms of subfield specificity for the two hippocampus-dependent tasks.

# CHAPTER V:
# GENERAL DISCUSSION

## 5.1. SUMMARY OF THE RESULTS

Sexual dimorphisms in performance of some behavioural tasks assessing hippocampus-dependent memory formation have been described in rodents (Jonasson, 2005; Kudo et al., 2004; Gresack and Frick, 2003; Wiltgen et al., 2001; Frick et al., 2000; Roof and Stein, 1999). Traditionally, these dimorphisms are interpreted as the result of organizational and activational effects of gonadal hormones (Frick et al., 2002; Gupta et al., 2001; Frick et al., 2000; Anagnostaras et al., 1998; Williams and Meck, 1991).

The consolidation of hippocampus-dependent memory requires *de novo* transcription, engaging activation of the transcription factor CREB. CREB activation can be mediated by different signalling pathways, including the CaM Kinase cascade (Soderling, 1999; Bito et al., 1996; Enslen et al., 1995). Combination of gene targeting approaches with behavioural and electrophysiological techniques has established the participation of members of the CaM Kinase cascade in synaptic plasticity and memory consolidation processes (Mizuno et al., 2006; Peters et al., 2003; Wei et al., 2002; Kang et al., 2001; Ho et al., 2000).

Peters and colleagues (Peters et al., 2003) generated a null mutant mouse for CaMKK$\beta$, an upstream activator of CaMKIV and CaMKI (Takemoto-Kimura et al., 2003; Anderson et al., 1998; Tokumitsu et al., 1995) which, in turn, is known to activate a number of transcription factors including CREB (Chow et al., 2005; Impey et al., 2002; Kane and Means, 2000). At the behavioural level, CaMKK$\beta$ male null mutants displayed delayed learning of the Morris Water Maze Task. This was accompanied by a failure to phosphorylate CREB after spatial training and by impaired late-phase LTP (L-LTP) in CA1 synapses which is dependent on

*de novo* transcription and protein synthesis (Nguyen et al., 1994). These results suggest that lack of CaMKK$\beta$ causes an impairment in the transcriptional events underlying memory consolidation for the MWM task. Remarkably, only males displayed the phenotype described above, while null mutant females were comparable to their WT counterparts in MWM performance and exhibited normal L-LTP (Mizuno et al., 2006). These findings provided evidence for sexual dimorphisms in the plasticity mechanisms underlying memory formation.

The work by Peters and colleagues paved the way for the project described in this thesis. The current project used the CaMKK$\beta$ mutant mouse line to fulfil two main objectives:

(1) To identify target genes regulated by CaMKK$\beta$ in male mice and whose transcriptional activation correlates with spatial learning.

(2) To use the insights provided by the transcriptional studies in male mice in order to investigate sexual dimorphisms in the molecular mechanisms underlying hippocampus-dependent memory formation.

The initial screening, performed by microarray analysis, compared transcriptional profiles between hippocampal tissue of WT and CaMKK$\beta$ null mutant male mice after training in the Morris Water Maze. Among a number of transcripts detected in the microarray analysis, three genes were confirmed to be differently regulated in the naïve male hippocampus of WT and CaMKK$\beta$ mice (Figs. 3.5 and 3.6). These genes encode a ubiquitin ligase (Arih1), and two multifunctional nuclear proteins (SRp20 and PSF). A fourth gene, GPI-anchor transamidase (Gaa1), was shown to be expressed in higher levels in the hippocampus of WT mice after training in the MWM but did not differ between

naïve and trained CaMKK$\beta$ mutants. As explained in chapter III, these genes were designated as "CaMKK$\beta$-regulated genes".

Transcriptional profiles of these genes were then characterized in null mutant females and compared between the sexes in WT naïve mice. These results pointed to (a) sex-specific requirement of CaMKK$\beta$ for basal expression in the hippocampus (Figs. 3.5 and 3. 6); (b) sex-specific basal levels of expression in WT mice (Fig. 4.1). Finally, the experiments focused on the regulation of these genes after training in hippocampus-dependent tasks in WT mice. For this purpose male and female mice were trained in two hippocampus-dependent tasks: the MWM and contextual fear conditioning. In male mice, three of the genes (Gaa1, PSF and SRp20) were upregulated thirty minutes after a probe trial in the MWM (Fig. 4.9), and thirty minutes after conditioning in a contextual fear conditioning paradigm (Fig. 4.13). However, assessment of the transcriptional profile of these genes in the female hippocampus, at the same time points revealed an upregulation of PSF mRNA and no differences in mRNA levels of Gaa1 and SRp20 between naïve and trained animals (Figs. 4.10 and 4.14). For the gene encoding the ubiquitin ligase (Arih), no statistically significant differences in mRNA levels were found between naïve and trained animals of either sex.

In summary, this work has identified novel molecular markers for sex-specific gene expression in the naïve hippocampus and after training in two hippocampus-dependent tasks (Table 5.1). These results suggest: (a) Sexual dimorphisms in experience dependent transcriptional outputs; (b) Coincidence of transcriptional outputs between tasks with distinct procedural demands.

The transcriptional analysis described above establishes correlations between regulation of the target genes and memory consolidation processes. However, to determine whether these are in fact "memory effector genes" would require *in vivo* disruption of normal gene function. The cellular functions of these target genes have been previously described. In the light of the idea that physical changes in synaptic morphology or properties represent the support of memory encoding, a potential function of these genes in memory consolidation was discussed in section 3.5.6. Possible explanations for sex differences in transcription induced by behavioural training will be discussed in section 5.2. The significance of sex differences in gene expression in the naïve hippocampus, in the light of memory consolidation will be discussed in section 5.3. The coincidence of transcriptional outputs in memory consolidation of hippocampus-dependent tasks that are known to engage distinct molecular pathways will be addressed in section 5.4. Potential improvements to the experimental design used in this study and alternative approaches to studying sex differences in memory consolidation at the molecular level will be discussed in sections 5.5 and 5.6 respectively.

**Table 5.1. Summary of the transcriptional studies described in chapters III and IV.** (Mean values per experimental group are indicated; Statistically significant differences are highlighted in bold font; *p<0.5, **p<0.01; ***p<0.001, for one-way ANOVA comparisons).

| Gene | | % mRNA relative to WT naïve | | | |
|---|---|---|---|---|---|
| | | Naïve CaMKK$\beta$ null mutants | Naïve females (relative to males) | Selective Animals in the MWM | After Training in Contextual Fear Conditoining |
| Arih1 | Males | **67%*** | **143%*** | 129% | 138%(p<0.10) |
| | Females | **180%*** | | 112% | 149%(p=0.12) |
| Gaa1 | Males | 111% | **70%***** | **149%*** | **119% *** |
| | Females | 94% | | 96% | 93% |
| PSF | Males | **190%**** | **60%***** | **139%*** | **142%**** |
| | Females | 124%(p=0.11) | | **145%**** | **127%*** |
| SRp20 | Males | **56%**** | **80%*** | **176%***** | **137%*** |
| | Females | 108% | | 106% | 94% |

## 5.2. SEX DIFFERENCES IN HIPPOCAMPAL TRANSCRIPTION INDUCED BY BEHAVIOURAL TRAINING

Gaa1, PSF and SRp20 are expressed at lower levels in the female naïve hippocampus. While PSF is regulated by training in the MWM and contextual fear conditioning in both sexes, Gaa1 and SRp20 are regulated in males only. Under the prevailing conditions, Gaa1 and SRp20 represent molecular markers for hippocampal activity in male but not in female mice.

Whether these transcriptional changes are mirrored at the protein level has not been tested, although the hypothesis that mRNA can, *per se*, have a role in memory consolidation can not be ruled out. In addition, evidence for a biological role for these gene products in memory consolidation is still missing. For these reasons, the possibility that Gaa1 and SRp20 represent epiphenomena, with no significance in memory consolidation processes, can not be ignored. Nevertheless, the work here described provides evidence for the occurrence of sex-specific transcriptional events triggered by training in hippocampus-dependent tasks. Possible causes for sex differences in hippocampal transcription in response to behavioural training will be addressed below and are summarized in figure 5.1.

### 5.2.1. DISTINCT KINETICS OF TRANSCRIPTIONAL ACTIVATION?

Sex-differences in the kinetics of transcriptional activation of these genes account for a failure to detect an upregulation at the 30 minute time point in females (Fig. 5.1A). A possible approach to test this hypothesis would be to try to establish a time course of gene transcription in the female hippocampus by

sacrificing the animals at different time points after conditioning. A transcriptional upregulation in females would be more likely to de detected after the 30 min time point as, in most studies the 30 min after conditioning has been the earliest time point after which changes in IEG expression have been detected.

Differences in the kinetics of activation of these genes could be accounted for by the engagement of distinct signalling transduction pathways. This hypothesis is supported by the following lines of evidence: (a) Gaa1 expression is regulated by CaMKK$\beta$ after MWM training in males, but not in naïve males or females; (b) SRp20 is regulated by CaMKK$\beta$ in the male, but not in the female naïve hippocampus, indicating that CaMKK$\beta$ is not required for basal expression of SRp20 in females; (c) Lack of CaMKK$\beta$ affects spatial memory consolidation in males but not in females (Mizuno et al., 2006); (d) SRp20 and Gaa1 are regulated after spatial training, in males but not in females, despite similar performances. Hence, it is likely that alternative signalling pathways are involved in male and female learning in the MWM.

The possibility that males and females engage distinct signalling pathways in hippocampus-dependent memory consolidation is more difficult to reconcile with the contextual fear conditioning data. This is because CaMKK$\beta$ is not required for contextual memory formation, but CaMKK$\beta$ regulated genes are regulated in an association-specific manner. Studies by Mizuno and colleagues (2006) established the requirement for CaMKK$\alpha$ in the consolidation of contextual memories in males but not in females. Taken together, these findings suggest that Gaa1 and/or SRp20 may be regulated by CaMKK$\alpha$ upon training in the contextual fear conditioning task. This hypothesis could be tested by comparing

hippocampal transcriptional profiles between CaMKKα null mutant and WT males after training in contextual fear conditioning.

Insights from the CaMKKα and CaMKKβ null mutant lines suggest that these kinases are not required for hippocampus-dependent memory consolidation in females, as far as the contextual fear conditioning and the MWM tasks are concerned. In females, signalling pathways other than the CaM kinase cascade can mediate the transcriptional mechanisms required for memory consolidation for the MWM and contextual fear conditioning tasks. For example, the use of pharmacological inhibitors in cultured pyramidal neurons has established that fast CREB activation is mediated by the CaMK cascade and a slower CREB activation is mediated by the MAPK cascade (Wu et al., 2001). If these kinetic differences also occur *in vivo* it is possible that the same transcriptional outputs would be produced by activation of either of the pathways, but the time point after which transcriptional differences could be detected should differ between the sexes.

### 5.2.2. DISTINCT TRANSCRIPTION FACTORS?

An additional possibility would be that not only the molecular pathways, but also the transcription factors engaged in memory consolidation differ between the sexes (Fig. 5.1B). For example, a sexually dimorphic impact of CREB mutations in spatial learning has been suggested by Hebda-Bauer and colleagues (2005), who demonstrated that learning of the MWM by CREBαδ hypomorphic mutants is affected by previous unsuccessful experiences in males but not in females. In addition, a study by Kudo and colleagues

demonstrated that CREB activation in hippocampal subfield CA1 is detectable in male rats but not female rats thirty minutes after conditioning in a contextual fear conditioning task (Kudo et al., 2004).

It is possible that other transcription factors display a sex-specific pattern of activation or are expressed in different levels in the hippocampus as it has been described for other brain structures (e.g. Coelho et al., 2005).

### 5.2.3. DISTINCT TRANSCRIPTIONAL OUTPUTS?

An alternative explanation would be that the target genes transcribed upon behavioural stimulation differ between the sexes. If any of these genes does indeed play a role in memory consolidation, it is conceivable that male-specific and female-specific memory effector genes exist.

These sex-specific gene products could subserve similar cellular functions and ultimately mediate the cellular modifications that constitute the bases of the memory engram (Fig. 5.1C). This would be a likely hypothesis for SRp20 as the SR family of proteins displays high redundancy. It would not be as likely for Gaa1 as this is the only member of the family so far identified. However, other members of the transamidase complex could be responsible for a higher GPI anchoring activity of proteins in females.

Alternatively, the "memory effector genes" may differ between the sexes which could partially explain differences in the signalling pathways activated (Fig. 5.1D).

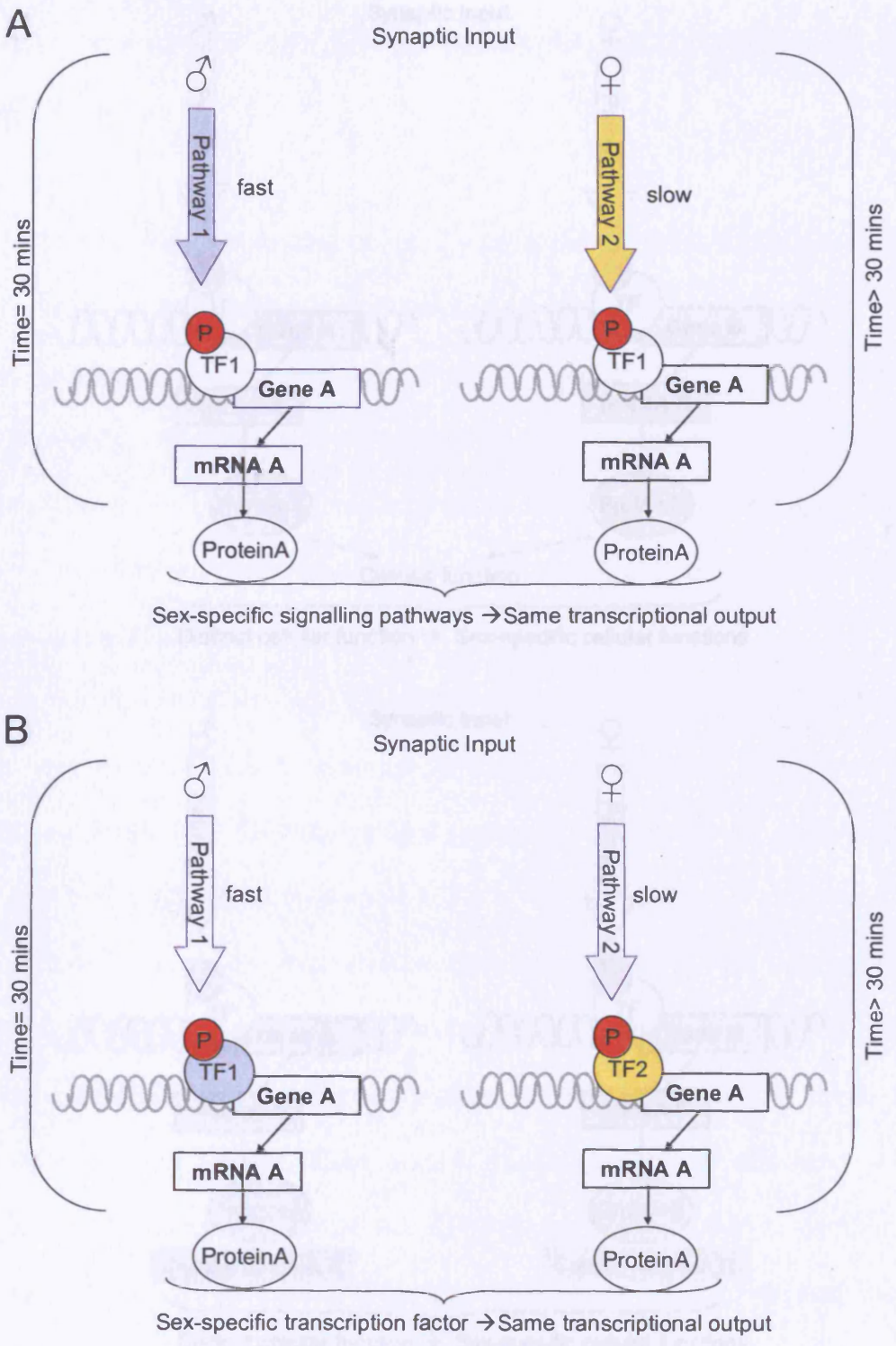



**Figure 5.1- Sex-specific hippocampal transcription induced by behavioural training (I).**
The sexes may produce the same transcriptional outputs but differ in the kinetics of transcriptional activation due to the engagement of **(A)** distinct signalling pathways or **(B)** distinct transcription factors. (TF-transcription factor; P-phosphate, as an exmpale of covalent modification).

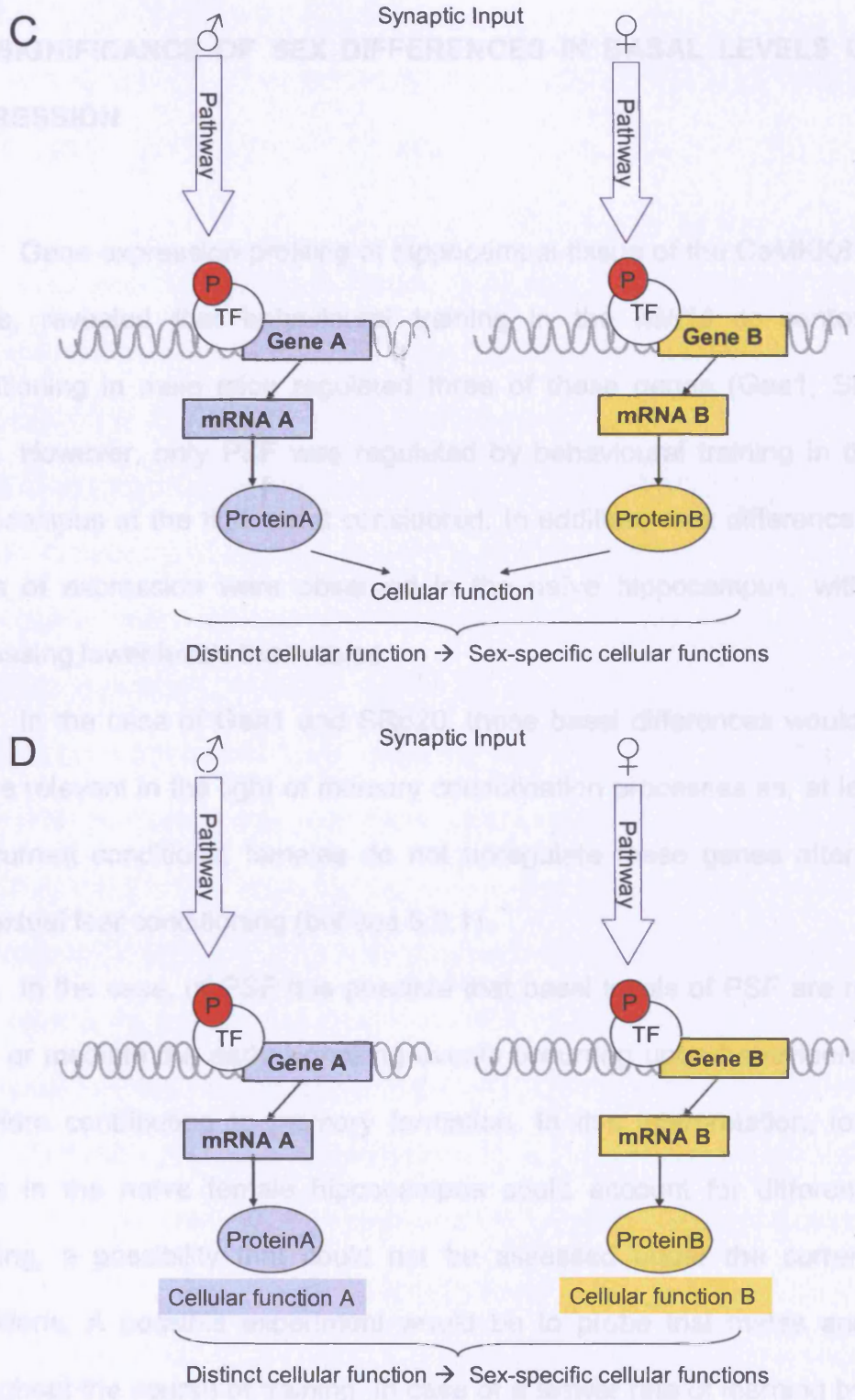



**Figure 5.1- Sex-specific hippocampal transcription induced by behavioural training (II).**
The genes regulated by behavioural training may differ between the sexes, but ultimately **(C)** accomplish the same cellular function or **(D)** accomplish different cellular functions (TF-transcription factor; P- phosphate, as an exmpale of covalent modification).

## 5.3. SIGNIFICANCE OF SEX DIFFERENCES IN BASAL LEVELS OF GENE EXPRESSION

Gene expression profiling of hippocampal tissue of the CaMKK$\beta$ regulated genes, revealed that behavioural training in the MWM or contextual fear conditioning in male mice regulated three of these genes (Gaa1, SRp20 and PSF). However, only PSF was regulated by behavioural training in the female hippocampus at the time point considered. In addition, sex differences in basal levels of expression were observed in the naïve hippocampus, with females expressing lower levels than males.

In the case of Gaa1 and SRp20, these basal differences would probably not be relevant in the light of memory consolidation processes as, at least under the current conditions, females do not upregulate these genes after MWM or contextual fear conditioning (but see 5.2.1).

In the case, of PSF it is possible that basal levels of PSF are required to ease or mediate the early signalling events occurring upon behavioural training, therefore contributing to memory formation. In this interpretation, lower basal levels in the naïve female hippocampus could account for different rates of learning, a possibility that could not be assessed under the current training conditions. A possible experiment would be to probe trial males and females throughout the course of training. In case of a slower rate of learning by females, revealed by worse performance in the probe trial, one could determine whether this was correlated with different levels of PSF expression.

## 5.4. COINCIDENT TRANSCRIPTIONAL OUTPUTS BETWEEN TWO HIPPOCAMPUS-DEPENDENT TASKS

A number of studies in mutant mice and pharmacological interventions have established the dissociation between the signalling pathways involved in the consolidation of spatial and contextual memories (reviewed in Mizuno and Giese, 2005). Surprisingly, the present study reports the coincidence of transcriptional outputs induced by spatial and contextual memory tasks (section 5.2.1). At least three non-mutually exclusive interpretations can be proposed: (a) Intracellular signalling cascades respond differently according to the nature of the hippocampus-dependent task, however the distinct signalling pathways eventually converge onto the same transcriptional outputs; (b) Coincident transcriptional events subserve the same purpose in both tasks, for example, regulation after MWM training may support encoding of non spatial information (e.g. related to emotional/aversive aspects of the task), or regulation upon contextual conditioning may support the encoding of spatial information which is only triggered after the experience of a noxious event; (c) Coincident transcriptional events occur for genes which are essential for memory encoding regardless of the nature of the memory in cause.

As mentioned in chapters III and IV, the current training protocol for the MWM, and the time point chosen to assess transcriptional profiles after MWM training, raises the possibility that memory reconsolidation may have been probed. In order to test this possiblility, two approaches could be used: (a) animals could be subjected to intensive training in the MWM over one day only,

and sacrificed at this stage of training in order to rule out the detection of molecular events associated with reconsolidation (as, for example, in: Leil et al., 2003; Cavallaro et al., 2002; Leil et al., 2002). (b) Alternatively, memory reconsolidation could be probed in the contextual fear conditioning task, as a number of studies point to regulation of different subsets of immediate early genes after memory consolidation and reconsolidation in this task (von Hertzen and Giese, 2005; Lee et al., 2004). The assessement of the transcriptional profile of the target genes under all those experimental conditions would reveal whether their expression can correlate with memory reconsolidation.

## 5.5. TROUBLE SHOOTING

The major limitation of the transcriptional scre.ening described in chapter III was the number of chips used per group. For this reason, the experimental design comprised four biological replicates per group (WT and CaMKK$\beta$ null mutants) of trained animals. An ideal experimental design would have included naïve and trained animals from both genotypes, permiting the screening for mutation induced changes, the identification of MWM training regulated genes in WT animals, and the identification of genes regulated by CaMKK$\beta$ in response to spatial training.

As discussed in chapter III, the transcriptional profile of the candidate genes described in the microarray analysis was firstly investigated in naïve mice of both genotypes by QPCR. The transcriptional profile of the "CaMKK$\beta$ regulated genes", was then investigated after training in the MWM in WT mice. For these reasons, the follow up procedure did not allow the direct confirmation of the

candidate genes revealed by microarray analysis and it is not possible to assess directly the false positivity rate of the array analysis.

Potential improvements to the experimental design in order to reduce sources of type I errors include: (a) the use of more biological replicates in order to reduce the contribution of within group variations; (b) the use of different cohorts of mice in order to reduce effects related to a single behavioural experiment (Levenson et al., 2004a); (c) the use of technical replicates to control the quality of each experimental step during sample preparation and hybridization; (d) choice of a higher cutoff value for transcriptional changes, at the expense of missing true transcriptional differences of small magnitude; (e) the choice of a more stringent significance level (lower p-value) (Unger et al., 2005; Levenson et al., 2004a; Benjamini et al., 2001).

## 5.6. FURTHER EXPERIMENTS TO IDENTIFY SEX SPECIFIC GENES INVOLVED IN MEMORY FORMATION

The current project was started under the finding that CaMKK$\beta$ is required for male long-term spatial memory formation in males but not in females. For this reason, the initial array analysis aimed at identifying CaMKK$\beta$ regulated genes in male animals. The transcriptional profile of these genes was then compared between the sexes in both naïve and trained animals. A number of alternative approaches could be used in order to identify sex-specific genes involved in memory formation.

The most straightforward approach would be to compare transcriptional profiles between the sexes after training in hippocampus dependent tasks.

The detection of sex differences at the behavioural level depends on task, training protocol and training apparatuses; and task parameters can be manipulated in order to favour or prevent the detection of sex differences at the behavioural level. The assessment of hippocampal transcriptional profiles would be equally in formative independently of finding the sex difference or not. For example if, like in the present study, sex differences are not detected at the behavioural level, but are detected at the molecular level, this raises the possibility that males and female engage distinct molecular pathways for memory encoding and consolidation. If on the contrary, sex differences are detected in behaviour, differences in transcriptional profiles could be interpreted as the cause for differences in behaviour.

## 5.7. CONCLUSION

Sexual dimorphisms in the performance of tasks assessing hippocampus-dependent memory have been intensively described in the human and rodent literature. These dimorphisms are classically attributed to the effects of gonadal hormones throughout development and during adulthood. In addition to the contribution of gonadal hormones, recent evidence points to the engagement of sex-specific signalling pathways in the transcriptional mechanisms required for memory consolidation. The results described in this thesis demonstrate, for the first time, that changes in gene expression induced by training in two hippocampus-dependent tasks differ between the sexes. These results suggest that differences in training-induced transcription may represent the molecular bases for sex difference in memory consolidation.

# APPENDICES
# AND
# REFERENCE LIST

## APPENDIX I

**Table A1-** QPCR of cDNAS for target genes. Primer sequences, concentrations, internal control transcripts and respective primer concentrations

| | Primer sequences (5'-3') | Primer conc Fwd/Rev(nM) | Internal control Fwd/Rev (nM) |
|---|---|---|---|
| **Star** | Starfwd: GCAGGACTCAGGACCTTGAAAG<br><br>Starrev: GAACGTAGCGAGGAACATGCT | 900/300 | HPRT 300/300 |
| **Gaa1** | Gaa1fwd:GGCCAACATTTAGCTACTCAGCAT<br><br>Gaa1rev:GCGAGCAGCGTCAACACA | 900/900 | HPRT 900/900 |
| **Serpina 3n** | Serpinfwd: CCCCGTGATAGTGCCCATGAT<br><br>Serpinrev: GAAAAGCTCCTCATCTCGGAAGT | 900/300 | GAPDH 300/300 |
| **brPTB** | PTBfwd: CTGCGTGGCTCGGTTCTT<br><br>PTBrev: CCTCAGTGACAATTCCGTCCAT | 900/300 | HPRT 900/900 |
| **GluR1** | GluR1fwd:CTGTGAATCAGAACGCCTCAAC<br><br>GluR1rev:ACCCGATGCCGTTCTTTTC | 900/900 | GAPDH 300/300 |
| **Iqgap1** | Iqgap1fwd:CGGAAGTGTCTCTCACGTTGAC<br><br>Iqgap1rev: GGTCCGAGCGTCCATCTCT | 900/900 | HPRT 300/900 |
| **Fmslike tyrosine kinase** | FMSlike fwd : GAAGGTACCGCAAGGAGAATC A<br><br>FMSlike rev : GGTGTGACGCAGCATCATG | 900/300 | GAPDH 300/300 |
| **Arih1** | Arih1fwd: GAGATGCAGCAGCACAACATG<br><br>Arih1rev: CACTGGCAGAGGACATCAACTG | 900/900 | GAPDH 300/300 |
| **PMP70** | pmp70 fwd: TGACTCGGAAGCACAGT<br><br>pmp70 rev: CCATGCCTCTTATCTCTCTGGTTAATA | 900/900 | HPRT 900/900 |
| **Dinein** | Dineinfwd:ACAATGGACAATCCCACCACTAC<br><br>Dineinrev: TGCTCCGCGCCTTCA | 900/900 | GAPDH 300/300 |
| **Srp20** | SRp20fwd:TGAGGATCCCCGAGATGCT<br><br>SRp20rev: CTTACACGGCAGCCACACAGT | 900/900 | HPRT 900/900 |
| **Ny2** | Ny2fwd: AGCAGGCACAGCATTGCA<br><br>Ny2rev: GTGTGAGCCAGCAAGTGAGATC | 300/900 | HPRT 900/900 |

| | | | |
|---|---|---|---|
| **Syn4** | Syn4fwd:GACAGAGCACGCAGAAAACATG<br><br>Syn4rev:CCACTGTGGGAATTTCATCGA | 900/900 | HPRT 900/900 |
| **U2af1** | u2af1fwd: GCTGTCCCCAGTAACTGACTTCA<br><br>u2af1rev:GCCCCCTCTTGTGCACTT | 300/300 | GAPDH 300/300 |
| **Bok** | bcl2rfwd: GGGAGTTTGTACGCAAGACCTT<br><br>bcl2rev:CTGACCACACACTTGAGGACATC | 900/900 | HPRT 900/900 |
| **PSF** | PSFfwd: GGAGTTCCACCAGCAACCAT<br><br>PSFrev: CTGCCCAAAGCGCTCAGT | 900/900 | HPRT 900/900 |
| **Pbx3** | pbx3fwd: GCGACGGCAGGAAGCA<br><br>pbx3rev:CTGGTCGGTGATGGTCATGA | 300/900 | HPRT 900/900 |
| **HPRT** | HPRT fwd: ATACAGGCCAGACTTTGTTGGATT<br><br>HPRT rev: TCACTAATGACACAAACGTGATTCAA | | |
| **GAPDH** | GAPDH fwd: CATTTCCTGGTATGACAATGAATACG<br><br>GAPDH rev: TCCAGGGTTTCTTACTCCTTGGA | | |

## APPENDIX II

**Table A2-** *In situ* hybridization. Oligonucleotide sequences and times of film exposure for the different mRNAs targeted.

| Target mRNA | Oligonucleotide sequence (5'-3') | Time Exposure |
|---|---|---|
| Arih1 | TCAGTCCTCAATGTACTCCCACAGATCTTTTTCATAGCCTTCATG | 7 days |
| Gaa1 | TTGAGCTTGCGGAACATGCCCTCCAGAGCCTTGCCCACTGTCGCCAAAT | 28 days |
| PSF | CTTTCTTCTCGTTGGCGTCTCATTTGTTCTTCCATCTCACGTTGGCGA | 7 days |
| SRp20 | GTGGTCCATAATAGCCAAAAGCCCGTTCTAATTCAGTCTTGTTTCCAT | 7 days |

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