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Ovarian steroids and their influence on hippocampal memory function and morphology

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Experiment 3

"Differential modulation of activity related to the anticipation of monetary gains and losses across the menstrual cycle," by J. Bayer, P. Bandurski, T. Sommer, 2013, *The European Journal of Neuroscience, 38*(10), p. 3519–3526. Copyright 2013 by the Federation of European Neuroscience Societies and John Wiley & Sons Ltd. Adapted with permission.

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Contents

Glossary and List of Abbreviations

ACC	anterior cingulate cortex
AI	aromatase inhibitor, drug that decreases estrogen synthesis
ALL	allopregnanolone, neuroactive metabolite of progesterone
AMPA	Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid, AMPA receptors are activated by glutamate and its analogs
ANOVA	analysis of variance
BDNF	brain-derived neurotrophic factor
BOLD	blood-oxygen-level dependent, measure used in magnetic resonance imaging
CA1-4	the four subdivisions of the cournu amonis area, i.e. hippocampal subregions
CEE	conjugate-equine-estrogen, compound of different estrogens used in hormone-replacement therapy
CLS	complementary learning systems model
CMT	cognitive map theory
CSF	cerebrospinal fluid
CYP19A1	gene coding for aromatase; variants on this gene can determine how effective the enzyme aromatase converts testosterone to estradiol
DARTEL	Diffeomorphic Anatomical Registration Through Exponentiated Lie Algebra, algorithm used for normalization of structural and functional

	brain images
DG	gyrus dentatus, hippocampal subregion
DLPFC	dorsolateral prefrontal cortex
DM	difference due to memory, approach that relates brain activity during encoding to subsequent memory performance
DNA	deoxyribonucleic acid
E2	17-beta estradiol, the most prominent estrogen in premenopausal women
EEM	emotional enhancement of memory
FDR	false discovery rate, statistical method to control for false-positive findings
fMRT	functional magnetic resonance imaging
FOV	field of view
FWE	family-wise error rate, statistical method to control for false-positive findings based on the gaussian random field theory
FWHM	full-width at half maximum, term used to describe size of a smoothing kernel
GABA	gamma-aminobutyric acid, a mostly inhibitory neurotransmitter
GLM	general linear model
GM	gray matter
GnRH	gonadotropin-releasing hormone, hormone that triggers the release of

	the gonadotropins luteinizing hormone and follicle stimulating hormone
GRF	gaussian random fields; the gaussian random field theory is the basis for family-wise error corrections in structural and functional neuroimaging
HRT	hormone-replacement therapy
HRF	hemodynamic response function
LTD	long-term depression
LTP	long-term potentiation
MNI	Montreal Neurological Institute; the MNI space defines a common target space for normalization
MPA	medroxyprogesterone acetate, artificial progesterone used for hormone-replacement therapy
mPFC	medial prefrontal cortex
MRI	magnetic resonance imaging
MTL	medial temporal lobe
MTT	multiple trace theory
NMDA	N-methyl-D-aspartate, neurotransmitter
OVX	ovariectomy/ovariectomized, i.e. removal of the ovaries leading to a drop in hormone levels
P4	progesterone, sex steroid
PET	positron-emission tomography
RBT	relational binding theory

rCBF	regional cerebral blood flow, measure used in positron emission tomography
ROC curve	receiver-operating characteristic curve, created by plotting hits versus false alarms across confidence levels (can be used for estimation of the process parameters recollection and familiarity)
RT	reaction time
SCT	standard-consolidation theory
SNP	single nucleotide polymorphism
SPM	statistical parametric mapping, software package for structural and functional image analyses
SVC	small volume correction, method to correct for multiple comparisons in structural and functional image analyses
TAM	tamoxifen, drug that acts through partial blockade of estrogen receptors
TAP	test of attentional performance, neuropsychological test-battery
TE	echo time, time between application of the excitation pulse and signal sampling (parameter of MR sequences)
TR	repetition time, time between two excitation pulses (parameter of MR sequences)
VBM	voxel-based morphometry, method to examine gray matter differences in structural brain images

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1 Summary

The ovarian steroids estradiol and progesterone play a critical role in the regulation of hippocampal plasticity. Estradiol has been shown to increase the growth of new spines and synapses and to facilitate hippocampal long-term potentiation. In line with findings on the cellular level, animal studies evidence beneficial effects of estradiol on hippocampus-dependent memory. Although much less investigated, progesterone can counteract several estradiol-related effects. Unfortunately, human studies about hormonal effects on the hippocampus and hippocampus-dependent memory are rare and highly equivocal. The present thesis aims to contribute data to the understanding of hormonal effects on the human hippocampus. For this purpose, four experiments were conducted assessing hippocampus-dependent memory performance as well as hippocampal structure and activity by means of magnetic-resonance tomography. Experiment 1 investigated the consequences of pharmacologically-induced decreases in estradiol synthesis. In this study, postmenopausal women suffering from hormone-sensitive breast cancer and age-matched controls were tested before onset of anti-hormone therapy and three to six months later. Behaviorally, anti-hormone therapy was associated with worse performance in a specific subdomain of hippocampus-dependent verbal memory. On the neuronal level, anti-hormone therapy increased neural responses in the anterior cingulate and the prefrontal cortex during a verbal source memory task. Results of Experiment 1 suggest that anti-hormone therapy impaired hippocampus-dependent verbal memory and increased prefrontal activity. Experiment 2 made use of a polymorphism associated to differences in estradiol serum levels in men. Hippocampal volume and hippocampus-dependent memory performance were examined across genotype groups within three independent cohorts, differing with respect to their genetic disposition to either high or low serum estradiol levels. Analysis of structural brain

images showed, that the genotype group with a disposition to high estradiol levels had higher volumes of the posterior hippocampus within both hemispheres. In contrast, hippocampus-dependent memory did not differ significantly between genotype groups. These results show that a genetic disposition to higher E2 levels is associated with higher macroscopic hippocampal volume in men. Experiments 3 and 4 examined women during two phases of their menstrual cycle. In both experiments, women were tested once during the early follicular phase, when estrogen and progesterone levels are low, and once during the luteal phase, in which estradiol is at a medium peak and progesterone is at its maximum. In Experiment 3, functional neuroimaging was recorded during encoding of emotional and neutral pictures in both cycle phases. Retrieval was conducted two days after encoding outside the scanner. Behavioral analyses yielded a significant valence by cycle interaction for recollection-based memory, which was mainly driven by decreases in recollection-based memory for negative pictures in the high- relative to the low-hormone phase. On the neuronal level, the hippocampus as well as prefrontal areas showed significant variations in their neural responses across menstrual cycle phases. Behavioral results of Experiment 3 suggest that hormones decrease the efficacy of negatively valenced arousal to stimulate hippocampus-dependent consolidation. Experiment 4 examined variations in hippocampal structure across the menstrual cycle. In line with existing literature, hippocampal volumes were higher when estradiol and progesterone were both low, compared with a cycle phase in which estradiol and progesterone were both high. Findings of Experiment 4 highlight the critical role of progesterone in the regulation of macroscopic hippocampal volume. In summary, present results underscore the variety of hormonal effects on the human memory system and their sensitivity to experimental factors. Furthermore, current results suggest that at least a part of the inconsistency in human literature can be accounted by not differeniating between hippocampus-dependent and -independent memory functions.

2 Introduction

Named after their seahorse-like shape, the hippocampi are located beneath the cerebral hemispheres deep within the medial temporal lobe (MTL). They are composed of the gyrus dentatus (DG) and the cornu amonis (CA) area (Andersen, 2007; Duvernoy, 2005). Based on morphological and cytoarchitectonic criteria, the CA region has been subdivided into the four sub-regions CA1 to CA4 (Andersen, 2007). As one of its outstanding features, the hippocampus has multiple connections to and from neocortical structures, making the hippocampus an ideal structure to integrate multimodal information (Andersen, 2007; Jeffery, 2007). Hippocampal neurons are well known for their unique capacity of learning and experience-related neuronal plasticity including long-term potentiation (LTP), synapto- and neurogenesis (Taupin, 2007)

The role of the hippocampus in memory has been investigated for more than five decades. A significant milestone was the observation that MTL resections are associated with severe memory deficits (Scoville & Milner, 1957). For instance, much insight was gained through studying the famous patient H.M., who developed a temporally graded retrograde and anterograde amnesia, i.e. experienced a nearly complete loss of declarative memory for events subsequent to his hippocampal resection and was unable to form new declarative memories. However, other types of memory such as skill learning remained surprisingly intact, suggesting that not all memory functions rely on the hippocampus (Cohen & Squire, 1980; Corkin, 1984, 2002; Milner, Corkin, & Teuber, 1968; Scoville & Milner, 1957)[1].

The hippocampus is particularly vulnerable to hazardous conditions. In fact, diseases like

1 It is now known, that not hippocampal damage alone but additional damage to surrounding areas was presumably the cause for the severity of his memory impairments (Corkin, Amaral, Gonzalez, Johnson, & Hyman, 1997; Squire, Amaral, & Press, 1990; Zolamorgan, Squire, & Amaral, 1986; Zolamorgan & Squire, 1986).

dementia, hypoxia but also mental conditions like depression or chronic stress are associated with decreased hippocampal volume (Fein et al., 2000; Gianaros et al., 2007; Jack et al., 2000; Sheline, Sanghavi, Mintun, & Gado, 1999). Because of its crucial role in human cognition, treatments aiming to protect hippocampal functions became an important field of research. One of the first hints that the sex steroid[2] 17-beta-estradiol (E2) might have beneficial effects on memory was reported by Caldwell and Watson (1952). The authors observed improved memory performance in a 75-year old woman after E2 injections. Later on, several studies suggested that E2 and progesterone (P4) modulate hippocampal structure and memory also in the young and healthy (Frye, Duffy, & Walf, 2007; Prange-Kiel & Rune, 2006; Protopopescu et al., 2008; van Wingen et al., 2007). Together with the discovery that hippocampal neurons synthesize E2 *de novo* from cholesterol, these findings gave rise to the idea that E2 not only protects hippocampal neurons from pathological conditions, but is even crucial for normal hippocampal functioning. Nevertheless, particularly results from human research are highly controversial and attest several known and unknown factors that modulate the rather small effects of hormones on hippocampal function. Therefore, the current thesis aims to contribute data on the mechanisms of hormonal effects on structure and functioning of the human hippocampus.

2 The terms sex steroids and ovarian steroids are used interchangeably in the present thesis, because both expressions are commonly used to refer to E2 and P4. However, it is of note, that the production of both hormones is not limited

3 Theoretical Background

3.1. Hippocampus and recognition memory

3.1.1 Psychological theories

The current chapter outlines the most influential theories of hippocampal function in recognition memory. Although some theories are primarily of historical value, they help to understand current research strategies and interpretations.

The *cognitive map theory* (CMT) of hippocampal function proposes that the hippocampus constructs and stores information about view-point independent space by forming associations between single environmental cues (O'Keefe & Nadel, 1978). Strong support for this theory comes from the discovery of pyramidal neurons in the hippocampus of rats that fire predominately in a restricted part of the environment (O'Keefe & Dostrovsky, 1971). These so-called 'place cells' are located in CA1 as well as CA3/4 and encode the rat's location independent of its orientation. In accordance with the CMT, rats with hippocampal lesions are selectively impaired in maze paradigms, when performance benefits from using a view-point independent spatial map (Morris, Garrud, Rawlins, & O'Keefe, 1982). It is of note, that the CMT understands the linguistic semantic map in humans as a modified version of the spatial map (Bloom, 1999; O'Keefe & Nadel, 1978). Whereas the spatial map represents spatial configurations with physical attributes, the semantic map represents spatial configurations in linguistic terms. It is still an ongoing debate, whether the hippocampus is disproportionally more involved in the storage of spatial than non-spatial information (e.g. Burgess, Maguire, & O'Keefe, 2002; Kumaran & Maguire, 2005).

As one of the most important concepts in memory research, the term 'consolidation' describes a time-dependent stabilization process, in which transient short-term memories are transferred into

persistent long-term memories (Burnham, 1903; Meinong, Müller, & Pilzecker, 1900; Ribot, 1887). Following this idea, the *standard-consolidation theory* (SCT) of hippocampal function assumes that repeated activation of hippocampal-cortical connections leads to a slow but progressive strengthening of cortical-cortical connections (Dudai, 2004; Frankland & Bontempi, 2005; Mcclelland, Mcnaughton, & Oreilly, 1995; Squire, 1992). The hippocampus thereby integrates information from distributed cortical nodes, each representing distinctive features of the event, and binds them into a coherent memory trace (Eichenbaum, 2004). Once the cortical-cortical connections have become sufficiently stable, retrieval is thought not to rely on hippocampal activation anymore (Squire & Alvarez, 1995). Biophysiological support is provided by the existence of neocortical projections to and from the hippocampus as well as the occurence of learning-dependent strengthening of synaptical connections known as LTP (Insausti, Amaral, & Cowan, 1987; Lavenex & Amaral, 2000). Further evidence for the SCT comes from patients with hippocampal damage such as H.M., showing a relatively intact remote declarative memory in combination with a severely impaired recent declarative memory (i.e. temporally graded retrograde amnesia; Marslen-Wilson & Teuber, 1975; Squire & Alvarez, 1995).

The *multiple trace theory* (MTT) of hippocampal function proposes that the hippocampus is primarily concerned with the recollection of autobiographical events, regardless of their age (Nadel & Moscovitch, 1997; Nadel, Samsonovich, Ryan, & Moscovitch, 2000). The hippocampal contribution to semantic memory is thought to decrease over time (Nadel & Moscovitch, 1997; Nadel et al., 2000). The MTT states further, that each time a specific memory content is retrieved, it is re-encoded and a new trace is formed (for a review, see Moscovitch, Nadel, Winocur, Gilboa, & Rosenbaum, 2006; Nadel et al., 2000). Consequently, this leads to the formation of more traces the older the memories are. The number of traces is assumed to be proportional to the probability of successful retrieval of the memory trace. On this ground, the MTT predicts that hippocampal lesion

size is correlated not only to the severity of retrograde amnesia but also to its temporal extent. Although much evidence from lesion and neuroimaging studies is in agreement with the MTT, criticism emerged especially from advocates of the *relational binding theory* (RBT; Moscovitch et al., 2006; Shimamura, 2003).

The RBT of hippocampal function states that the hippocampus is specifically involved in linking memory representations into a relational framework (Eichenbaum, 2004; Shimamura, 2003). In contrast to the MTT, the RBT understands hippocampal involvement into semantic and episodic memory not as qualitatively different. In accordance with the SCT, it is assumed that activation of hippocampal-cortical connections strengthens cortical-cortical connections. The degree of consolidation of cortical-cortical connections depends on the number of reactivations of the respective memory representations. Although the RBT proposes that retrieval can be facilitated through activation of hippocampal-cortical connections as associative links, retrieval of remote memories is not thought to necessarily rely on hippocampal activation. Thus, the hippocampus *can* contribute to the access as well as the retrieval of recent and remote memories. In fact, several studies report memory deficits in patients with hippocampal lesions for events 10 to 20 years prior to their surgery (for a review, see Nadel & Moscovitch, 1997; Rempel-Clower, Zola, Squire, & Amaral, 1996). The assumption that the hippocampus contributes to retrieval of recent but also very remote autobiographic memories has also been supported by studies employing functional imaging (for review, see Cabeza & St Jacques, 2007).

The *complementary learning systems* (CLS) model states that the hippocampus contributes to recognition memory through its ability to perform a process known as 'pattern separation' (Norman & O'Reilly, 2003). The term pattern separation refers to the 'process of transforming similar representations or memories into highly dissimilar, non-overlapping representations' (Bakker,

Kirwan, Miller, & Stark, 2008, p. 1640). It enables the organism to discriminate among similar stimuli such as targets and lures. The more similar stimuli are, the more pattern separation is needed to store them as distinct memory traces. In fact, the CA3 region and the DG are able to convert similar input firing patterns into less similar, and thereby less overlapping, output firing patterns (Bakker et al., 2008; Deng, Aimone, & Gage, 2010). In concordance with the above mentioned theories, the CLS model assumes that successful recognition of items together with its learning context requires hippocampal involvement. According to the CLS model, the neocortex, as a poor pattern separator, makes a qualitatively different contribution to memory than the hippocampus (Norman, 2010).

Although theories do not agree whether hippocampal involvement is moderated by memory content, remoteness or stimulus material, *they converge on the view that the hippocampus is critically involved in the formation and storage of coherent memory representations made up of single associations between stimuli*. The CLS model further specifies that the hippocampus is particularly involved in increasing the distinctiveness between memory representations. It is important to note, that episodic memory, which is constituted of configurational associations between information about times, places and subjective thoughts and feelings, is also associative in nature (Tulving, 1984). Today, the involvement of the hippocampus in associative memory is well evidenced by numerous studies employing functional magnetic resonance imaging (fMRI) or behavioral testing in patients with hippocampal lesions (Chua, Schacter, Rand-Giovannetti, & Sperling, 2007; Davachi, Mitchell, & Wagner, 2003; Davachi & Wagner, 2002; Eldridge, Knowlton, Furmanski, Bookheimer, & Engel, 2000; Giovanello, Verfaellie, & Keane, 2003; Henke, Buck, Weber, & Wieser, 1997; Kroll, Knight, Metcalfe, Wolf, & Tulving, 1996; Yonelinas et al., 2002). A further refinement to the definition of hippocampus-dependent memory will be discussed in the following chapter.

3.1.2 Theoretical basis of the current thesis: experimental research strategies and a current view on hippocampal functioning

Assessing hippocampus-dependent memory in animals

The present chapter outlines how hippocampus-dependent memory is typically assessed in animals. Animal research is crucial for the current thesis, because human research does not provide sufficient data to build specific hypotheses about hormonal influences on the hippocampus.

Research on hippocampal functioning in animals is highly influenced by the CMT. Therefore, hippocampus-dependent memory is usually quantified by assessing behavioral performance in spatial memory tasks. One of the most popular paradigms that is sensitive to hippocampal lesions is the *Morris Water Maze* task (Logue, Paylor, & Wehner, 1997; Morris, Garrud, Rawlins, & O'Keefe, 1982; Morris, 1981). In this paradigm, the animal has to find the previously learned location of a hidden or unhidden platform in opaque water. Hippocampal lesions particularly impair memory performance when the platform is not visible (for a review, see D'Hooge & De Deyn, 2001). Hippocampal contributions to performance in the Morris Water Maze task have been observed during acquisition, consolidation and retrieval. Findings from animal studies employing spatial memory tasks are therefore used in the present thesis to examine the influences of E2 and P4 on hippocampus-dependent memory.

Assessing hippocampus-dependent memory in humans – Dual process models

Previous chapters highlighted the role of the hippocampus in a broad range of relational memory functions, among which are processes supporting spatial and episodic memory (Abrahams et al., 1999; Burgess et al., 2002; Maguire, Frackowiak, & Frith, 1996; Montaldi & Mayes, 2010). The present chapters summarizes experimental approaches that aim to further parcel out the

contributions of hippocampus-dependent from –independent memory to performance in recognition memory tasks.

Dual process models refine previous theories about hippocampus-dependent memory by proposing that memory performance can be supported by the two qualitatively different processes *recollection* and *familiarity* (Yonelinas, 1994). Recollection is defined as the successful retrieval of a memory item together with contextual information (e.g. the learning context). Familiarity is understood as the successful recognition of an item as having been encountered before, but without recollecting any further information about the learning context. One central assumption of Yonelinas's model is that the hippocampus particularly mediates recollection, while familiarity is assumed to rely primarily on the perirhinal cortex (Brown & Bashir, 2002; Yonelinas et al., 2002; Yonelinas, Dobbins, Lazzara, & Knight, 1998). This assumption is supported by numerous studies combining behavioral techniques for the estimation of recollection and familiarity with neuroimaging and lesion approaches (Bowles et al., 2010; Daselaar, Fleck, & Cabeza, 2006; Staresina, Fell, Do Lam, Axmacher, & Henson, 2012; Turriziani, Serra, Fadda, Caltagirone, & Carlesimo, 2008; Yonelinas et al., 2002). The two processes differ not only with respect to their neural correlates, but also by the way information is retrieved from memory. Yonelinas' model defines familiarity as a continuous process reflecting memory strength, or in other words a process in which a memory signal is compared with a criterion. For example, when the memory signal for a previously learned word exceeds a subjective criterion, the participant perceives the item as a target word. Recollection in contrast is understood as a categorical or threshold retrieval process, in which information about the learning context is either retrieved or not (Yonelinas, Aly, Wang, & Koen, 2010). Therefore, memory judgments based on recollection are always characterized by a high degree of confidence. If recollection fails, memory decisions rely on the degree of familiarity. Because simple item recognition tasks can be solved by either recollecting target words together

with their learning context, but also by judging their degree of familiarity, overall memory performance does not serve as a clear indicator of hippocampal function in these tasks.

Several process-estimation methods exist that aim to quantify the contribution of recollection familiarity to overall memory performance (Yonelinas, 2002). For instance, inclusion/exclusion tasks require the subject to learn two word lists with different learning contexts (Jacoby, 1991; Prull, Dawes, Martin, Rosenberg, & Light, 2006). In the retrieval phase, subjects have to decide whether a specific item occurred in a specific learning context ('to-be-included') or not ('to-be-excluded'). In this task, recollection is reflected by the correct retrieval of a to-be-included word together with its learning context, whereas item memory can be a product of both processes. Other techniques are based on introception rather than objective memory performance. These techniques make use of recognition tests with confidence ratings, in which subjects have to judge on a 6-point scale ranging from 'very sure new' to 'very sure old' how sure they are about their old/new decision. The estimation of familiarity and recollection in those paradigms is based on the assumptions of the *signal detection theory* under the usage of *receiver-operating characteristic* (ROC) curves. ROC curves are functions that relate the proportions of hits and false alarms across confidence levels, starting with the items that were remembered with the highest confidence (Yonelinas, 1994). The intercept of the z-transformed ROC curve serves as a measure of discriminability, while the slope of the z-transformed ROC curve is a measure of the symmetry of the untransformed ROC curve. If performance in a memory task would rely only on familiarity, the ROC curve would by symmetric or in other words a signal detection process. If the criterion changes, the symmetry of the ROC curve would remain stable as this would affect hit rate and false alarm rate proportionally. In contrast, recollection specifically affects the number of old items that elicit high confidence answers, producing changes in the shape of the ROC curve. As another approach, source memory tasks are used, in which items are presented with varying contextual features such as time and color.

Hippocampus-dependent memory is assumed to be reflected by the retrieval of an item together with its corresponding feature, whereas item-memory can be supported equally well by hippocampus-independent as well as -dependent processes. Source memory tasks have the drawback, that item (e.g. apple) and source (e.g. red) can be encoded as unitized objects so that these tasks can be also solved by hippocampus-independent processes (Diana, Yonelinas, & Ranganath, 2008; Staresina & Davachi, 2009).

Although still controversies exist about the assumptions stated by the dual-process model (Jeneson, Kirwan, Hopkins, Wixted, & Squire, 2010), the current thesis follows the terminology and the theoretical implications of Yonelinas's dual process model. Consequently, the terms recollection and familiarity are used to refer to hippocampus-dependent or hippocampus-independent memory, respectively. Despite the profound actions of E2 and P4 on cellular process in the hippocampus (see 3.4), no attempt has been made to study the impact of E2 and P4 on recollection or familiarity. The experimental part of the current thesis therefore presents data concerning hormonal influences on the two processes obtained by different techniques. Additionally, associative memory is assessed through verbal source memory tasks, in which words are presented with varying contextual features (i.e. location and color).

Emotional enhancement of memory as an indicator of emotional modulation of hippocampal consolidation

Beyond the role of the hippocampus in memory for neutral events, there is considerable evidence that the hippocampus participates in the emotional modulation of memory as well as in non-mnemonic emotional functions (for a review, see Fanselow & Dong, 2010). The current paragraph focuses on the well described emotional enhancement of memories (EEM), i.e. the memory advantage for memories that are emotionally charged above memories with neutral contents.

The memory advantage of emotionally charged relative to neutral events relies upon the interplay between hippocampus, amygdala and prefrontal cortices (for a review, see Dolcos et al., 2012). Prefrontal areas, the hippocampus and the amygdala contribute differentially to the EEM, i.e. to distinct aspects and at different stages of emotional memory formation. When an emotional stimulus is encountered, a network between the amygdala and cortical areas rapidly initiates emotional processing and the allocation of attentional resources (Davis & Whalen, 2001; Pessoa, 2008). The elaborate cognitive processing of stimulus valence is then mediated by a prefrontal-parietal network (Canli, Zhao, Desmond, Glover, & Gabrieli, 1999; Dolcos, LaBar, & Cabeza, 2004; Kensinger & Schacter, 2006; Mickley & Kensinger, 2008). After the initial processing, emotional arousal leads to enhanced hippocampal consolidation of arousing stimuli via the amygdala and increased noradrenergic neurotransmission (Huff, Miller, Deisseroth, Moorman, & LaLumiere, 2013; McGaugh, 2004; Roozendaal, Nguyen, Power, & McGaugh, 1999; Strange & Dolan, 2004). Consequently, the effect of emotional arousal on memory requires a consolidation-delay between encoding and retrieval (Schwarze, Bingel, & Sommer, 2012; Sharot, Verfaellie, & Yonelinas, 2007).

Obviously, EEM effects do not serve as a good model of *pure* hippocampus-dependent processes since other brain areas also play critical roles. However, functional imaging and estimation of the process parameters recollection and familiarity make it possible to investigate the role of the hippocampus and its hormonal modulation in EEM effects.

3.2. Neurobiological basis of memory

Naturally, hormonal influences on hippocampal memory can only occur if the neurobiological basis of memory provides targets for hormonal actions. The following chapter reviews current

knowledge about neurobiological substrates of memory. It is important to note, that when behavioral performance is used to investigate memory performance, it is usually a result of various memory processes, i.e. recollection and familiarity, and different memory stages, i.e. acquisition, consolidation and retention, which are likely supported by different neurobiological mechanisms.

3.2.1 Processes involved in memory acquisition and consolidation

The acquisition and consolidation of memories requires activity-dependent synaptic plasticity, or in other words a learning-dependent enhancement of the connection between synapses (Martin, Grimwood, & Morris, 2000). Varieties of functional synaptic plasticity involve LTP and long-term depression (LTD). Moreover, structural changes at neurons such as synapto- and spinogenesis as well as the remodeling of spines and synapses can alter transmission efficacy and in turn affect memory acquisition and consolidation.

LTP occurs when synapses are repeatedly or simultaneously activated within 100 ms, and results in an enhancement of future synaptic transmission (Bliss & Lomo, 1973; Gustafsson & Wigström, 1988; Hebb, 2002; Levy & Steward, 1983). The best known form of hippocampal LTP is induced through activation of N-methyl-D-aspartate (NMDA) receptors (Abel & Lattal, 2001). NMDA-dependent LTP occurs for example at Schaffer collaterals in CA1, perforant path neurons onto CA3 and interconnecting CA3 neurons (Yeckel & Berger, 1998). LTP at mossy fiber synapses onto CA3 neurons are NMDA-independent and rely for instance on the activation of β-adrenergic receptors (Huang & Kandel, 1996). Many other processes such as activation of Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and metabotropic glutamate receptors participate in LTP, providing further targets for hormonal actions (Bashir, Jane, Sunter, Watkins, & Collingridge, 1993; Lu et al., 1997).

LTP can be subdivided into an early and a late phase, which are suggested to correspond to the acquisition and consolidation of memory (Bach et al., 1999; Yan-You Huang, Nguyen, & Kandel, 1996). In the early phase of LTP, lasting about 1 to 3 hours, an influx of calcium to the postsynaptic neuron leads to modification of existing enzymes. The experimental inhibition of early LTP induction is accompanied by impairments in the acquisition of hippocampus-dependent memory, such as spatial memory or contextual fear conditioning (Brigman et al., 2010; Fanselow, Kim, Yipp, & De Oca, 1994; Watanabe, Himi, Saito, & Abe, 1992). The later and more persistent phase, lasting more than 8 hours, relies on transcription synthesis of new proteins and is probably the underlying mechanism of memory consolidation (Frey, Frey, Schollmeier, & Krug, 1996; Frey, Krug, Reymann, & Matthies, 1988; Kelleher, Govindarajan, Jung, Kang, & Tonegawa, 2004; Montarolo et al., 1986; Sutton & Schuman, 2006). In fact, transient inhibition of messenger ribonucleid acid (mRNA) and protein synthesis block memory consolidation but not acquisition (Castellucci, Blumenfeld, Goelet, & Kandel, 1989; Crow & Forrester, 1990; Tully, Preat, Boynton, & Delvecchio, 1994). Similarly, genetic manipulations of protein synthesis alter late phase LTP and cause behavioral changes in memory consolidation but not acquisition (Miller et al., 2002; Costa-Mattioli et al., 2005). In addition to protein synthesis, the consolidation of memory also requires the reactivation of NMDA and AMPA receptors (Riedel et al., 1999; Shimizu, Tang, Rampon, & Tsien, 2000). Specific suppression of LTP maintenance, achieved via inhibition of a particular enzyme, even leads to forgetting of previously established spatial memory (Pastalkova et al., 2006).

LTD results in a lasting decrease in synaptic transmission (Levy & Steward, 1979; Lynch, Dunwiddie, & Gribkoff, 1977). Similar to LTP, NMDA, metabotropic glutamate and AMPA receptors participate in LTD (Bashir et al., 1993; Malenka & Bear, 2004; Martin et al., 2000; Stanton, 1996). LTD in CA1 has been found to be facilitated by the exploration of environments with novel objects and is suppressed during exploration of empty novel environments (Kemp &

Manahan-Vaughan, 2004; Lemon & Manahan-Vaughan, 2006; Manahan-Vaughan & Braunewell, 1999). Moreover, blockade of LTD impairs, whereas stimulation of LTD improves spatial reversal learning, which is interpreted as LTD being involved in the processing of new information (Dong et al., 2013). Selective inhibition of LTD, leaving LTP unchanged, also causes attenuated consolidation in a spatial memory task (Ge et al., 2010). Altogether, hippocampal LTD is most critical for memory acquisition as it mediates the exploration and processing of new information. LTD in the perirhinal cortex has been speculated to be a substrate of familiarity (Brown & Bashir, 2002).

As said above, memory is also related to morphological changes at neurons, which are inducible by activation of NMDA receptors and LTP (Constantine-Paton & Cline, 1998; Geinisman, 2000). Training in spatial and associative memory tasks increases spine density at basal dendrites of pyramidal CA1 neurons in rats (Leuner, Falduto, & Shors, 2003; Moser, Trommald, & Andersen, 1994; Moser, Trommald, Egeland, & Andersen, 1997). Conversely, application of a NMDA receptor antagonist during memory acquisition reduces hippocampal dendritic spine density and impairs LTP, LTD and memory performance (Brigman et al., 2010). Moreover, several studies observed training-induced synaptogenesis and structural modulation of existing synapses (for a review, see Bailey & Kandel, 1993; Moser et al., 1994; Ramírez-Amaya, Escobar, Chao, & Bermúdez-Rattoni, 1999).

As one of the most fascinating phenomena in neuroscience, the DG gives birth to new neurons even in the adult human brain (Eriksson et al., 1998). Neurogenesis comprises several sub-processes such as cell proliferation, differentiation, cell survival, maturation and integration. Although thousands of new neurons proliferate from progenitor cells in the DG each day, only few survive until full integration into the neuronal network (Shors, Anderson, Curlik, & Nokia, 2012). Evidence

accumulates that learning increases the survival probability of newborn cells (Gould et al., 1999; Leuner et al., 2004; Waddell and Shors, 2008; Curlik and Shors, 2011). Experimental reduction of neurogenesis impairs performance in several hippocampus-dependent memory tasks (Dupret et al., 2008; Jessberger et al., 2009; Saxe et al., 2006; Shors, Townsend, Zhao, Kozorovitskiy, & Gould, 2002; Snyder, Hong, McDonald, & Wojtowicz, 2005; Winocur, Wojtowicz, Sekeres, Snyder, & Wang, 2006; Zhang, Zou, He, Gage, & Evans, 2008). In humans, the neurogenic capacity of hippocampal neurons is associated with episodic memory performance before and after epilepsy surgery (Coras et al., 2010). Although results are controversial regarding which memory stage recruits neurogenesis the most, it has been speculated that hippocampal neurogenesis plays a central role in pattern separation (Deng et al., 2010; Sahay et al., 2011). This is based on the observation that the physiological properties of newborn neurons vary with age, so that a richer set of neuronal combinations exists for encoding new memories and separate them from others. To sum up, hippocampal neurogenesis makes important contributions to memory but the exact mechanisms are far from being understood.

3.2.2 Processes involved in memory retrieval

Experimental inhibition and reactivation of hippocampal neurons during retrieval showed that recent and remote recall relies upon reactivation of a specific neuronal ensemble within the hippocampus (Goshen et al., 2011; Liu et al., 2012). As memory retrieval does not require forming synaptical connections, it does not rely on hippocampal NMDA receptors, protein kinases or protein synthesis (Bourtchouladze et al., 1998; Lattal & Abel, 2000; Steele & Morris, 1999). Nevertheless, experiments in transgenic mice revealed that memory retrieval shares at least some processes with memory acquisition and consolidation such as the involvement of specific cellular proteins (Mansuy et al., 1998). On the other hand, certain exchange proteins play a specific role in memory retrieval

but not acquisition or consolidation (Ostroveanu, van der Zee, Eisel, Schmidt, & Nijholt, 2010).

Altogether, many cellular mechanisms are involved in memory acquisition, consolidation and retrieval. Theoretically, *targets for hormonal actions on memory could be provided by all processes, which are involved in the induction and maintenance of LTP and LTD, protein synthesis, synaptogenesis and neurogenesis*. Beyond direct actions on these cellular processes, hormones could interact with other neurotransmitter systems that influence neurobiological substrates of memory. The following chapters will cover knowledge about cellular actions of E2 and P4 at hippocampal neurons.

3.3. Synthesis and action pathways of ovarian steroids

The present chapter summarizes synthesis pathways of E2 and P4 and how these sex steroids affect cellular processes. These findings might help to appraise the influence of natural or experimentally produced peripheral hormonal fluctuations on hormone concentrations in the hippocampus. Moreover, it could help to infer under which circumstances hormones might modulate hippocampus-dependent memory functions.

3.3.1 Synthesis of E2 and P4

The amount of E2 and P4 in the brain is a mixture of centrally and peripherally synthesized hormones (Lars et al., 2009; Pardridge & Mietus, 1979). Peripherally, E2 and P4 are produced in the gonads, the skin as well as adipose and bone tissues (for a review, see Nelson & Bulun, 2001; for a review, see Strauss & Barbieri, 2009). Central hormone synthesis is carried out by various brain areas including the hippocampus (Bixo, Andersson, Winblad, Purdy, & Bäckström, 1997; Fester et al., 2006; Frye & Walf, 2004; Kominami et al., 2004; Kretz et al., 2004; Lars et al., 2009).

In fact, hippocampal neurons synthesize E2 and P4 *de novo* from cholesterol, leading to a hippocampal E2 concentration that can be considerably higher than in serum (Hojo et al., 2009; Kato & Kawato, 2013). Interestingly, peripheral and hippocampal hormone synthesis are not independent from each other, but are tightly coupled via gonadotropin-releasing hormone (GnRH) (Frye, Paris, & Rhodes, 2009; Prange-Kiel et al., 2008; Rhodes & Frye, 2004). The mechanism by which central and peripheral syntheses of P4 are synchronized is still unclear.

The first step in the production of E2 and P4 is the conversion of cholesterol to pregnenolone by cholesterol side-chain cleavage enzyme, which is located at the inner mitochondrial membrane. Therefore, cholesterol has to be first transported to the inner mitochondrial membrane. This rate-limiting step in steroid genesis is mediated by the steroid acute regulatory protein (Miller & Auchus, 2011). Pregnenolone is in turn converted by the enzyme 3β-hydroxysteroid dehydrogenase to P4 (Payne and Hales, 2004). After several further conversion steps, P4 is converted to testosterone which is then transformed to E2 by the enzyme *aromatase*.

3.3.2 Action pathways of E2

E2 acts via genomic and rapid, nongenomic pathways on hippocampal cells (McEwen & Alves, 1999; Nadal, Díaz, & Valverde, 2001; Yang et al., 2010). The genomic effects are exerted through binding on classical estrogen receptors, which are located mainly in the nucleus and cytosol of the cell. Once activated by E2, the estrogen receptors bind to estrogen response elements of several genes or interact with another DNA-bound transcription factor. This results in an altered rate of transcription of several genes, followed by alterations in protein synthesis (Foy, Baudry, & Thompson, 2004; Mangelsdorf et al., 1995). The classical estrogen receptor subtypes ER-α and ER-β are both expressed in the human adult hippocampus in pyramidal cells and the gyrus dentatus (DG; González et al., 2007; Österlund, Gustafsson, Keller, & Hurd, 2000; Österlund, Keller, &

Hurd, 1999; Tee et al., 2004).

Fast, non-genomic pathways are initiated at the cell membrane and are, for instance, mediated by classical and membrane estrogen receptors (Maggiolini & Picard, 2010; Migliaccio et al., 1996). Non-genomic actions account for effects of E2 that occur too rapid (<10 minutes) to be attributed to slow genomic pathways which take hours to days (Foy et al., 2004). In fact, non-genomic pathways of E2's action have a rapid onset varying between seconds and minutes, are not blocked by transcriptional inhibitors and their effects appear to be rather transient (Beyer, Pawlak, & Karolczak, 2003; Charlier, Cornil, Ball, & Balthazart, 2010). Most likely, several genomic and non-genomic actions of E2 account in parallel for E2's effects on hippocampal function (Spencer, Waters, Romeo, et al., 2008).

3.3.3 Action pathways of P4

Like E2, P4 acts via genomic and non-genomic pathways on hippocampal cells. In the genomic pathway, P4 binds at classical nuclear P4 receptors which results in a modulation of gene transcription (for a review, see Brinton et al., 2008). P4 receptors exist in the two major isoforms PR-A and PR-B, both occurring in several brain areas including the hippocampus (Camacho-Arroyo et al., 1998). Fast non-genomic actions of P4 are mediated through binding at P4 membrane receptors or other receptors of other neurotransmitters such as gamma-aminobutyric acid (GABA; Pang, Dong, & Thomas, 2013; Wu, Gibbs, & Farb, 1990). Moreover, indirect actions are exerted through conversion to the potent GABA-A agonist allopregnanolone (ALL; Fodor, Bíró, & Maksay, 2005; Majewska, Harrison, Schwartz, Barker, & Paul, 1986).

3.4. Ovarian steroids and the hippocampus of animals

The following chapters highlight the role of E2 and P4 in the regulation of cellular processes at hippocampal neurons. In line with cellular studies, behavioral animal studies show beneficial effects of E2 on spatial memory, whereas the role of P4 is much less clear.

3.4.1 Actions of E2 on neuronal substrates of memory

Evidence from animal studies show that E2 stimulates several mechanisms of hippocampal neuronal plasticity that underlie memory formation. The present chapter presents six mechanisms by which E2 might affect hippocampus-dependent memory.

First, E2 could improve memory consolidation by stimulation of LTP. E2 increases hippocampal LTP *in vivo* and *in vitro*, likely mediated by an E2-stimulated increase in NMDA signaling (Córdoba Montoya & Carrer, 1997; Foy et al., 1999; Good, Day, & Muir, 1999; Smith & McMahon, 2006; Vierk et al., 2012; Warren, Humphreys, Juraska, & Greenough, 1995; Wei, Yongxiang, & Jinhuang, 2000). Modulations of LTP have been shown across the estrous cycle and after application of aromatase inhibitors or E2 replacement. Furthermore, the stimulation of LTP has been shown to coincidence with improvements in spatial memory performance (Pulito et al., 2008).

Second, E2 might affect memory acquisition through modulation of LTD. Evidence exists that E2 can both stimulate and reduce LTD at hippocampal cells (Day & Good, 2005; Desmond, Zhang, & Levy, 2000; Good et al., 1999; Shiroma, Yamaguchi, & Kometani, 2005; Zamani, Desmond, & Levy, 2000). One explanation for discrepant findings is the fact, that application of E2 reduces NMDA receptor-dependent LTD, whereas it increases metabotropic glutamate receptor-dependent

LTD (Shiroma et al., 2005).

Third, E2 could improve hippocampus-dependent memory via induction of morphological changes at hippocampal neurons. One of the first evidences for actions of E2 on hippocampal cells was the observation that dendritic spine and synapse density in the CA1 region covary with the estrous cycle (Woolley, Gould, Frankfurt, & McEwen, 1990; Woolley & McEwen, 1992). Deprivation of E2, as a result of gonadectomy or application of an aromatase inhibitor, decreases spine and synapse density in rats and monkeys (Gould, Woolley, Frankfurt, & McEwen, 1990; Hao et al., 2003; Kretz et al., 2004; Leranth, Petnehazy, & MacLusky, 2003; Rune, Lohse, Prange-Kiel, Fester, & Frotscher, 2006; Woolley & McEwen, 1993). Systemic E2 replacement brings spine and synapse density back to control levels. Interestingly, hippocampus-derived E2 is much more effective in the maintenance of spine and synapse density than peripheral E2 (Prange-Kiel & Rune, 2006; Rune et al., 2006). Beneath stimulating the growth of new spines and synapses, E2 has the potential to remodel immaturate hippocampal spines into maturated mushroom-shaped spines (Li et al., 2004). In fact, E2-related morphological changes at hippocampal neurons are positively associated with performance in spatial memory tasks (Frick, Fernandez, & Bulinski, 2002; Li et al., 2004; Liu et al., 2012; Wallace, Luine, Arellanos, & Frankfurt, 2006).

Forth, E2 could influence memory through the modulation of neurogenesis. E2 not only promotes the plasticity of existing neurons, but also enhances cell proliferation and the survival of newborn neurons in the adult hippocampus (Banasr, Hery, Brezun, & Daszuta, 2001; Galea, Spritzer, Barker, & Pawluski, 2006; Ormerod, Lee, & Galea, 2003, 2004b; Tanapat, Hastings, Reeves, & Gould, 1999). Application of an aromatase inhibitor to hippocampal slice cultures even shows that a certain amount of E2 is necessary to maintain normal cell proliferation and survival (Fester et al., 2006). Because the critical amount of E2 exceeds the concentration in serum, these findings again confirm

the crucial role of hippocampus-derived E2. In rodents, the E2-stimulated increase in the survival of new cells is accompanied by better performance in a spatial memory task (Ormerod et al., 2004b). In ovariectomized (OVX) non-human primates, prolonged cyclic and continuous E2 treatment leads only to a marginally significant increase in several markers of neurogenesis (Kordower, Chen, & Morrison, 2010). Moreover, the effects of E2 on neurogenesis are rather transient and depend heavily on the treatment schedule (Barker & Galea, 2008; Ormerod et al., 2003; Tanapat, Hastings, & Gould, 2005). Thus, although effects of E2 on neurogenesis do occur, it is questionable whether they play a role in prolonged effects of E2 on hippocampal function and structure in humans.

Fifth, E2 could modulate memory through its interactions with other neurotransmitter systems or signaling pathways. The stimulation of NMDA signaling by E2 is only one of the many interactions between E2 and other neurotransmitter systems (Spencer, Waters, Romeo, et al., 2008). For instance, E2 interacts with GABA, acetylcholine, glutamate and serotonin in the hippocampus (Farr, Banks, & Morley, 2000; Gibbs, Gabor, Cox, & Johnson, 2004; Mize, Poisner, & Alper, 2001; Murphy, Cole, Greenberger, & Segal, 1998). Moreover, E2 influences several signaling pathways and neurotrophins such as the brain-derived neurotrophic factor (BDNF) which play a crucial role in E2's effects on neuronal plasticity (Sawai et al., 2002; Spencer, Waters, Romeo, et al., 2008; Zhou, Zhang, Cohen, & Pandey, 2005).

Sixth, E2 could maintain memory functions by enhancing neuroprotection. Hazardous conditions such as ischemic stroke, exposition to neurotoxic agents or Alzheimer's disease can lead to cell loss in the hippocampus and cognitive deficits (Rothman & Olney, 1986; West, Coleman, Flood, & Troncoso, 1994). In animal models, E2 prevents apoptosis, reduces neuronal inflammation and stimulates restorative processes like spino-, synapto- and neurogenesis (for a review, see Azcoitia, Arevalo, Nicola, & Garcia-Segura, 2011; Li et al., 2011).

Altogether, animal studies clearly demonstrate that E2 has beneficial effects on numerous cellular substrates of memory and could contribute to the maintenance of intact memory functions through the stimulation of neuroprotective and neurorestorative processes.

3.4.2 Actions of P4 on neuronal substrates of memory

Surprisingly few studies investigated the actions of P4 alone on neuronal substrates of memory. In contrast to E2, P4 can support but also impair neuronal correlates of memory. As such, P4 could also modulate behavoral memory performance in both directions.

For one thing, P4 could impair memory functions through inhibition of LTP. In fact, application of P4 to CA1 hippocampal slice cultures results in a dose-dependent decrease of LTP magnitude and baseline synaptic transmission (Foy, Akopian, & Thompson, 2008; Ito, Skinkle, & Hicks, 1999). P4 does not alter LTD in hippocampal slice cultures (Foy et al., 2008).

In contrast, P4 could improve or maintain memory functions via stimulation of structural neuronal plasticity and neuroprotection. P4 treatment in OVX animals increases synapse density, cell number and several synaptic proteins but not spine density (Choi et al., 2003; Murphy & Segal, 1996; Silva, Mello, Freymüller, Haidar, & Baracat, 2000). Moreover, P4 stimulates cell proliferation *in vivo* and *in vitro* in the adult rat hippocampus (Liu et al., 2009, 2010). P4 alone reduces apoptosis, decreases neuronal inflammation and initiates restorative processes after stroke and traumatic brain injury (Stein, 2008).

Similar to E2, P4 interacts with several neurotransmitter systems that are involved in memory processes. One of the best known interactions is mediated by the impact of P4 or ALL on the GABAergic system, which accounts for various effects of P4 on hippocampal cells (Wu et al., 1990). Moreover, P4 interacts with glutamate, acetylcholine and serotonin in the hippocampus

(Benmansour, Piotrowski, Altamirano, & Frazer, 2009; Benmansour, Weaver, Barton, Adeniji, & Frazer, 2012; Dazzi, Sanna, Cagetti, Concas, & Biggio, 1996; Kim, Cho, Choi, Lee, & Jang, 2011; Nilsen & Brinton, 2002). Additionally, P4 activates several signaling pathways (for a review, see Brinton et al., 2008; Nilsen & Brinton, 2003). In contrast to E2, P4 most likely does not influence hippocampal BDNF and NMDA receptor function (Bergeron, deMontigny, & Debonnel, 1996; for a review, see Brinton et al., 2008; Cyr, Ghribi, & Paolo, 2000).

All in all, P4 alone acts on several neuronal substrates of hippocampal memory. Because P4 acts inhibiting on some but stimulating on other neuronal correlates of memory, predictions about consequences of fluctuations in P4 on behavioral memory performance are difficult to make.

3.4.3 Actions of E2 plus P4 on neuronal substrates of memory

Even more complicated than the effects of P4 alone, is the biological basis for potential effects of *combined* E2 and P4 fluctuations on memory performance. P4 alone not only antagonizes some actions of E2, but also interacts with E2 itself in various ways. For instance, P4 decreases the amount of E2 receptors, whereas E2 increases the amount of P4 receptors as well as central P4 synthesis (Camacho-Arroyo, Guerra-Araiza, & Cerbon, 1998; Jayaraman & Pike, 2009; for a review, see Micevych, Soma, & Sinchak, 2008). Morover, indirect interactions occur for example in GABAergic neurotransmission that is stimulated by P4 but down-regulated by E2 (Murphy and Segal, 2000). These interactions are relevant especially for studies employing the natural estrous cycle, since peaks in E2 are either accompanied or preceded by peaks in P4.

As stated above, P4 alone has detrimental effects on LTP. However, it seems that P4 has additive effects on LTP when it is preceded by a peak in E2 (Warren et al., 1995). This is inferred from the finding, that the LTP amplitude is highest in the afternoon of proestrus, a phase that is characterized

by a peak of E2 in the morning and a peak of P4 in the afternoon. Moreover, P4 decreases excitability in the CA1 region.

With respect to structural changes, interactions of P4 and E2 depend on time as well as the order of hormone peaks. More precisely, spine density is highest during proestrus when E2 and P4 peak subsequently and drops sharply during estrus when E2 and P4 are both low (Woolley et al., 1990; Woolley & McEwen, 1993). The drop in spine density is abolished, when a P4 antagonist is given during proestrus. This effect can be explained by the biphasic action of P4. Whereas P4 initially augments the E2-stimulated increase in spine density, it leads to a much sharper decrease after a longer time period (Gould et al., 1990). Simultaneous exposure to P4 and E2 fully blocks the E2-stimulated increase in spine density and leads to a decrease in several synaptic markers (Choi et al., 2003; Murphy & Segal, 1996, 2000). On the subject of neurogenesis, simultaneous application of E2 and P4 increases the proliferation of hippocampal cells to a similar extent as E2 alone (Kordower et al., 2010; Liu et al., 2010). In contrast, cell proliferation is decreased when P4 is given subsequent to E2 (Tanapat et al., 2005).

Overall, P4 exerts additive and antagonistic actions on E2-stimulated plasticity of hippocampal neurons. One important modulating factor is the sequence of hormonal treatments. It is therefore important to consider the timing of hormonal peaks when investigating the influence of ovarian steroids on the hippocampus.

3.4.4 Actions of E2 and P4 on spatial memory

The present chapter reviews studies investigating the influence of hormone treatment in OVX animals and hormonal peaks across the estrous cycle on behavioral hippocampus-dependent memory performance. Fitting to cellular studies, behavioral data imply beneficial effects of E2 on

spatial memory performance but are inconsistent with respect to P4.

Consistent with cellular effects of E2 on hippocampal neurons, E2 enhances performance in spatial memory tasks in OVX animals during the acquisition as well as the consolidation phase (Gibbs et al., 2004; Gresack & Frick, 2006; Harburger, Bennett, & Frick, 2007; Li et al., 2004; Luine, Richards, Wu, & Beck, 1998; Packard & Teather, 1997; Packard, 1998; Ping, Trieu, Wlodek, & Barrett, 2008; Sandstrom & Williams, 2004). The effects depend on the dose and duration of E2 treatment, with low compared to high doses (Gresack & Frick, 2006; Rissanen, Puoliväli, van Groen, & Riekkinen Jr, 1999) and longer compared to shorter E2 treatments being more effective (Hodgson, Meddle, Christians, Sperry, & Healy, 2008; Luine et al., 1998). Interestingly, E2 specifically improves hippocampus- but not striatal-dependent memory performance (Davis, Jacobson, Aliakbari, & Mizumori, 2005).

Mirroring cellular effects of P4, literature is inconsistent concerning behavioral effects on memory. Acute and chronic administration of P4 or ALL enhances hippocampus-dependent spatial memory consolidation in OVX rats and mice (Frye et al., 2007; Frye, Koonce, & Walf, 2010; Frye, Llaneza, & Walf, 2009; Frye & Walf, 2008a, 2008b; He, Yang, Zhai, Shao, & Li, 2011). Similar to E2, the beneficial effects of ALL on memory performance depend on the doses, with low doses exerting beneficial but high doses detrimental effects (Frye & Sturgis, 1995). Higher *natural* levels of P4 in the hippocampus are associated with better performance in a spatial memory task (Paris, Walf, & Frye, 2011). It is important to note, that several studies failed to find improved hippocampus-dependent spatial memory after P4 treatment (El-Bakri et al., 2004; Harburger, Pechenino, Saadi, & Frick, 2008; Lewis, Orr, & Frick, 2008; Sandstrom & Williams, 2001).

Not surprising, studies on the association between OVX and the estrous cycle with changes in hippocampus-dependent memory performance are equivocal, too. Whereas some studies indicate

better spatial memory during high compared to low hormone phases of the estrous cycle (Frick & Berger-Sweeney, 2001; Reddy & Kulkarni, 1999; Walf, Koonce, Manley, & Frye, 2009), others report reversed or no differences (Berry, McMahan, & Gallagher, 1997; Lacreuse, Verreault, & Herndon, 2001; Pompili, Tomaz, Arnone, Tavares, & Gasbarri, 2010; Spencer, Waters, Milner, & McEwen, 2008; Warren & Juraska, 1997). One reason for the divergency in study results might be, that rats shift from a striatal-dependent to a hippocampus-dependent strategy when hormones are high, which can be beneficial in some but hindering in other spatial tasks (Korol, Malin, Borden, Busby, & Couper-Leo, 2004; McElroy & Korol, 2005; Quinlan, Hussain, & Brake, 2008). Similar to the variation of spatial memory performance across the estrous cycle, OVX leads to decreases in spatial memory performance in some studies (El-Bakri et al., 2004; Hammond, Mauk, Ninaci, Nelson, & Gibbs, 2009; Sarkaki, Amani, Badavi, Safahani, & Aligholi, 2008; Sato et al., 2003; Su, Sripanidkulchai, Hu, Wyss, & Sripanidkulchai, 2012; Wallace et al., 2006), but not in others (Singh et al., 1994; Wilson et al., 1999; Voytko, 2000). When memory impairments after OVX have been found, they were rather related to decreases in E2 than P4 levels (El-Bakri et al., 2004; Hammond et al., 2009; Martin, Jones, Simpson, & van den Buuse, 2003).

As observed for cellular effects of E2 and P4, different treatment schedules (e.g. sequences or dosages) lead to diverging hormonal effects on memory performance. For instance, performance in a spatial memory task, which was insensitive to E2 or P4 alone, was impaired after administration of E2 followed by P4 (Chesler & Juraska, 2000). Conversely, simultaneous application of P4 and E2 can either block E2-related improvements in memory but can also lead to similar improvements as E2 alone, depending on the dose of P4 (Bimonte-Nelson, Francis, Umphlet, & Granholm, 2006; Frye et al., 2007; Gibbs, 2000; Harburger et al., 2007; Sato et al., 2004).

On the whole, little doubt is left that both hormones affect hippocampus-dependent spatial

memory under particular circumstances. In general, E2 alone improves memory and stimulates the use of a hippocampus-dependent learning strategy. Although much less studied, it is likely that P4 has also the potential to improve certain types of hippocampus-dependent memory. It is important to note that ALL, as a potent GABA agonist, also exerts actions on other parts of the brain such as the amygdala (Akwa, Purdy, Koob, & Britton, 1999) and could thereby influence memory performance through modulation of affective processes.

3.4.5 Actions of E2 and P4 on emotional memory

Emotional memory in animals is most often studied by employing fear conditioning paradigms. In these tasks, an unconditioned stimulus, like an air puff or a foot shock, is paired with a conditioned stimulus, such as a context and/or a cue (Curzon, Rustay, & Browman, 2009). Freezing responses during acquisition, retrieval and/or extinction serve as dependent measures. While fear conditioning with a contextual cue (e.g. a chamber) depends on hippocampal functioning, fear conditioning with a single cue (e.g. a light) depends more on the amygdala.

In contrast to the influences of E2 on spatial memory, E2 decreases consolidation and fascilitates extinction in contextual fear conditioning (Day, Sung, Logue, Bowlby, & Arias, 2005; Graham & Milad, 2013; Gupta, Sen, Diepenhorst, Rudick, & Maren, 2001; Markus & Zecevic, 1997). This has been shown for the estrous cycle, OVX, genetic manipulations and the administration of estrogen receptor agonists or E2. It has to be emphasized, that E2 specifically affects the memory component of contextual fear conditioning and does not modulate initial freezing responses to the unconditioned stimulus (Day et al., 2005). P4 administered alone increased memory in contextual fear conditioning, suggesting opposing effects of the two hormones (Frye & Walf, 2008c, 2010).

3.5. Research strategies in humans

Based on animal studies, there is a strong supposition that human hippocampal structure and function is affected by variations in ovarian steroids. However, human research in this field is complicated by difficulties in the operationalization of hormonal status as an independent variable as well as in the assessment of hippocampal structure and function as dependent variables. The following chapters will discuss common research approaches in humans.

3.5.1 Hormonal status as an independent variable

Most studies in animal models administer E2 or P4 to young OVX animals or slice cultures. This has the advantage that hormone levels are under tight experimental control. In human research, the *administration of hormones* is associated with considerable efforts and potential medical risks, so that it is rarely employed in healthy young subjects. Existing human administration studies differ with respect to administration routes from animal studies, with more indirect routes (e.g. patches) in humans producing considerably higher variance than in animals. Moreover, animal administration studies are usually conducted in young OVX animals, while human research is mostly conducted on postmenopausal women or naturally cycling young women. The closest correspondence between human and animal research is given by studies administering oral E2 replacement to young women that underwent pharmacological or surgical menopause. Pharmacological menopause is induced by GnRH agonists like Lupron, which are used for the treatment of diseases such as endometriosis and uterine leiomyoma (Filicori & Flamigni, 1988). However, only a small number of women are treated with GnRH agonists and even fewer receive hormone-replacement in the form of E2. Moreover, this approach has the caveat that most of these subjects have hormonal diseases which could bias study results. Few studies exist in which elderly postmenopausal women are treated with E2. Here, concerns have been raised whether the sensitivity towards hormonal fluctuations remains

intact after long phases of hormonal deprivation (for a review, see Bimonte-Nelson, Acosta, & Talboom, 2010; Inagaki, Kaneko, Zukin, Castillo, & Etgen, 2012). Administration of hormones to naturally cycling women is also problematic, because the effects of exogenous hormone manipulation could be modulated by fluctuations in endogenous hormone levels.

Research on the consequences of *hormone-replacement therapy* (HRT), mostly consisting of conjugate-equine-estrogen (CEE) plus a progestin such as medroxyprogesterone acetate (MPA), provides the highest number of publications in the whole research field. The high availability of data, in part acquired from big study samples, is undoubtedly the major advantage of this approach. However, CEE is a compound of equilin (i.e. horse estrogen), estrone and other estrogens, which are acting considerably different from E2 (Barha, Dalton, & Galea, 2009; Jin, Jin, Zhang, Chen, & Huang, 2005; Whittaker, Morgan, Dean, Cameron, & Lind, 1980; Wroolie et al., 2011). Moreover, MPA has been shown to impair memory in aged OVX rats to a greater extent than natural P4 (Braden et al., 2010). Beneath pharmacological concerns, HRT studies often employ standard neuropsychological tests for memory assessments. These tests have been designed as indicators of clinically relevant memory changes, but might not be sensitive enough to detect subtle changes in hippocampus-dependent memory processes (Berga, 2008). For these reasons, it is highly questionable whether HRT studies contribute much to the understanding how E2 and P4 affect the human hippocampus.

The *inhibition of aromatase,* preventing the conversion of testosterone to E2, is an elegant approach because it inhibits peripheral as well as central E2 synthesis (Biegon et al., 2010; Overk et al., 2012). Moreover, it does not affect P4 so that declines in E2 can be studied in isolation. In humans, aromatase inhibitors (AI) such as Letrozole, Anastrozole or Exemestane are the gold standard for adjuvant endocrine treatment of estrogen receptor-positive breast cancer in

postmenopausal women (Coates et al., 2007; Mouridsen et al., 2001; Winer et al., 2005). Studies employing Letrozole or Anastrozole therapy are most useful, because Exemestane possesses mild andronergic properties that could affect hippocampal function on their own (Ariazi et al., 2007). Another agent used for breast cancer therapy is tamoxifen (TAM), which works through partial blockade of estrogen receptors. However, TAM has also agonistic effects on estrogen receptors, so that the effects of TAM on E2-stimulated cellular mechanisms in the hippocampus are unclear (Ernst et al., 2002; Gallo & Kaufman, 1997). Similar to HRT studies, the AI approach often provides data from large studies. Unfortunately, many studies suffer from methodological problems such as missing baselines or control groups and employ paradigms with insufficient sensitivity for changes in hippocampal function. *Experiment 1* of the current thesis therefore investigated postmenopausal breast-cancer patients and age-matched controls at baseline and 3 to 6 months after the onset of AI therapy (or waiting in controls) with a number of hippocampus-dependent memory tests as well as functional and structural imaging.

Genetic approaches for studying hormonal effects in animals include the experimental manipulation of specific proteins such as the knockout of aromatase or estrogen receptor expression (Day et al., 2005; Pierman, Tirelli, Douhard, Baum, & Bakker, 2006). This allows for example to study the influence of specific receptor subtypes or consequences of aromatase deprivation on brain development. Because these severe mutations are luckily rare in nature, human research rather makes use of common genetic variants like single-nucleotide polymorphisms. Most of these genetic variants are not linked to diseases, but are associated with small interindividual differences in hormonal metabolism. This approach is non-invasive, can be easily applied to big study samples and does not rely on highly variable hormonal fluctuations. Moreover, it allows studying the influence of long-term differences in hormone levels on the hippocampus in populations with relatively stable hormone levels (e.g. men). As a drawback, this approach is not useful to study the

effects of acute changes in hormone levels. *Experiment 2* investigated hippocampal structure and hippocampus-dependent memory in three cohorts of healthy young men that were genotyped for a polymorphism associated with serum E2 levels in men.

The use of the *natural menstrual cycle* provides a non-invasive opportunity to study endogenous hormonal fluctuations in women. However, women using oral contraceptive have a different hormonal profile and must thus be considered separately (Becker, 2005; Fleischmann et al., 2010). In practice, the length of cycle phases is highly variable across but also within subjects, so that between-subject studies have to be interpreted with big caution especially when no hormone levels have been assessed (Becker, 2005; Fehring et al., 2006; Small, et al., 2010). Moreover, sharp increases and decreases in E2 or P4 as well as additional variations in luteinizing hormone can have effects on the central nervous system on their own (Hausmann et al., 2000; Berry et al., 2008; Ossewaarde et al., 2010). In *Experiments 3 and 4*, a within-subject design was used to compare hippocampal function and structure in the early follicular phase, where both hormones are low, with the luteal phase, in which E2 is at a medium and P4 is at its peak. These phases have relatively stable hormone levels and are not biased by variations in luteinizing hormone.

3.5.2 Assessment of hippocampal structure and function as dependent variables

Theoretically, the closest correspondence between human and animal research should be provided by applying structural and functional imaging. Differences in neuronal macroscopic structure as measured by structural magnetic resonance imaging (MRI) are likely the product of alterations in spine density, cellular shape or cell number (Fields, 2011; Zatorre et al., 2012). Assessment of brain activation patterns through fMRI or positron-emission tomography (PET), mirrors changes in neuronal metabolism (Bailey, Townsend, Valk, & Maisey, 2006; Logothetis, Pauls, Augath, Trinath, & Oeltermann, 2001). A closer inspection of the relationship between MRI signals and

cellular processes is provided in the general methods part of the current thesis.

When studying hippocampal activity or behavior as a measure of hippocampal processing, paradigms are needed which clearly depend on hippocampal functioning. So far, no study has yet investigated hormonal influences on human memory by using dual process approaches or other methods that allow separating hippocampus-dependent from -independent memory. This is problematic, because even patients with extended hippocampal lesions can show normal recognition memory (Bowles et al., 2010; Vann et al., 2009). To put it another way, impairments in hippocampus-dependent memory functions can be overseen when hippocampus-dependent and -independent memory processes are not seperated. In hormonal research, hippocampal functions are often assessed through spatial memory tasks in animals and verbal memory tasks in humans. This is highly challenging, not only because it forbids the direct comparison between animal and human research, but also because there is considerable variation of hippocampal involvement in verbal memory paradigms across tasks, subjects and studies (Loring et al., 1991; for a review, see Schacter & Wagner, 1999). Additionally, many studies utilized tasks with a strong working memory component, that indeed can recruit hippocampal areas involvement, but relies more on prefrontal processes (Monk et al., 2002; Stern, Sherman, Kirchhoff, & Hasselmo, 2001).

The experimental part of the present thesis therefore investigates hormonal influences on the human hippocampus by applying neuroimaging and behavioral memory tasks. The behavioral tasks employed in the current study allow disentangling hippocampus-dependent from –independent memory.

3.6. Ovarian steroids and the hippocampus of humans

The present chapters discuss results from human research with respect to hormonal actions on the hippocampus and memory. Exepct from the chapter on emotional memory, only studies using either verbal or spatial memory tasks that do not entail a strong working-memory component are reported.

3.6.1 Hormonal effects on hippocampal activation and metabolism

Until now, no study has yet directly investigated whether E2 or P4 modulate memory-related hippocampal activity. The suppression of E2 and P4 production by the GnRH agonist lupron leads to changes in prefrontal but not hippocampal activity during the encoding phase of a verbal memory task in young women (Craig et al., 2007). Neuronal activity during the recognition phase was unaffected by hormonal treatment. As the main effect of memory was not associated with hippocampal activity, it remains unclear whether the hippocampus was critically involved in this task at all. Hippocampal glucose mechanism under rest did not differ significantly between the early follicular phase, in which E2 and P4 are low, compared to the high hormone luteal phase, in which E2 and P4 are both high (Reiman, Armstrong, Matt, & Mattox, 1996). The hormonal manipulations in both studies lead to concomitant changes in E2 and P4 levels, null findings with respect to hippocampal activity or metabolism are therefore in line with the finding that certain actions of E2 and P4 can cancel each other out (Choi et al., 2003; Murphy & Segal, 2000).

HRT in elderly women was associated with higher hippocampal/parahippocampal regional cerebral blood flow (rCBF) as well as increased blood-oxygen-level dependent (BOLD) responses during the encoding and recognition of words (Gleason et al., 2006; Maki & Resnick, 2000; Maki et al., 2011; Resnick, 1998a). These results are in line with the finding that E2 increases hippocampal functional plasticity in animals, and therefore imply that E2 and CEE share at least some cellular actions on the hippocampus (Vierk et al., 2012). CEE users had higher hippocampal rCBF than

CEE plus progestin users, suggesting that CEE and progestins compensate one another (Silverman et al., 2011). However, several studies failed to find a relationship between short-term HRT in postmenopausal women and hippocampal BOLD response or rCBF during the encoding of words and under rest (Eberling, Wu, Tong-Turnbeaugh, & Jagust, 2004; Persad et al., 2009; Rasgon et al., 2005). A part of this variance in study designs could by accounted by the 'limited time-window hypothesis', claiming that beneficial effects of HRT can only be exerted within a short time period after menopause (Erickson, Voss, Prakash, Chaddock, & Kramer, 2010; Maki, 2006). Advocates of this theory believe that after a certain period with low hormone levels the brain looses its sensitivity to hormonal changes.

3.6.2 Hormonal effects on hippocampal macroscopic morphology

In line with the stimulation of spino- and synaptogenesis by E2, macroscopic hippocampal volumes are larger during phases of high E2 levels across the menstrual cycle and the menopausal transition (Protopopescu et al., 2008; Goto et al., 2011). In support of antagonistic actions of E2 and P4, parahippocampal/hippocampal volumes were higher in women during the low-hormone early follicular phase compared to women in the luteal phase in which E2 and P4 are both high (Pletzer et al., 2010).

Similar to studies on the effects of HRT on hippocampal activation and metabolism, some studies attest positive effects of HRT on hippocampal volume while others do not. In fact, several studies suggest larger hippocampal volumes in postmenopausal women who are current or past HRT users compared to postmenopausal women who never have had HRT (Boccardi et al., 2006; Eberling et al., 2003; Erickson et al., 2005; Lord, Buss, Lupien, & Pruessner, 2008). Likewise, women using HRT have lower markers of membrane turnover and glia content but higher marker of cholinergic activity as well as muscarinic receptors in the hippocampus (Ernst et al., 2002; Norbury et al., 2007;

Robertson et al., 2001; Smith et al., 2011). However, numerous studies failed to replicate positive effects of HRT on hippocampal volume or cellular markers. Again, these null findings connect to the hypothesis of a limited time-window of opportunity after menopause (Eberling et al., 2004; Erickson et al., 2010; Espeland et al., 2009; Lord et al., 2008; Low et al., 2006; Raz, Rodrigue, Kennedy, & Acker, 2004; Robertson et al., 2001).

3.6.3 Hormonal effects on memory

Administration studies show that E2 improves verbal memory in young women but are inconsistent with respect to postmenopausal women. For instance, transdermal E2 treatment over 30 days in healthy young women lead to marginal improvement in verbal memory (Bartholomeusz et al., 2008). Likewise, add-back E2 improved verbal memory in young women who underwent pharmacologically or surgically induced menopause (Phillips & Sherwin, 1992a; Sherwin, 1988; Sherwin & Tulandi, 1996). Natural fluctuations in E2 across the menstrual cycle or the menopausal transition are positively associated with verbal and spatial memory in some studies (Hogervorst, De Jager, Budge, & Smith, 2004; Protopopescu et al., 2008; Solis-Ortiz & Corsi-Cabrera, 2008), but not in others (Phillips & Sherwin, 1992b; Resnick, 1998a). Resembling data collected in premenopausal women, E2 alone and E2 plus a progestin can improve verbal memory in postmenopausal women (Baker et al., 2012; Duka, Tasker, & McGowan, 2000; Krug, Born, & Rasch, 2006; Linzmayer et al., 2001; Wolf et al., 1999). On the other hand, there are numerous reports of a missing association between E2 treatment and verbal memory in post- and perimenopausal women (Almeida et al., 2006; for a review, see Eef Hogervorst & Bandelow, 2010; Joffe et al., 2006; Kocoska-Maras et al., 2011; LeBlanc, Neiss, Carello, Samuels, & Janowsky, 2007).

Similar to findings about E2 administration in postmenopausal women, studies examining

cognitive effects of AI therapy are contradictory, too. A total of four studies compared memory performance in patients treated with AIs to healthy controls without any medical treatment. In a rather small prospective clinical study, breast cancer patients treated with Anastrozole showed a significant decline in verbal memory in comparison to unmedicated healthy controls (Collins, Mackenzie, Stewart, Bielajew, & Verma, 2009). A cross-sectional study on 22 postmenopausal women aged 75 to 80 years found marginal worse in visuo-spatial memory but not verbal memory in the AI group compared to controls (Nattinger et al., 2013). In a sub-study of a big clinical trial (i.e. IBIS-II), 227 healthy postmenopausal women at high risk of breast cancer were administered Anastrozole or placebo in a randomized and double-blind manner (Jenkins et al., 2008). Women were tested before randomization, as well as 6 and 24 months after onset of the AI therapy with a number of psychometric assessments including verbal memory tests. No difference was found on any measure. Other studies using mixed patient samples (AI, TAM, chemotherapy) or comparing different treatment groups also delivered highly inconsistent results. In fact, there is some indication for poorer memory of Anastrozole compared to TAM users (Bender et al., 2007) as well as worse verbal memory in a mixed patient group (AI, TAM) compared to healthy controls (Shilling, Jenkins, Fallowfield, & Howell, 2003). Others report little or no differences between endocrine treatments (mixed AI and TAM) and healthy controls in memory performance (Breckenridge, Bruns, Todd, & Feuerstein, 2012; Hedayati, Alinaghizadeh, Schedin, Nyman, & Albertsson, 2012; Lejbak, Vrbancic, & Crossley, 2010; Schilder et al., 2010), or even higher overall cognitive scores of women on Letrozole compared to women on TAM (Ribi et al., 2009).

P4 or ALL, given to premenopausal women, impeded verbal memory but likewise enhanced subjective reports of fatigue and confusion (Freeman, Purdy, Coutifaris, Rickels, & Paul, 1993; Freeman, Weinstock, Rickels, Sondheimer, & Coutifaris, 1992; Kask, Backstrom, Nilsson, & Sundstrom-Poromaa, 2008). A concomitant drop in E2 and P4 levels, caused by Lupron therapy or

surgical menopause, impaired verbal memory in previously premenopausal women (Phillips & Sherwin, 1992a; Sherwin, 1988; Sherwin & Tulandi, 1996; Varney et al., 1993). However, two other studies failed to replicate the association between Lupron therapy and verbal memory (Owens, Matthews, & Everson, 2002; Schmidt et al., 2013). The natural concomitant peak of E2 and P4 during luteal phase was not associated to changes in verbal memory performance (Mordecai, Rubin, & Maki, 2008; Solis-Ortiz & Corsi-Cabrera, 2008).

Similar to the effects of HRT on hippocampal structure and function, the effects of HRT on memory performance are conflicting. In detail, although evidence exists for a positive effect of HRT on verbal memory in postmenopausal women and male-to-female transsexuals, larger clinical studies often fail to find an association between HRT and verbal memory (Greendale et al., 2009; Jacobs et al., 1998; Kampen & Sherwin, 1994; Low et al., 2006; Miles, Green, Sanders, & Hines, 1998; Resnick, 1998b). A review including 27 studies on the effects of HRT on verbal memory, reported that 26% of the tests yielded negative effects, 37 % null effects and only 37 % positive effects (Eef Hogervorst & Bandelow, 2010). Therefore, it is highly questionable whether HRT has an effect above chance on verbal memory at all. Again, those studies are often challenged by experimental confounds and paradigms that do not aim to separate different memory processes from each other.

3.6.4 Hormonal effects on the emotional enhancement of memory

Until now, hormonal effects on declarative EEM have been only investigated inter-individually (Ertman, Andreano, & Cahill, 2011; Felmingham, Fong, & Bryant, 2012). Ertman and colleagues report that women in the second half (i.e. after ovulation until menstruation) of the menstrual cycle recalled more pictures with negative contents than women in the first half (i.e. menstruation to ovulation) of the menstrual cycle. The recall of negative pictures was positively associated to P4

levels during encoding across subjects. In the second study, women were grouped according to individual P4 level into a high and a low P4 group. Here, the recall of negative pictures did not differ between the two groups (Felmingham, Fong, & Bryant, 2012). Postmenopausal HRT use was associated to higher subjective arousal but not alterations in EEM effects (Pruis, Neiss, Leigland, & Janowsky, 2009).

On the whole, E2 increases macroscopic hippocampal volume and can improve verbal memory in young women. Much higher inconsistencies emerge for hormonal manipulations in postmenopausal women, of which some might be accounted by decreased sensitivity towards hormonal influences after menopause. Although much less studied, P4 alone can impair memory, most likely caused by the sedative effects of P4. It remains unclear whether inconsistencies are related to unreliable or non-existent effects of E2 and P4 on hippocampal function in humans or whether they are rather the result of methodological issues. Although the current thesis will not provide the ultimate answer to this question, the use of MRI and clearly hippocampus-dependent paradigms should clarify at least some issues in this field.

4 Research questions

1. *Effects of E2 deprivation on hippocampal functions in postmenopausal women*: Does a decrease in the production of E2 after AI therapy have adverse effects on hippocampus-dependent memory, macroscopic structure and hippocampal BOLD responses in postmenopausal women suffering from breast cancer (*Experiment 1)*?

2. *Effects of E2 on the hippocampus in men*: Is a genetic disposition to higher E2 serum levels associated with differences in hippocampal macroscopic structure and hippocampus-dependent memory in men *(Experiment 2)*?

3. *Effects of endogenous hormone fluctuations on emotional memory in young women*: Does behavioral performance and neural activity in an emotional memory task, as a specific type of hippocampus-dependent memory, vary across the menstrual cycle when a within-subject design is used *(Experiment 3)*?

4. *Effects of endogenous hormone fluctuations on hippocampal volume in young women*: Can changes in hippocampal volumes across the menstrual cycle be replicated and do they correlate to hormone levels in saliva? *(Experiment 4)*

5 General Methods

Methods employed in the current thesis examine hormonal influences on the human hippocampus. On the behavioral level, paradigms are used that assess hippocampus-dependent memory based on a prominent dual-process model (Yonelinas, 2002; see chapter 3.1.2.). On the neuronal level, MRI is utilized to examine differences in hippocampal activity and structure. The following chapters briefly describe the physical background of MRI as well as preprocessing and analysis methods applied in the current thesis. Furthermore, the last chapter discusses the problem of multiple testing in voxel-based neuroimaging.

5.1. Physical and physiological background of magnetic resonance imaging

MRI is a powerful tool to examine brain structure and brain activity in vivo. Since studies concerning hormonal influences on the hippocampus are much more equivocal on the behavioral than on the cellular level, imaging data are of particular importance as they are closer to neuronal processes. The present chapter summarizes current knowledge about physical and physiological processes in MRI.

MRI uses the characteristics of hydrogen nuclei, which are positively charged and rotate around their axis. This so-called 'spin' produces a magnetic field with a specific orientation. During MR examination of the human brain, the head of the volunteer is placed in a strong magnetic field. As a result, protons that are normally aligned in a random manner become aligned parallel or anti-parallel to the external magnetic field (Schild, 1990). Because more protons are aligned parallel than anti-parallel to the magnetic field, the net magnetization is *longitudinal* to the magnetic field. Moreover, under the force of the external magnetic field, the spins wobble independently from each

other across the shape of a cone; a movement that is termed 'precession'.

When appropriate radio-frequency pulses are applied to the brain, the alignment of the protons changes again to an anti-parallel orientation and the precessing is synchronized. This introduces a new *transversal magnetization* and decreases the longitudinal magnetization. The change from one net magnetization state to the other evokes an electric current in the receiver coil. When the radio-frequency pulses are switched off, the protons align parallel to the external magnetic field again and proton precession returns to its desynchronized state. As a consequence, the transversal net magnetization disappears and the longitudinal magnetization becomes stronger. The time it takes for the protons to return to their parallel alignment is termed 'T1 relaxation time'. The time it takes for proton precession to returns in their desynchronized state and thereby lowering the transversal magnetization is termed 'T2 relaxation time'. The third time constant that is acquired is the 'T2* relaxation time', that describes the time it takes until a further decay of transversal magnetization, as a result of external field inhomogeneities, and spin-spin relaxation is accomplished (Chavhan, Babyn, Thomas, Shroff, & Haacke, 2009). Since different tissue types have different magnetic characteristics, they also possess different T1 and T2 relaxation times. The assessment of T1 and T2 relaxation times via the receiver coil thus allows describing certain tissue types in terms of signal intensities. Functional MRI preferably uses T2*-relaxation times, because they capture field inhomogeneities due to metabolic changes better than T2 relaxation times (Ogawa, Lee, Kay, & Tank, 1990).

Spatial coding of MRI signals is accomplished by the use of spatial gradients. Brain slices are selected one after the other in descending, ascending or interleaved order. Slice selection is possible, because a gradient slope exists in the external magnetic field, making different slices resonant to HF pulses of different frequencies. Finer spatial coding within a given slice is enabled

by two further gradients within each slice. Fourier transforms are used to calculate the spatial origin of a MRI signal.

5.2. Preprocessing, analysis and evaluation of structural brain images

Voxel-based morphometry (VBM) is a whole-brain, semi-automatic and unbiased technique for characterizing local differences in gray matter (GM). The VBM8 toolbox for the software package Statistical Parametric Mapping (SPM) version 8 provides preprocessing workflows for cross-sectional and longitudinal structural imaging data. In the current thesis, structural three-dimensional T1-weighted images with a resolution of 1 mm in all three dimensions were used.

5.2.1 Cross-sectional preprocessing

In short, cross-sectional VBM preprocessing involves segmentation, registration to 'Montreal Neurological Institute' (MNI) space and normalization of T1 images. In detail, raw T1 images are first segmented into GM, white matter and cerebrospinal fluid (CSF). Segmentation is conducted in order to improve normalization procedures and to restrict statistical analyses on GM. One major advantage of VBM8, compared with other software packages, is that segmentation does not rely on a priori information of tissue probabilities (Rajapakse, Giedd, & Rapoport, 1997). Segmentation results in an image for each tissue type, containing estimates for the proportion of the pure respective tissue type in every voxel (e.g. the amount of GM in a hippocampal voxel; Tohka, Zijdenbos, & Evans, 2004). After segmentation, denoising algorithms are applied that improve the signal-to-noise ratio, remove isolated voxels of a specific tissue type and close holes in a cluster of connected voxels of one tissue type (Coupé, Hellier, Prima, Kervrann, & Barillot, 2008; Rajapakse et al., 1997). In order to calculate group comparisons and to enable the use of anatomical atlases,

individual structural brain images are normalized to MNI space. In VBM8, an existing 'Diffeomorphic Anatomical Registration Through Exponentiated Lie Algebra' (DARTEL) template in MNI space (http://www.brain-development.org) integrated in the VBM8 toolbox is used as a reference for this purpose.

In the course of spatial normalization, brain regions are contracted and expanded to fit into reference space using affine transformations. If volume rather than concentration changes are of interest, it is recommended to modulate normalized images because normalization changes the total amount of GM in structural images. For this purpose, VBM8 provides the 'non-linear' or 'non-linear only' modulation algorithms. Both modulation types scale the normalized images by the amount of contraction/expansion applied during normalization, so that the total amount of GM is the same as in the raw T1 images. The two modulation types differ with respect to the need for using the total intracranial volume as a covariate in subsequent statistical analyses to correct for different brain sizes. When applying 'affine and non-linear' modulation, the choice for or against correction for brain size depends on the hypothesis to be tested (i.e. relative vs. absolute volume). The examination of relative volume requires the correction for different brain sizes, whereas the analysis of absolute volumes does not. When the 'non-linear only' modulation is used, it is not necessary to correct for individual brain sizes. This is because the algorithm used in 'non-linear only' modulation produces images that are equivalent to 'affine and non-linear' modulated images corrected for different brain sizes. In other words, while the correction for different brain sizes is conducted through including the total intracranial volume to the statistical model in the case of 'affine and non-linear' modulation, algorithms correcting for different brain sizes are applied directly to the images in the course of 'non-linear only' modulation. While differences on the basis of unmodulated images are understood as density differences, differences on the basis of modulated images are interpreted as volume differences (Ashburner & Friston, 2000; Good et al., 2001).

Finally, GM segments are spatially smoothed, i.e. the value at each voxel is replaced by a weighted average of itself and neighboring voxels (Brett, Penny, & Kiebel, 2003). Reasons for spatial smoothing are the improvement of the signal-to-noise ratio, the requirement of the GLM of spatially normally distributed error terms and the assumption that nearby voxels possess similar biological characteristics. To calculate group statistics, preprocessed T1 images are fed into second-level models.

5.2.2 Longitudinal preprocessing

The longitudinal preprocessing workflow of VBM8 is similar to the cross-sectional one. The biggest difference is the use of equal normalization parameters for the two time points. In detail, images from the second measurement point are aligned to the first measurement point. Then, individual mean images are calculated from the two measurement points. Normalization parameters are estimated on these mean images, so that the same normalization parameters are applied to images of the two measurement points. The longitudinal processing stream does not involve modulation, because normalization parameters are the same for both time points so that modulation would have little effect on the results. However, in mixed designs including within- as well as between-group factors, the total intracranial volume should be used as a covariate to correct for different brain sizes between groups.

5.2.3 Validity of VBM findings

VBM reliably differentiates between young subjects, healthy elderly subjects and patients suffering from Alzheimer's disease, of which the latter two groups both show histologically confirmed alterations in neuronal structure (Creasey & Rapoport, 1985; Good et al., 2001; for a review, see Matsuda, 2012; Smith, 2002). Moreover, VBM is as sensitive as manual segmentation to detect hippocampal volume differences between depressive and healthy subjects (Bergouignan et

al., 2009). Additionally, VBM-detected gray matter increases in response to physical or cognitive training coincidence with elevations in synapto- or spinogenesis (Black, Isaacs, Anderson, Alcantara, & Greenough, 1990; Draganski et al., 2006; Driemeyer, Boyke, Gaser, Büchel, & May, 2008; Leuner et al., 2003; Moser et al., 1994; Trachtenberg et al., 2002).

Above coincidences in cellular and VBM findings, the underlying cellular substrates of gray matter differences detected via VBM are still not clarified. Candidate mechanisms include the structural changes of spines and dendrites, neurogenesis, gliogenesis, synaptogenesis and vascular changes (Fields, 2011; Zatorre, Fields, & Johansen-Berg, 2012). Several researchers believe that neurogenesis can be rather excluded as an underlying mechanism, because the total increase in the number of new neurons is relatively small (Couillard-Despres & Aigner, 2011; Czéh & Lucassen, 2007). Very few studies attempted to draw a direct link between gray matter differences detected via MRI and the underlying cellular events. Literature currently suggests that different cellular substrates underlie macroscopic gray matter changes depending on the experimental manipulation. For instance, training-induced gray-matter changes detected via MRI correlated with a marker of neuronal process remodeling, but neither with neurogenesis nor the number of neurons or astrocytes (Lerch et al., 2011). However, in rats with hippocampal damage due to cardiac arrest, GM changes detected via VBM correlated positively with the number of neurons (Suzuki et al., 2013).

In sum, VBM appears to be a valid method to detect hippocampal gray-matter differences between different time points or study groups. Nonetheless, results have to be interpreted considering knowledge from animal studies about the cellular effects of E2 and P4.

5.3. The blood oxygen level dependent response

The previous chapters outlined the use of MRI to quantify differences in hippocampal structure. However, MRI not only allows assessing different neuronal tissue types, but can be also used as a marker of neural activity in the brain.

Neural activity is associated with increased oxygen consumption, which is satisfied by an increased local blood flow, the *hemodynamic response.* This local blood flow is so high, that the oxygen level in a given brain area even exceeds the actual needs. Therefore, the local relative amount of oxygenated haemoglobin increases. Because oxygenated haemoglobin differs from deoxygenated haemoglobin with respect to magnetic characteristics, MR signal intensities are different for brain areas with high compared to low neural activity (Frith & Friston, 1997). In detail, the reduction in deoxygenated haemoglobin results in an increased field homogeneity, which in turn leads to longer T2* times. These activity-related hemodynamic changes are called BOLD-responses. Notably, BOLD signals not only reflect changes in blood flow but are also affected by blood volume and oxygenation (for a review, see Heeger & Ress, 2002).

BOLD responses typically drop down just after stimulus onset (initial dip) and go up 2 seconds after stimulus onset. The maximum is reached at 4 to 8 seconds after stimulus onset, followed by a small undershooting. Finally, baseline levels are reached at 20 to 30 seconds after stimulus onset (for a review, see Heeger & Ress, 2002; Logothetis et al., 2001). The typical BOLD-response is mathematically described by the *hemodynamic response function* (HRF), which can be further partitioned in three gamma functions. Derivatives of the HRF can be used to model onset (first derivative) and width of the HRF (second derivative).

Studies on primary sensory cortices of monkeys and humans agree that fMRI signals reflect local field potentials rather than neuronal spiking (i.e. action potentials; Logothetis et al., 2001; for a

review, see Ojemann, Ojemann, & Ramsey, 2013). Local field potentials arise from extracellular voltage within a certain radius, dominantly produced from activity at dendritic synapses on pyramidal cells (Lindén et al., 2011).

5.4. Preprocessing, analysis and evaluation of functional brain images

As mentioned above, functional imaging allows assessing BOLD responses as a measure of neuronal activity in vivo. Because event-related neuronal responses have to be separated from general neuronal activity, reliable event-related BOLD responses require repeated recording of neural activity associated to a given event. Therefore, different stimuli of a certain type (i.e. words, images of one valence) are repeatedly presented. In the current thesis, functional brain images are recorded during the encoding of words and images. This chapter describes a number of preprocessing steps that were applied to the raw T2*-weighted brain images in the respective studies. Preprocessing of functional MR images is conducted in order to improve data quality and to bring brain images into a common space. Usually, these prepropressing steps are referred to as *slice-timing* (temporal correction), *realignment with or without unwarping* (movement correction), *normalization* (adjustments to a standard brain template) and smoothing.

In the present thesis, slice-timing is performed as the first step in functional image preprocessing. Slice-timing corrects for the fact that slices are sequentially acquired, so that brain activity in a specific brain area not only varies as a function of the stimulus, but also of slice position. Therefore, slice-timing temporally shifts signal intensities of each slice to a specified reference slice using a Fourier transform. In order to avoid high interpolation errors, the reference slice is set to the middle slice in the current thesis. Next, realignment corrects for head movements by applying rigid body transformations, so that all images are translated and rotated to a common position and orientation.

However, certain types of head movements can interact with the magnetic field ('susceptibility-by-movement interaction') and lead to image distortions that are not corrected by rigid body transformations. For this purpose, unwarping algorithms are applied that correct for these image deformations on the basis of observed variance and estimated head movements. In the two following steps, spatial information gained from individual structural T1 images is used to further improve the normalization procedure. At first, structural images are coregistered to mean functional images. Coregistered structural images are then segmented into different tissue classes. Next, tissue-class images for gray and white matter are used within the DARTEL toolbox, to create a structural group template and individual flowfields. The group template and flowfields are utilized for normalization of functional images to MNI space. For visualization purpose, these flowfields can be additionally used to normalize structural images, which are then averaged to create a group overlay. Finally, all functional images are spatially smoothed.

5.5. Statistical analyses of functional imaging data

As stated above, functional brain images are recorded during encoding but not retrieval of stimuli. For analysis, brain images are *post hoc* categorized, depending on whether the prevailing stimulus has been subsequently remembered or not. BOLD responses during encoding of subsequently remembered stimuli are then contrasted against BOLD responses during encoding of subsequently forgotten stimuli, resulting in a 'Difference-due-to memory' (DM) index of memory-related BOLD responses. The DM index can then be used to compare memory-related BOLD responses across groups or time points. DM paradigms have the advantage that BOLD-responses are not confounded by performance-related influences, as it would be the case when fMRI would be acquired during retrieval (Staresina & Davachi, 2006).

In the present thesis, statistical analyses of neuroimaging data were based on the application of the general linear model (GLM) to each voxel, so that a separate GLM is specified and estimated for every single voxel. The GLM assumes, that the observed data Y equal the linear combination of (i) the constant β_0 plus (ii) the explanatory variables X, weighted by certain parameters β, plus (iii) an error term ε; i.e. $Y = \beta_0 + \beta*X + \varepsilon$. Thus, Y contains intensity values for every single voxel across all scans. The β parameters weight a matrix containing the predictory variables X to optimally predict Y. The variable X is also known as design matrix, that usually dummy codes stimulus conditions. The number of betas usually equals the number of conditions. The constant β_0 is the arithmetic mean of all signal intensities within the respective voxel across all scans recorded in a session.

In practice, the GLM is first applied to the single-subject data; also known as *first-level analysis*. For this purpose, a design matrix is created that specifies whether a particular scan belongs to a specific stimulus type or not. Each regressor (i.e. column of the design matrix) thus specifies at which time point a given stimulus has been presented. The estimation of beta parameters is a three-step procedure. First, the regressors are convolved with the HRF, transforming the dummy coded onset regressors into parameters that model the expected signal. Regression analysis is used to estimate β parameters for the predictory variables X. This first 'data GLM' explains variance in the observed values Y to the greatest amount possible, with the result that the residual term is minimized. Second, the residual term of the 'data GLM' is used to estimate a GLM for residuals related to temporal auto-correlations (auto-regressive model). This auto-regressive model plus a high pass filter are applied to the data to fulfill the sphere city assumption. Third, in a second 'data GLM' the beta parameters are estimated again using the pre-whitened and filtered data. Model estimation results in as many beta-images as regressors, each containing information about how strongly a particular condition affected BOLD responses within each voxel. Resulting beta images

can then be taken to the group level (*second-level analysis*). Alternatively, beta images can be contrasted against each other on the first level, in order to use single-subject contrast images for statistical analyses on the group level. Contrasts are row vectors that specify a weight for every beta image. For instance, when the design matrix contains one vector for subsequently remembered words and one vector for forgotten words, a contrast of [1 -1] would result in a contrast image that is brighter at voxels which show higher signal intensities for remembered compared to forgotten stimuli. The latter approach has the advantage that the design matrix on the group level is less complicated and provides the possibility to compute correlations of a particular contrast with all kind of variables (e.g. a questionnaire score). Nevertheless, the resulting group level model is rather inflexible, as prevailing contrasts have to be defined on the single-subject level in advance.

Estimation of the group level model is performed in a three-step procedure, similar to the first level analysis. Here, individual beta or contrast images are used to set up a model that explains variance across and within subjects with pre-specified regressors. On the group level, the auto-regressive model corrects for dependencies or unequal variances between conditions, subjects or groups. Beta images on the second level contain information on how strongly the particular condition affects activity within each voxel *across all subjects*. Statistical hypothesis are tested by applying contrasts to the second-level model. Results can be visualized by overlaying color-scaled *t* or *F*-values onto a T1 brain template or a structural mean T1 image.

5.6. The multiple testing problem in neuroimaging

Whenever more than one statistical test is calculated, researchers are faced with a potential inflation of alpha errors, i.e. the increased probability of false positive results. The more tests are calculated, the worse the problem usually becomes. Since *t*-tests are calculated for every single

voxel in voxel-wise MRI analysis, the problem of multiple testing and the resulting inflation of alpha errors are quite severe. However, conventional correction methods such as Bonferroni-Correction, based on the sum of every single *t*-test/voxel, are too conservative for MRI analysis. This is because the number of *independent* data points is far less than the number of voxels due to preprocessing steps and biological factors. Therefore, SPM provides two different approaches to correct for multiple testing in MRI data analysis. For one, the 'Family Wise Error Rate' (FWE) employs the 'Gaussian Random Fields' (GRF) theory to correct for multiple testing under consideration of local smoothness estimation (i.e. spatial correlation). In contrast to the classical Bonferroni-Correction, this method does not assess the likelihood of false positives relative to the number of every single *t*-Test or voxel. Instead, the *GRF* estimates the number of *independent t*-tests based on relative local smoothness levels (Brett et al., 2003). Application of the GRF requires that the data meet several assumptions, for instance that the smoothness kernel is at least 2 to 3 times the voxel size so that the data 'approximate the behavior of a random continuous field' (p. 430, Nichols & Hayasaka, 2003). An alternative approach to correct for multiple testing is the 'False Discovery Rate' (FDR). In contrast to FWE-corrections, FDR is not based on the number of all calculated *t*-tests, but on all discoveries that were made. FDR corrections thus adapt to signal intensities in the sense, that small signals make the correction more liberal and higher signals make the correction more conservative. In the current thesis, the more commonly used FWE approach is applied to whole brain analyses and analyses within a predefined region of interest ('Small Volume Correction', SVC). SVC's correct significance only for the number of *t*-tests that were carried out within a restricted set of voxels. It therefore requires conclusively justified assumptions in which regions certain processes will occur.

6 Experiments

6.1. Experiment 1: Aromatase inhibition in postmenopausal breast cancer patients

6.1.1 Theoretical Background

In female OVX animals, the drop in E2 synthesis due to inhibition of aromatase leads to a dramatic decline in spine- and synapse density at hippocampal neurons and inhibits hippocampal LTP (Frotscher et al., 2004; Vierk et al., 2012). Moreover, animal studies clearly show a positive association between E2 and hippocampus-dependent spatial memory (Davis et al., 2005; Gibbs et al., 2004; Korol et al., 2004). It thus stands to reason, that AI therapy in postmenopausal women might lead to impairments in hippocampus-dependent memory functions. Evidence for memory impairments as a consequence of AI therapy was delivered by studies with small sample sizes, but a sub-study of the clinical IBIS-II trial with a larger sample failed to replicate these findings (Collins et al., 2009; Jenkins et al., 2008; Nattinger et al., 2013). The latter study is not only characterized by a relatively large sample size but also a randomized placebo-controlled design, so that results are less likely biased by unwanted influences compared with the other two studies (for details on the study design, see chapter 3.6.3). Cognitive performance was monitored by means of a battery of neuropsychological tests in the IBIS-II sub-study, among which was the Californian Verbal Learning Test that detects memory deficits in patients with frontal lesions or after anterior medial temporal lobe resection (Alexander, Stuss, & Fansabedian, 2003; Davies, Bell, Bush, & Wyler, 1998). Nevertheless, strong evidence from animal research as well as concerns about the sensitivity of neuropsychological test-batteries to detect subtle changes in memory functions (Berga, 2008), suggest that further research is necessary to examine the influence of AI therapy on the human hippocampus.

Next to differences in study designs, the variance between study results could be related to factors that mask overt memory performance and are influenced by sample characteristics or testing conditions. It is known that organisms are able to compensate for shortcomings in neuronal processes under particular circumstances. Compensatory processes have been reported on the cellular, systems and behavioral level. Compensation on the cellular level has been observed in response to age-related hippocampal synapse loss, after which remaining synapses showed increased electrical responsiveness to synaptic activation (Barnes & McNaughton, 1980). On the systems level, recruitment of prefrontal areas has been shown to compensate for inhibition of the hippocampus in animals (Goshen et al., 2011). In humans, changes in the pattern of neuronal activation compensated for age-related decline in memory performance (Cabeza, Anderson, Locantore, & McIntosh, 2002). Behaviorally, compensation can emerge from adaptive strategy switches, such as the switch from a place-strategy when hippocampal efficacy is boosted by high E2 levels, to a response-strategy when E2 is low (McElroy & Korol, 2005). With respect to humans, elderly subjects use more semantic clustering than young subjects in free recall memory tests, a finding that has been interpreted as a compensatory strategy to overcome increases in task difficulty (Golomb, Peelle, Addis, Kahana, & Wingfield, 2008). Whereas compensatory mechanisms on the cellular level should not differ between study samples, behavioral compensatory mechanisms can be highly influenced by factors such as the motivation of study participants (Carretti, Borella, Zavagnin, & De Beni, 2011; Park & Reuter-Lorenz, 2009; Verhaeghen, Martin, & Sedek, 2012). A possible explanation for controversies regarding the influence of AI therapy on memory could be that the participants' motivation in smaller studies is higher than in studies with larger samples due to the closer contact to a single experimenter.

The current study therefore employed sensitive behavioral memory tasks as well as neuroimaging in order to uncover changes in memory processes that can not be detected by means of standard

neuropsychological memory tests. For this purpose, postmenopausal breast cancer patients and age matched healthy controls, were both tested at baseline and 3 to 6 months after onset of AI therapy or a waiting period, respectively. Potential shifts from recollection-based recognition memory relying on hippocampal recruitment, to the rather hippocampus-independent familiarity-based recognition memory were thus made visible. In detail, an inclusion-exclusion task was used, in which recollection-based memory is characterized by successful recognition of a word together with its learning context (i.e. a specific encoding list). Second, a visual memory task with confidence ratings was used that allows estimation of familiarity and recollection on the basis of ROC curves. Functional imaging was used to detect potential compensatory mechanisms or other changes on the neuronal level. More precisely, neuronal activity was monitored during the encoding of words and colors, for which memory was assessed by means of a free recall and a recognition memory test. Potential structural hippocampal changes were investigated through structural MRI.

6.1.2 Material and methods

Subjects

Twenty-two postmenopausal patients aged 62 to 75 years (M = 68.09, SD = 4.59) suffering from hormonal-sensitive breast cancer with an indication for neoadjuvant aromatase inhibitor therapy (AI group) and 26 postmenopausal controls aged 56 to 79 years (M = 68.19, SD = 7.33), who did not receive aromatase inhibitors (C group), completed the current study.

With the exception of three subjects, all subjects of the AI group received a standard daily dose of 2.5 mg Letrozole during the whole study period (i.e. after baseline testing). One patient started to take Letrozole and switched to Anastrozole (1 mg daily) after 6 weeks because of medical side effects. Two patients received Anastrozole during the whole study period. One participant from the

control group had a ductal carcinoma in situ, and underwent surgery as well as subsequent radiotherapy. Another participant from the C group received a mastectomy as a treatment for invasive breast cancer. Both participants were grouped as controls, because they did not receive any further pharmacological treatments.

Two controls were under HRT during the study period. Ten patients and nine controls had a history of HRT, but were free of HRT at the time of baseline testing. In total, 18 patients and 20 controls underwent MRI; of these 13 patients and 17 controls accomplished functional fMRI. Table 1 summarizes final sample sizes for all measures.

Informed consent was obtained in a manner approved by the institutional review board at University Medical-Center of Hamburg Eppendorf, and subjects were paid financial compensation for their participation.

Table 1. Number of subjects included in final analyses (Experiment 1).

Task/Measure	AI Group	C Group	Sum
Total	20	25	45
Test of Attentional Performance	20	25	45
Stroop Task	17	24	41
Inclusion/Exclusion Task	20	24	44
Word-Color Association Task	17	23	40
Visual Memory Task	20	25	45
Voxel-based Morphometry	15	16	31
fMRI	13	14	27

Note. AI Group = patient group receiving aromatase inhibitor therapy. C Group = control group. Total = number of subjects who completed both measurement points. fMRI = (functional) magnetoresonance imaging.

Procedure

For the AI group, potential study participants were acquired during the breast consultation-hour of the University Medical-Center of Hamburg Eppendorf and the Breast Center Hamburg International

at the Jerusalem Hospital. For the C group, potential study participants were acquired during breast consultation-hour and via advertisement. An initial telephone conversation ensured that subjects had no history of neurological diseases and were willing to participate. Three controls had severe problems to understand instructions of the memory tasks during baseline measurement ($T_{baseline}$), so that they were not invited to the second measurement (T_{post}). One control did not complete the second measurement because she was afraid to become infected with Enterohemorrhagic Escherichia Coli that was prevalent in Germany at this time.

All participants completed two measurement points spread across four days (Figure 1). $T_{baseline}$ was scheduled after breast surgery and before onset of AI therapy in the AI group. T_{post} was scheduled so that subjects from the AI group had received at least three months of AI therapy (M = 127.77, SD = 26.14). On the first testing day ($T_{baseline\_1}$), all participants accomplished a brief standard dementia screening using a German version of the DemTect, to ensure that no subject showed signs of dementia or mild cognitive impairment (Kalbe et al., 2004). Afterwards, subjects performed the inclusion-exclusion task, two sub-tests of the 'Test of Attentional Performance' battery (TAP; Zimmermann & Fimm, 2002) and a visual memory task. Demographic variables were assessed via questionnaires filled out at home. On the second testing day ($T_{baseline\_2}$), subjects performed the word-color association task while lying in the scanner and underwent a structural MRI scan. Participants who did not undergo fMRI performed the word-color association task outside the scanner. Aside from the dementia screening, all tests were repeated during the second measurement (T_{post_1} and T_{post_2}).

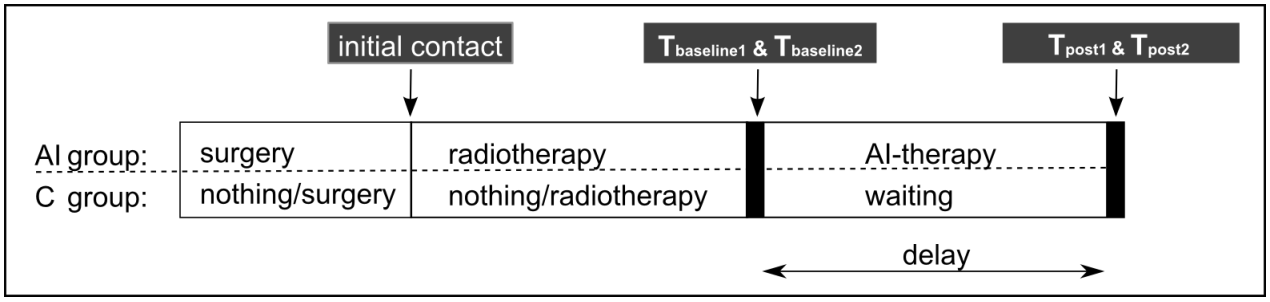

Figure 1. Design of the present study (Experiment 1). Dark gray boxes illustrate timing of the initial contact and the measurement points. White boxes depict the time line of medical treatments within the patient group (AI group) and the control group (C group). All participants visited the institute on two occasions before onset of the AI therapy or a waiting period ($T_{baseline1}$ and$T_{baseline2}$) and on two occasions afterwards (T_{post1} and T_{post2}).

Design and analysis of behavioral paradigms

Results of all behavioral analysis were considered significant at a threshold of $p < .05$. No outliers were removed because of the small sample sizes, except for one subject for which false alarms exceeded hit rates in the word-color association task (see below). However, to prevent false positive findings, significant group by time interactions were confirmed by non-parametric analyses.

Inclusion-exclusion task

The verbal source memory task was adapted from Prull and colleagues (2006). During this task, participants have to encode two word lists (Figure 2). After encoding, they are informed which list is defined as the *to-be-included list* and which list is defined as the *to-be-excluded list.* During retrieval, subjects should respond 'no' to words that were either on the to-be-excluded list or new, and 'yes' to words which were on the to-be-included list.

For the present study, nouns with word frequencies of 5 to 11 per million and word lengths of 4 to 10 characters were selected from the German CELEX database (Baayen, Piepenbrock, & Van Rijn, 1993). Nouns were excluded when they were emotionally charged, difficult to pronounce,

orthographically similar or potentially unknown to people from lower educational levels. A resulting stimulus set of 408 words was randomly assigned to one of 12 word lists, each containing 30 words. Testing on each occasion (i.e. $T_{baseline\_1}$ and T_{post_1}) consisted of two study-test blocks (Figure 2). Each block began with the presentation of the words from list 1, with each word presented separately in the middle of a computer screen. Participants were required to read each word aloud, and memorize every word together with its list number. During encoding, subjects were unaware which list would serve as the to-be-included list later on. After presentation of each word list, subjects performed a distractor task ('arrow task') for about 3 minutes to clear working memory content. In this task, participants had to indicate the pointing direction of an arrow via button press. Pointing direction switched every second between the left and right side in pseudo-randomized order. Please see Figure 2 for details on the timing of the encoding phase.

Before retrieval phase, subjects were informed which list has been chosen as the to-be-included list in this block (e.g. list 1). During retrieval, the previously studied items (i.e. list 1 and list 2) intermixed with words from a new list were presented in pseudo-randomized order. For each word, subjects were asked whether the word was on the respective to-be-included list (e.g. 'Was this word presented on list 1?'). Participants answered 'yes' by moving a cursor via button-presses, when they thought that the word belonged to the respective to-be-included list. They answered 'no', when they thought that the item has been presented previously but was on the to-be-excluded list or that the word was new. Timing of the retrieval phase was self-spaced. The sequence of the to-be-included list assignment to either list 1 or list 2 was pseudo-randomized, but all subjects were tested on both lists. Two practice blocks prior to the whole experiment ensured that participants understood the task.

In this task, the value for *recollection* represents the successful retrieval of a word together with its

learning context (i.e. list 1 or list 2). *Familiarity* represents the recognition of the word without their learning context. At first, the proportions of 'yes' answers to to-be-included items (correct), to to-be-excluded (incorrect) and to new items (incorrect) were calculated. Recollection values were calculated by subtracting the proportion of 'yes' answers to to-be-excluded-items from the proportion of 'yes' answers to to-be-included-items, i.e. R = p(yes|oldincl) - p(yes|oldexc). Familiarity values were calculated by applying the formula F = p(yes|oldincl)/ (1 - R), which is a rearrangement of the formula that describes the contributions of recollection and familiarity to the total probability of 'yes' answers to to-be-included items, i.e. p(yes|oldincl) = R+ F(R-1). A more detailed description of the theoretical basis for the calculation of the process parameters is given in the article of Prull and colleagues (2006). Although potentially confounded by motor abilities, median reaction times (RT) of 'yes' answers to to-be-included items were used as a further indicator of memory strength.

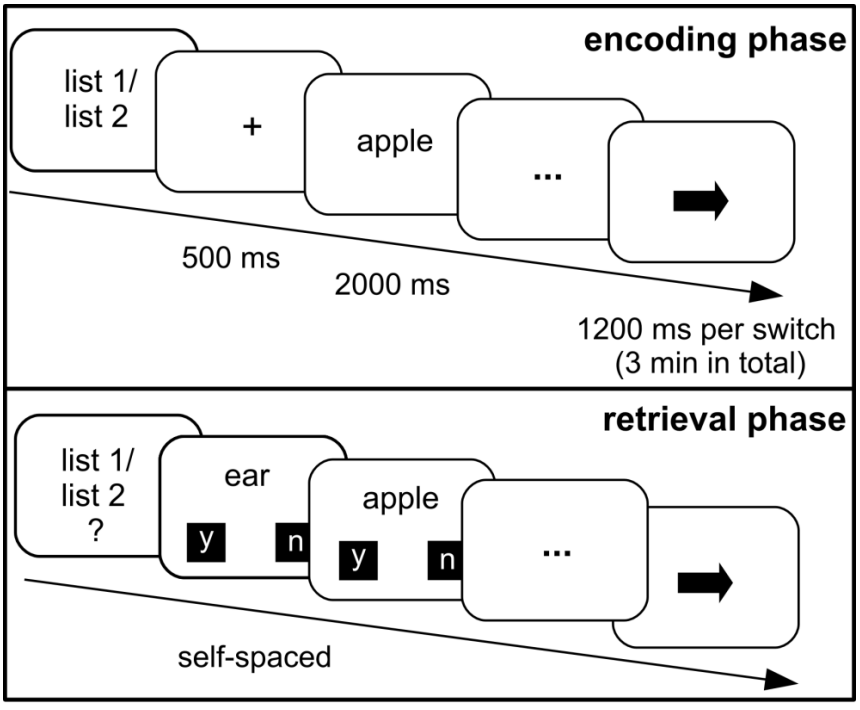

Figure 2. Design and timing of the inclusion-exclusion task (Experiment 1).

Visual memory task

The visual memory task was adapted from Howard and colleagues (2006). In this task, participants encode a number of pictures. During retrieval, target pictures are intermixed with new pictures and participants have to a make old/new decision for every picture.

In the present study, a total of 1024 pictures depicting scenes from various travel destinations were chosen randomly from a picture set provided by Howard and colleagues (2006). Pictures were randomly assigned to 8 lists, each containing 128 pictures in pseudo-randomized order for each participant. Two lists were used as target lists for $T_{baseline}$, 2 lists were used as lure lists for $T_{baseline}$, the other 4 lists were used as target and lure lists for $T_{post.}$

Each measurement consisted of two study-test blocks. During encoding, each trial began with a fixation cross lasting about 500 ms. Next, a picture was presented in the middle of the screen for 1500 ms. Subjects were instructed to watch each picture attentively and memorize it. A total of 128 pictures were presented in pseudo-randomized order during encoding in each block. During retrieval, subjects saw the previously studied pictures intermixed with 128 lure pictures in pseudo-randomized order. For each item, participants were asked to judge whether they thought that the picture has been shown before and how confident they were about their decision by moving a cursor via button presses on a 6-point Likert scale, ranging from 'very sure old' to 'very sure new'. They were encouraged to use the full range of the scale. Timing of the test phase was self-spaced. A practice block ensured that participants understood the task.

Because the range of confidence judgments was not sufficient to estimate process parameters on the basis of receiver-operator characteristics, hit rates of high confidence answers minus false alarm rates of high confidence answers (very sure old/new) were used as an indicator of recollection. Hit rates of low confidence answers minus false alarm rates of low confidence answers (sure old/new,

maybe old/new) were used as indicators of familiarity. For this purpose, the hit rates on each confidence level (i.e $HR_{very\ sure}$, HR_{sure}, HR_{maybe}) were calculated by dividing the number of correct answers on the respective confidence level by the total number of choices for the respective confidence level. The proportions of false alarms (i.e $FA_{very\ sure}$, FA_{sure}, FA_{maybe}) were calculated similarly. Corrected hit rates were then calculated by subtracting the false alarm rates from the hit rates, e.g. $HRcor_{very\ sure} = HR_{very\ sure} - FA_{very\ sure}$. To simplify analyses, corrected hit rates for the two lower confidence levels (sure, maybe) were averaged and will be referred to as corrected hit rates for low confidence responses. Median RT's of correct high and low confidence answers were used as a second indicator of memory strength.

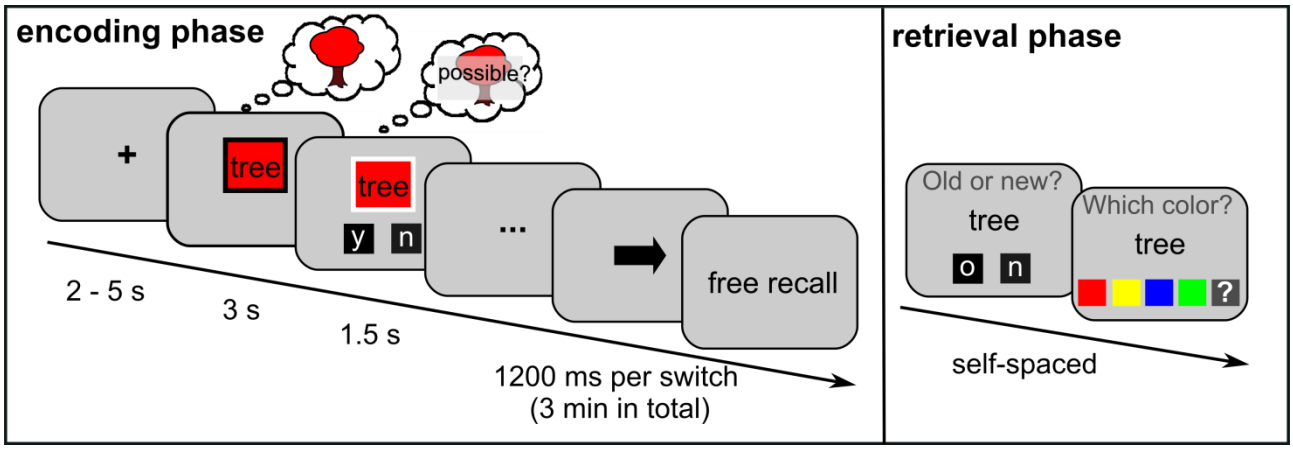

Figure 3. Design of the word-color association task (Experiment 1).

Word-color association task (fMRI paradigm)

The fMRI paradigm was adapted from Staresina and Davachi (2006). In this task, subjects encode words presented in front of a colored square. After encoding, subjects are asked to freely recall as many words as possible. In an additional recognition test, previously presented words are intermixed with new words and subjects have to make old/new decisions for every word. For words that are classified as new, participants are asked to indicate with which color the respective word

has been presented.

In the present study, the entire task consisted of three runs, each containing an encoding, distractor task, free-recall and retrieval phase. All parts of this task were carried out while partipants lay in the scanner, but fMRI was recorded only during the encoding phase. Because pilot tests revealed that the original task is too long and demanding for elderly subjects, two modifications were made to the original paradigm. First, timing of trial presentation was shortened to minimize the time subjects had to lie in the scanner. Second, while in the original paradigm the arrow task was performed after each trial as an active baseline task, this task served only as a distractor task after the encoding phase to clear working memory content in the present study.

Material for this task consisted of a total of 459 nouns, with word lengths ranging from 4 to 11 characters and a frequency of 1 to 421 per million were selected from the German CELEX database (Baayen et al., 1993). Care was taken that words used for the current task did not occur in any other task of the present study. Imageability and concreteness ratings were available for 206 nouns and ranged from 1 to 18.4 for imageability (M = 14.31; SD = 3) and -0.2 to 19.4 for concreteness ratings (M = 14.06; SD = 3.66). Words were randomly distributed on 3 lists of 92 targets and 3 lists of 61 lures for each subject and measurement in pseudo-randomized order. Word lists were matched for word imageability and frequency. Additionally, one of four colors (i.e. red, green, blue or yellow) was assigned in pseudo-randomized order to each target word, with the restriction that the same color did not occur more than two times in a row.

Every trial began with a fixation cross with a jittered duration of about 2 to 5 seconds (Figure 3). Next, each target word was presented separately for 3 seconds in front of a colored square surrounded by a black frame. Subjects were instructed to create a vivid mental image of the respective object in the given color (e.g. a red tree). Next, the frame of the color square turned from

black to white, indicating that the subject should now judge via button press whether this word-color combination was 'plausible' or 'implausible' (e.g. whether a red tree could exist in real life or not) within the remaining 1.5 seconds. Total trial length ranged from 6.5 to 9.5 seconds. After encoding, subjects performed the arrow task for about 3 minutes to clear working memory content. Subjects were then asked to freely recall as many previously learned words as possible. After free-recall, the 92 targets words together with the 61 new words were presented separately in the center of the screen. For each given word, subjects had to indicate via button press whether they thought that the item has been presented in the previous encoding block ('old') or not ('new'). For words judged as 'old', subjects were additionally asked to indicate the color on which the word was presented, by choosing between four possible colors through moving a cursor via button presses. To prevent guessing, subjects were asked to choose a fifth answer option (i.e. a question mark), in case they were not able to remember the color of the respective item. Timing of the retrieval phase was self-spaced. A practice block outside the scanner ensured that participants understood the task.

The paradigm allowed categorization of target words into four categories, based on memory performance in the free recall and recognition phase: *(1) items that were freely recalled (FC), (2) items that were remembered together with the correct color (IC), (3) items that were remembered without the correct color (II) and (4) items that were forgotten (MS)*. Items that were both freely recalled and remembered together with their correct color were categorized as FC items. Median RT's for old-new hits (collapsed across II and IC items) were used as a further indicator of memory strength. Reaction times (RT's) of color decisions were not used because the number of required button presses depended on the chosen color. Repeated-measures analyses of variance (ANOVA's) were conducted on FC and IC rates, d-prime of II items and RT's. In order to reduce the number of

statistical tests and because of the lack of a clear hypothesis, plausibility ratings were not analyzed statistically. One control[3] was excluded from further analyses because the false-alarm rate exceeded the hit rate (see Table 1 for remaining sample sizes).

Neuropsychological tests of executive functioning

To assess potential changes in attention, subjects performed the two sub-tests 'alertness' and 'divided attention I (auditive/visual)' from the TAP battery (Zimmermann & Fimm, 2002). The alertness sub-test comprised four runs, in which subjects were instructed to press a button as soon as a cross appeared on the computer screen. In runs 2 and 3, a tone preceded the cross with a variable delay between tone and cross ('tone condition'). No tone was presented in runs 1 and 4 ('no tone condition'). Data from the two runs of the same condition were averaged. Mean RT's of correct responses within the no tone condition were used to assess general processing speed. The difference in RT between the tone and no tone condition was used as a measure of phasic alertness, with faster responses in the tone condition signaling greater phasic alertness. These two variables will be referred to as *general processing speed score* and *phasic alertness score.* In the divided attention sub-test, subjects had to simultaneously monitor the occurrence of a visual pattern (i.e. four small crosses) and an auditive pattern (i.e. two identical tones in a row). They were instructed to press a button as soon as they detected one of the two patterns. The sum of omissions and false responses, collapsed across the auditive and visual conditions, was used as a measure of how well subjects can divide or switch attention. This variable will be referred to as *attentional switch score.*

To test the ability of attentional allocation, the color-word interference sub-test from the

3 This subject did not undergo fMRI scanning.

Nürnberger Altersinventar was used (Fleischmann & Oswald, 1999). This task is a modified version of the Stroop test (Stroop, 1935), in which subjects have to name the color of congruent (e.g. the word red printed in red) and incongruent color-words (e.g. the word red printed in yellow). The dependent variable derived from this task is the *interference score,* i.e. the difference in reading speed between the congruent and incongruent condition. Data from four subjects (three patients, one control) were missing for the Stroop task.

Acquisition and preprocessing of structural MRI

High-resolution T1-weighted structural MR image were acquired by using a standard 3D-MPRAGE sequence (TR 2300 ms, TE 2.89 ms, flip angle 9°, 1 mm slices, field of view 256 x 192; 240 slices). T1 images were preprocessed and analyzed using the VBM8 toolbox for SPM8 (Wellcome Department of Imaging Neuroscience, London, UK) running under Matlab R2009a (Mathworks, Inc, Natick, MA, USA). The default longitudinal preprocessing approach integrated into the VBM8 toolbox was used (see 5.2.2). Preprocessed GM segments were smoothed with a full-width at half maximum (FWHM) Gaussian kernel of 8 mm for all directions. A sample homogeneity check provided by the VBM8 toolbox classified three patients and two controls as outliers in brain structure (see Table 1 for remaining sample size).

Individual GM segments were included in a design matrix with the factors group (AI vs. controls) and time point ($T_{baseline}$ vs. T_{post}). Total intracranial volume was added as a covariate to correct for different brain sizes between groups. To examine whether AI therapy reduced hippocampal volumes, contrast weights of [1 -1] were assigned to the two time points of the AI group and contrast weights of [-1 1] were assigned to the two time points of the control group, resulting in a contrast vector of [1 -1 -1 1]. Results of all analyses were considered significant at p = .05, corrected for multiple comparisons at the entire scan volume and within an anatomical mask of the

hippocampus (Amunts et al., 2005).

Acquisition and preprocessing of functional MRI

Event-related functional MRI was performed on a 3 T system (Siemens Trio) with an echo planar imaging T2*-sensitive sequence in 40 contiguous axial slices (2 mm thickness with 1 mm gap; TR, 2380 ms; TE, 25 ms; flip angle, 80°; field of view, 204 x 204; matrix 102 x 102).

Functional MRI data were preprocessed and analyzed using Statistical Parametric Mapping (SPM8; Wellcome Department of Imaging Neuroscience, London, UK) running under Matlab R2009a (Mathworks, Inc, Natick, MA, USA). To prevent biases due to spin saturation, the first five functional images were discarded. All functional images were slice time corrected, realigned and unwarped. Individual structural T1 images were coregistered to functional images and segmented using the 'New Segment' algorithm provided by SPM8. The DARTEL toolbox was used to normalize structural and functional images to MNI space. Finally, images were smoothed with a FWHM Gaussian kernel of 8 mm for all directions. Functional imaging data from two controls had to be excluded from further analysis due to excessive head movements (see Table 1 for remaining sample size).

Event-related BOLD responses were analyzed employing the GLM as implemented in SPM using a mass univariate approach. On the individual subject level, eight separate regressors for the factors memory (FC, IC, II, MS) and time point ($T_{baseline}$,T_{post}) plus a regressor containing all rating events during encoding were created for each measurement point by convolving the onset regressors with the canonical HRF.

On the second level, individual beta images for both groups and time points were included, resulting in a design matrix with 16 regressors. For the linear effect of memory-success type (i.e.

FC > IC > II > MS), a weight of +1.5 was assigned to FC trials, a weight of +0.05 was assigned to IC trials, a weight of -0.5 was assigned to II trials and a weight of -1.5 was assigned to MS trials. To test whether AI therapy lead to an increase in neural activity related to encoding success, weights of [+1.5, +0.5, -0.5, -1.5] were assigned to FC, IC, II and MS trials at $T_{baseline}$ in the AI group, weights of [-1.5, -0.5, +0.5, +1.5] were assigned to FC, IC, II and MS trials at T_{post} in the AI group and reversed weights were assigned to the control group, i.e. [-1.5, -0.5, +0.5, +1.5] for $T_{baseline}$ and [+1.5, +0.5, -0.5, -1.5] for T_{post}. The opposite contrast was applied to test whether AI therapy lead to a decrease in neural activity.

Results from all analyses were considered significant at $p < .05$, corrected for multiple comparisons at the entire scan volume and within the reduced search volumes of interest, i.e. anatomical masks of the hippocampus and prefrontal areas (Amunts et al., 2005; Tzourio-Mazoyer et al., 2002).

6.1.3 Results

Behavioral Results

Sample Characteristics

One control and two patients had DemTect scores below 12, raising the suspicion of a mild cognitive impairment in these participants. All three participants were excluded from further analysis. Two-sample *t*-test showed that groups did not differ significantly with respect to age, $t(43) = -0.92$, $p = .364$ or delay between measurement points, $t(43) = -0.40$, $p = .69$ ($M_{AI} = 161.15$ days, $SD_{AI} = 38.16$; $M_C = 161.15$ days, $SD_C = 38.16$). Furthermore, groups did not show significant

differences in age at first pregnancy, $t(34) = 1.18$, $p = .246$, age at menopause, $t(40^4) = -.41$, $p = .849$, years since menopause, $t(40) = -1.18$, $p = .663$, and age at menarche, $t(41^5) = 0.35$, $p = .727$. Moreover, groups did not differ with respect to HRT (current, past, never), $\chi(2, N = 45) = 2.19$, $p = .334$, educational level, $\chi(5, N = 45)=2.22$, $p = .818$, or the number of nulliparous women[6], $\chi(1, N = 45) = 0.19$, $p = .663$. See Table 2 for descriptive statistics of sample characteristics.

Inclusion-Exclusion Task

Repeated measures ANOVA with the factors time point ($T_{baseline}$, T_{post}) and group (AI, control) were calculated separately for recollection and familiarity. Recollection did not vary significantly between time points, $F(1,42) = 0.187$, $p = .668$, or groups, $F(1,42) = 0.00$, $p = .995$, but showed a significant time by group interaction, $F(1,42) = 11.096$, $p = .002$ (Figure 4). Planned paired *t*-tests showed that recollection decreased with marginal significance in the AI group, $t(20) = 1.79$, $p = .089$, and increased significantly in the C group, $t(24) = -3.05$, $p = .006$. Further exploration revealed that 11 members of the AI group and seven controls had lower recollection at T_{post} compared to $T_{baseline}$. Confirming results from the parametric analysis, a non-parametric χ^2-test of association revealed significant differences in the number of worse performers between the two groups, $\chi(1, N = 44) = 5.53$, $p = .019$.

Familiarity was marginally higher at T_{post} compared with $T_{baseline}$, $F(1,42) = 3.95$, $p = .053$, and marginally higher in the AI than the control group, $F(1,42) = 2.86$, $p = .098$. The time by group interaction did not approach significance, $F(1,42) = 0.82$, $p = .37$. Difference scores ($T_{baseline} - T_{post}$)

4 Age at menopause was missing from 3 women.

5 Age at menarche was missing from 2 women.

6 The term 'nulliparous' is used for women who never had a child.

of recollection, $r(18) = .045$, $p = .851$, or familiarity, $r(18) = .143$, $p = .159$, did not correlate significantly with the amount of days since AI therapy start in the AI group. With respect to RT's, a repeated measures ANOVA revealed that subjects were faster at T_{post}, $F(1,42) = 10.81$, $p = .002$, but did not yield a main effect of group, $F(1,42) = 0.20$, $p = .657$, or a time by group interaction, $F(1,42) = 0.80$, $p = .375$. Means and standard deviations of positive responses to to-be-included, to-be-excluded and new items as well as RT's are presented in Table 3.

Table 2. Characteristics of the final sample (Experiment 1).

	AI Group		C Group	
	M	*SD*	*M*	*SD*
Age*	67.05	4.35	68.68	6.92
Delay (in Days)*	161.15	41.92	164.28	44.86
Age at First Pregnancy	26.07	4.20	24.12	5.31
Age at Menopause	50.05	7.02	50.45	6.69
Years since Menopause	17.00	7.79	18.18	9.44
Age at Menarche	13.50	1.20	13.35	1.58
HRT Use (in Case Numbers)				
Current	0		2	
Past	10		9	
Never	10		14	
Nulliparity (in Case Numbers)	3		5	
Years of Education				
9 Years	7		6	
10 Years (without Final Exam)	2		3	
10 Years (with Final Exam)	8		11	
13 Years (without Final Exam)	0		1	
13 Years (School) and uncompleted Studies (University)	0		1	
13 Years (School) and completed Studies (University)	3		3	

Note. AI Group = patient group receiving aromatase inhibitor therapy. C Group = control group. * = Means and standard deviations slightly deviate from values mentioned in the methods section, due to the exclusion of three subjects with possible mild cognitive impairment.

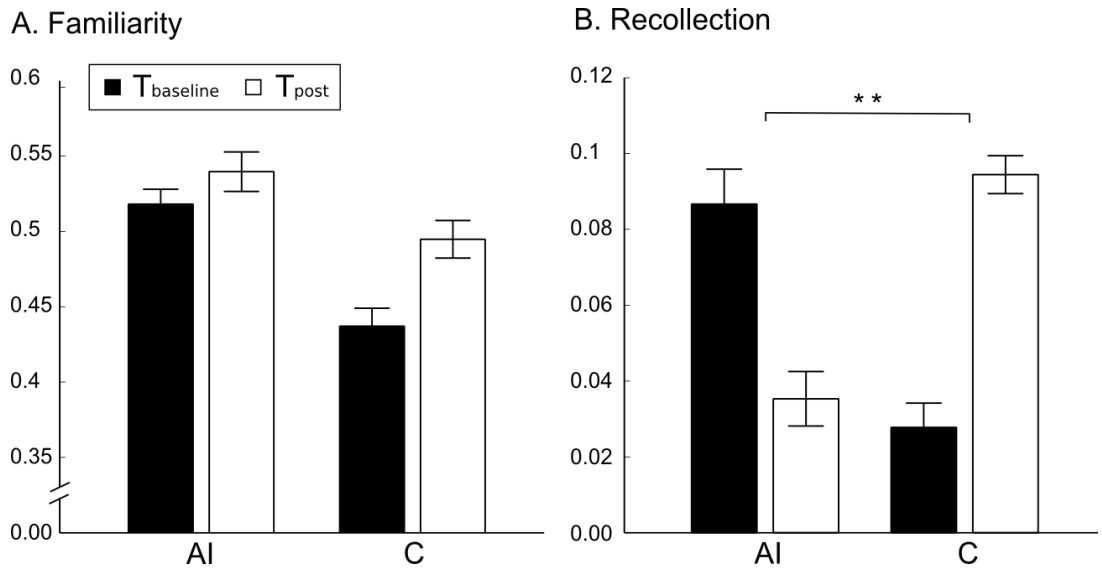

Figure 4. Process estimates of the inclusion-exclusion task (Experiment 1). Black bars represent familiarity or recollection in the patient group before and after onset of aromatase inhibitor therapy (AI) and the control group (C). Black bars represent familiarity (left) and recollection (right) at $T_{baseline}$ (i.e. before onset of aromatase inhibitor therapy), white bars represent parameter estimates at Tpost. A. Familiarity showed a marginally significant main effect of group, with higher values in the patient group receiving aromatase inhibitors (AI) compared with the control group (C). B. Recollection showed a significant group by time interaction (** = $p < .01$), with a marginally significant decrease in recollection within the AI group and an increase in the C group.

Visual memory task

Repeated measures ANOVA's with the factors time point ($T_{baseline}$, T_{post}) and group (AI, control) were calculated separately for high and low confidence corrected hit rates and RT's. High confidence corrected hit rates revealed a significant main effect of group, $F(1,43) = 10.74$, $p = .002$, with better performances in the AI than the control group (see Table 3). There was neither a significant main effect of time, $F(1,43) = 1.48$, $p = .231$, nor a significant group by time interaction, $F(1,43) = 0.07$, $p = .788$. Analysis of low confidence corrected hit rates did not reveal significant effects of group, $F(1,43) = 0.31$, $p = .582$, time, $F(1,43) = 0.77$, $p = .385$, or a group by time interaction, $F(1,43) = 2.56$, $p = .117$. Reaction times for correct high, $F(1,43) = 4.38$, $p = .042$, and low confidence responses, $F(1,43)=8.14$, $p = .007$, showed a significant main effect of time, with

faster RT's at T_{post} (Table 4). The main effect of group was neither significant for RT's of correct high, $F(1,43) = 1.73$, $p = .195$, nor low confidence responses, $F(1,43) = 2.12$, $p = .153$. Similarly, the time by group interaction was neither significant for RT's of correct high, $F(1,43) = 1.10$, $p = .301$, nor low confidence responses, $F(1,43) = 0.26$, $p = .611$. Means and standard deviations of hit rates, false alarm rates and RT's are presented in Table 4.

Word-color association task

Repeated measures ANOVA with the factors time point ($T_{baseline}$, T_{post}) and group (AI, control) were calculated separately for each item condition (i.e. FC, IC, d-prime of II) and RT's. Analyses revealed better performance at T_{post} compared with $T_{baseline}$ for FC rate, $F(1,38) = 16.60$, $p < .001$, IC rate, $F(1,38) = 4.78$ $p = .035$, d-prime for II items, $F(1,38) = 7.57$, $p = .009$, and RT's of correct old-new decisions, $F(1,38) = 8.85$, $p = .005$ (Table 5). The main effect of group was not significant for FC rate, $F(1,38) = 0.32$, $p = .573$, IC rate, $F(1,38) = 1.92$, $p = .174$, d-prime for II items, $F(1,38) = 0.01$, $p = .038$, or RT's of correct old-new decisions, $F(1,38) = 0.89$, $p = .350$. Similarly, the time by group interaction was not significant for FC rate, $F(1,38) = 0.12$, $p = .737$, IC rate, $F(1,38) = 1.53$, $p = .224$, d-prime for II items, $F(1,38) = 0.04$, $p = .839$, or RT's of correct old-new decisions, $F(1,38) = 0.90$, $p = .348$. Means and standard deviations for hit rates within each item condition, d-prime and RT's are presented in Table 5.

Neuropsychological tests of executive functions

Repeated-measures ANOVA with the factors time point and group were calculated separately for the general processing speed score, the phasic alertness scores, the number of errors made in the divided attention task and the interference score of the Stroop task. General processing speed scores showed a statistical trend towards significance for the main effect of group, $F(1,43) = 3.86$, $p =$

.056, with slower reactions in the control (M = 323.08, SD = 68.65) than in the AI group (M = 288.03, SD = 54.89). There was neither a significant main effect of time, $F(1,43) = 0.61$, $p = .806$, nor a significant time by group interaction, $F(1,43) = 0.21$, $p = .885$. The phasic alterness score did not show significant main effects of time, $F(1,43) = 0.14$, $p = .708$, or group, $F(1,43) = 0.02$, $p = .896$, nor a significant time by group interaction, $F(1,43) = 1.01$, $p = .321$. The attentional switch score did not reveal significant main effects of time, $F(1,43) = 0.00$, $p = .964$, or group, $F(1,43) = 2.05$, $p = .159$, nor a significant time by group interaction, $F(1,43) = 2.65$, $p = .111$. Similarly, the interference score of the Stroop task did neither yield significant main effects of time, $F(1,39)= 0.34$, $p = .563$, nor group, $F(1,39) = 1.96$, $p = .170$, nor a significant interaction, $F(1,39) = 0.66$, $p = .656$. Means and standard deviations for measures of executive functions are presented in Table 6.

Results of structural and functional MRI

With respect to structural MRI data, the interaction term testing for a decline in hippocampal volumes due to AI-therapy did not show significant hippocampal clusters. It is of note, that even the use of a very liberal threshold of $p < .01$ (uncorrected) did not show significant decreases in hippocampal volume after AI therapy. Likewise, whole brain analysis did not show significant findings in other brain regions surviving a threshold of $p < .05$, corrected for the whole brain.

Analysis of functional MRI data revealed a significant cluster within the right hippocampus associated to the linear contrast testing for encoding success, $x = 22$, $y = -30$, $z = -6$; $Z = 3.54$, $p_{SVC} = .032$ (Figure 5, A.). A cluster within the left hippocampus did not survive SVC, $x = -20$, $y = -32$, $z = -4$; $Z = 2.90$, $p_{SVC} = .185$. The interaction term, testing for changes in neural activity due to AI therapy, showed that hippocampal BOLD-responses were not significantly modified by AI-therapy. In contrast, a cluster within the right prefrontal cortex covering the ACC, $x = 14$, $y = 28$, $z = 32$; $Z = 4.71$, $p_{SVC} < .001$, and the dorsolateral prefrontal cortex (DLPFC), $x = 12$, $y = 28$, $z = 34$; $Z = 4.31$,

p_{SVC} = .003, showed higher BOLD-responses for encoding success in patients after AI-therapy relative to controls (Figure 5, B.). No other brain regions showed significant main effects or interactions surviving a threshold of $p < .05$, corrected for the whole brain or the reduced search volumes.

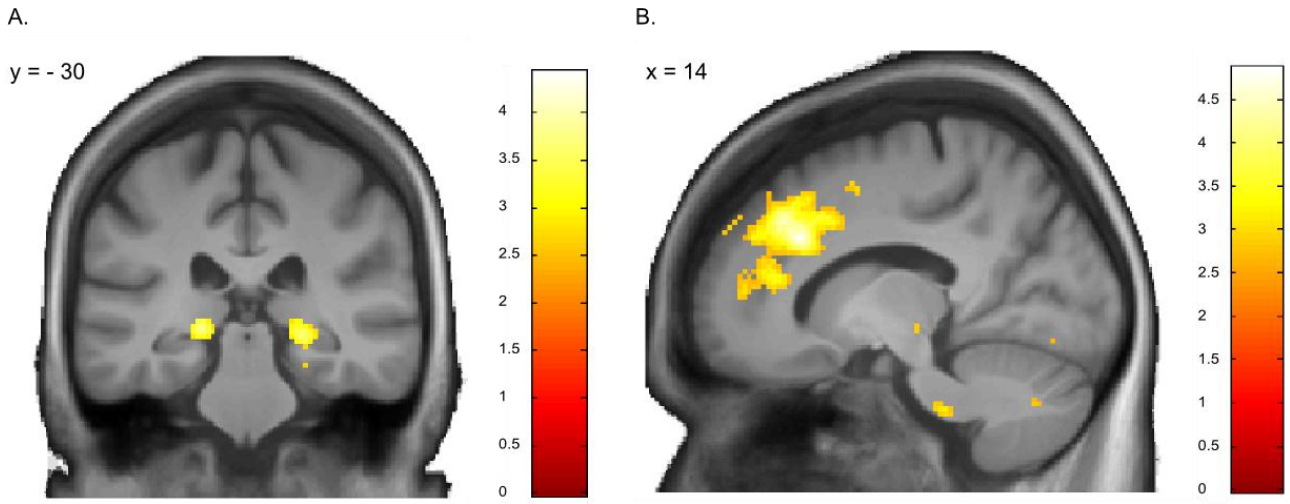

Figure 5. Statistical maps of functional neuroimaging (Experiment 1). A. Statistical map of the linear contrast of encoding success. Encoding success was associated to a significant cluster within the right hippocampus; a cluster within the left hippocampus did not survive small volume correction. B. Statistical map of the time by group interaction, i.e. AI group ($T_{baseline} < T_{post}$) x Control group ($T_{baseline} > T_{post}$). A cluster in the prefrontal cortex, covering the right anterior cingulate and the right dorsolateral prefrontal cortex, showed a significant group by time interaction. An uncorrected threshold of $p < .005$ was chosen for visualization purposes.

Table 3. Behavioral performance in the inclusion-exclusion task (Experiment 1).

		Inclusion		Exclusion		New		Recollection		Familiarity		RT	
		M	*SD*	*M*	*SD*	*M*	*SD*	*M*	*SD*	*M*	*SD*	*M*	*SD*
AI Group	$T_{baseline}$	0.558	0.117	0.472	0.110	0.207	0.109	0.109	0.091	0.518	0.113	2570.38	1028.94
	T_{post}	0.551	0.159	0.516	0.121	0.197	0.115	0.085	0.063	0.540	0.149	2212.13	774.61
C Group	$T_{baseline}$	0.462	0.139	0.437	0.138	0.199	0.143	0.062	0.051	0.448	0.134	2820.21	1310.04
	T_{post}	0.551	0.133	0.460	0.148	0.226	0.154	0.105	0.052	0.502	0.147	2193.50	688.85

Note. AI = Patient group before ($T_{baseline}$) and under (T_{post}) aromatase inhibitor therapy. C = Control group. The first three columns (Inclusion, Exclusion, New) represent means and standard deviations for the proportions of yes answers to to-be-included items (correct), to-be-excluded items (incorrect) and new items (incorrect). The last column shows mean and standard deviations for reaction times (RT) of yes-answers to to-be-included items.

Table 4. Behavioral performance in the visual memory task (Experiment 1).

		Hits		FA		$Hits_{cor}$ (HC)		$Hits_{cor}$ (LC)		RT (HC)		RT (LC)	
		M	*SD*	*M*	*SD*	*M*	*SD*	*M*	*SD*	*M*	*SD*	*M*	*SD*
AI Group	$T_{baseline}$	0.710	0.130	0.245	0.093	0.431	0.148	0.291	0.143	2947.00	777.08	4619.04	1485.26
	T_{post}	0.720	0.160	0.220	0.103	0.458	0.165	0.308	0.177	2644.25	455.66	4173.47	1222.86
C Group	$T_{baseline}$	0.665	0.321	0.300	0.266	0.291	0.143	0.031	0.031	3246.72	1119.34	4215.10	1400.01
	T_{post}	0.615	0.180	0.272	0.161	0.308	0.177	0.017	0.047	3146.02	1473.25	3574.30	1132.79

Note. AI = Patient group before ($T_{baseline}$) and under (T_{post}) aromatase inhibitor therapy. C = Control group. The first two columns show means and standard deviations for total hit (Hits) and false alarm rates (FA) across all confidence levels. The last four columns represent means and standard deviations for corrected hit rates ($Hits_{cor,}$ i.e. hit rate - false alarm rate) and reaction times (RT) for high confidence (HC) and low confidence (LC) responses.

Table 5. Behavioral performance in the word-color association task (Experiment 1).

		FC		IC		II		False-Alarm Rate		D-Prime (II)		RT (IC, II)	
		M	*SD*	*M*	*SD*	*M*	*SD*	*M*	*SD*	*M*	*SD*	*M*	*SD*
AI Group	$T_{baseline}$	0.039	0.031	0.400	0.119	0.338	0.103	0.102	0.082	2.360	0.575	2436.324	452.875
	T_{post}	0.072	0.063	0.447	0.112	0.285	0.115	0.122	0.084	2.500	0.467	2209.029	564.362
C Group	$T_{baseline}$	0.050	0.036	0.369	0.126	0.332	0.106	0.115	0.100	2.336	0.598	2737.370	1013.064
	T_{post}	0.077	0.057	0.382	0.109	0.311	0.107	0.110	0.089	2.498	0.573	2296.870	667.772

Note. AI = Patient group before ($T_{baseline}$) and under (T_{post}) aromatase inhibitor therapy. C = Control group. FC = Rate of freely recalled words. IC = Rate of words that were remembered together with their respective color. II = Rate of words that were remembered without their respective color. The last two columns represent mean and standard deviations for d-prime calculated on the basis of II items, and RT's for correct old-new decisions collapsed across II and IC items.

Table 6. Behavioral performance in tasks of executive functions (Experiment 1).

		General Processing Speed		Phasic Alertness		Attentional Switch Score		Interference Score	
		M	*SD*	*M*	*SD*	*M*	*SD*	*M*	*SD*
AI Group	$T_{baseline}$	288.03	54.89	10.37	29.56	1.20	0.94	17.12	5.88
	T_{post}	291.00	33.55	6.99	27.65	0.93	0.86	18.71	9.54
C Group	$T_{baseline}$	323.08	68.65	5.87	30.30	1.49	1.16	21.75	9.56
	T_{post}	323.84	78.50	13.33	29.59	1.75	2.11	21.96	13.01

Note. AI = Patient group before ($T_{baseline}$) and under (T_{post}) aromatase inhibitor therapy. C = Control group. The first three present means and standard deviations of the two sub-tests 'alertness' and 'divided attention I (auditive/visual)' from the 'Test of Attentional Performance' battery. The column 'Interference Score' shows means and standard deviations of the color-word interference subtest from the Nürnberger Altersinventar.

6.1.4 Discussion

The current study showed a significant group by time interaction for recollection in a specific sub-domain of verbal memory, with a marginal decrease in the AI group and an increase in recollection in the C group. Familiarity in the same domain, visual memory, associative memory of color-word associations and measures of attention remained unaffected by AI-therapy. Furthermore, AI therapy did not modulate hippocampal volumes or hippocampal activity associated to successful verbal encoding. However, AI therapy increased neural responses associated to successful verbal encoding in the right ACC and DLPFC.

Behaviorally, consequences of AI therapy were restricted to recollection in the inclusion-exclusion task, in which subjects had to memorize associations between words and their learning context (i.e. occurrence on list 1 or 2). This finding is consistent with beneficial effects of E2 on hippocampus-dependent, but not other forms of memory (Davis et al., 2005; Korol et al., 2004). On the cellular level, this could be explained by decreased neuronal plasticity at hippocampal neurons as a consequence of extremely low hippocampal E2 concentrations due to AI therapy (Frotscher et al., 2004; Vierk et al., 2012). Moreover, present results mirror findings of impaired recollection but preserved familiarity in elderly relative to young subjects in a similar task (Anderson et al., 2008). However, changes in hippocampal efficacy are not the only valid account for current findings. Recollection is also supported by prefrontal areas, which are sensitive to the influences of E2 as well (Anderson et al., 2008; Rugg, Henson, & Robb, 2003; Sárvári et al., 2010). The prefrontal cortex exerts top-down control of encoding processes and is involved in the specification and elaboration of retrieval cues (for reviews, see Ranganath, 2010; Simons & Spiers, 2003). However, existing literature and present data on executive functions speak against this assumption. It has been shown that the frontal composite of neuropsychological testing correlates with familiarity but not

recollection, whereas the temporal composite correlates positively with recollection but not familiarity (Prull et al., 2006). Additionally, executive functions were not influenced by AI therapy in the present study. Most indications thus speak to decreases in hippocampal efficacy as a cause for the present pattern of behavioral results.

Except of the decrease in recollection described above, AI therapy did not significantly affect any other behavioral measure used in the present study. Specifically, memory for the successful encoding of word-color associations was not affected by AI therapy. Interestingly, previous studies do not show significant hippocampal activity for successful word-color binding in this task, which seems to stand in contrast to the crucial role of the hippocampus in the binding of separable item features (Eichenbaum, 2004; Staresina & Davachi, 2006; Staresina et al., 2012). The missing link between associative memory and hippocampal activity in this task has been explained by the concept of *unitization*. In this task, subjects are explicitly instructed to imagine an object *in* a specified color (e.g. a red tree), so that the color becomes an item-feature of the respective object. As a result, the participants memorize the colored object as a unified whole, and do not have to store word-color associations. These unitization processes rely more on surrounding cortical regions than the hippocampus itself (Diana et al., 2008; Staresina & Davachi, 2006, 2009). Thus, AI therapy most likely did not affect memory for color-word associations because performance does not rely so much on hippocampal functioning in this specific paradigm.

AI therapy did not significantly influence behavioral performance in the verbal free recall test. Free recall has been long proposed to be supported by recollection, because it requires the retrieval of qualitative information about the learning context (Mandler, 1980). In fact, recollection and successful free recall share the recruitment of the hippocampus and the prefrontal cortex, but free recall recruits further brain areas such as the DLPFC (Alkire, Haier, Fallon, & Cahill, 1998;

Henson, Rugg, Shallice, Josephs, & Dolan, 1999; Staresina & Davachi, 2006; Strange, Otten, Josephs, Rugg, & Dolan, 2002; Yonelinas, Otten, Shaw, & Rugg, 2005). Consistent with this, dissociations in behavioral performance between free recall and recollection have been reported elsewhere (Dobbins, Kroll, Yonelinas, & Liu, 1998; Mandler, 1980). Moroever, the two memory functions differ with respect to the availability of retrieval cues. Whereas retrieval cues are given by the task itself in the case of recognition memory, subjects have to generate or find retrieval cues during free recall. As such, free recall depends much more on organizational processes during encoding and retrieval than recognition memory. The DLPFC is involved in organizational processes such as semantic clustering, i.e. the clustered recall of words that are semantically related (for a review, see Blumenfeld & Ranganath, 2007; Gershberg & Shimamura, 1995). Likewise, the DLPFC is positively associated to free recall but not recollection in recognition memory tests (Henson et al., 1999; Yonelinas et al., 2005). Thus, one might argue that proper prefrontal functioning rather than hippocampal functioning is critical for performance in the free recall memory test. Hence, AI therapy most likely did not affect memory performance in the free recall test because it relied more on prefrontal function.

On the neuronal level, there were no changes in hippocampal activation. However, AI therapy increased BOLD responses associated to encoding success in the ACC and DLPFC. Considering these results on the neuronal level together with preserved behavioral performance, one might argue that the enhanced activation of prefrontal areas reflects some sort of compensatory activity. In fact, compensatory activity in prefrontal areas is a common finding in research about cognitive decline in aging and Alzheimer's disease (Bäckman et al., 1999; Cabeza et al., 2002; Gould et al., 2006; Grady, Bernstein, Beig, & Siegenthaler, 2002; Gutchess et al., 2005). Specifically, elderly subjects with intact memory performance, show decreased activity in the medial temporal lobe, but enhanced prefrontal activity compared with young subjects (Gutchess et al., 2005). Moreover,

medial temporal lobe and prefrontal cortex showed a negative association in elderly subjects. More direct evidence has been reported in rats, where optogenetic hippocampal inhibition was compensated by activity within the ACC (Goshen et al., 2011). It is thus conceivable, that enhanced activity in the ACC and DLPFC mirror compensatory mechanisms in patients under AI therapy.

The current data do not allow differentiating compensatory mechanisms on the systems, cellular or behavioral level. The present paragraph discusses two potential compensatory mechanisms that could explain the finding of increased activity in the DLFPC and the ACC after AI therapy. As stated above, the DLPFC mediates organizational processes during encoding and recall of memory (for a review, see Blumenfeld & Ranganath, 2007). Therefore, enhanced recruitment of the DLPFC suggests that patients after AI therapy used organizational encoding strategies more intensely compared with controls. Fitting to this idea, elderly subjects tend to use more semantic associations than young subjects during free recall, a potential compensatory mechanism for memory deficits (Golomb et al., 2008). The ACC mediates performance monitoring and has been found to be activated in tasks that are perceived as cognitively difficult (Barch, Braver, Sabb, & Noll, 2000; MacDonald, Cohen, Stenger, & Carter, 2000; Paus, Koski, Caramanos, & Westbury, 1998). Increased activation within the ACC could thus be interpreted as an increase in perceived task difficulty. Although highly speculative, this mechanism could serve as a signal to initiate further compensatory mechanisms (e.g. through activation of the DLPFC). Unfortunately, we did not assess subjective task difficulty, which would allow testing this hypothesis by means of a psycho-physiological interaction. In sum, increased activity in the ACC and the DLPFC during successful encoding might signal a perceived increase in task difficulty and an amplified use of organizational processes after AI therapy.

AI therapy did not significantly decrease hippocampal volumes, a result that stands in clear

contrast to animal studies showing profound spine and synapse loss after AI administration in the hippocampus of OVX animals (Kretz et al., 2004; Prange-Kiel & Rune, 2006; Rune & Frotscher, 2005). Existing results from VBM studies imply that this method can detect structural changes related to hormonal fluctuations within the hippocampus, so that present null findings are most likely not attributable to the method used for image analyses (Goto et al., 2011; Pletzer et al., 2010; Protopopescu et al., 2008). Present findings connect to numerous studies that failed to find a positive association between HRT and hippocampal volumes (Eberling et al., 2004; Low et al., 2006; Raz et al., 2004). The missing link between HRT and hippocampal volume has been explained by the existence of a limited time-window, in which hippocampal neurons uphold their sensitivity to E2 after menopause (Erickson et al., 2010; Maki, 2006; Maki et al., 2011). More specifically, HRT appears to be most effective with respect to hippocampal volume when initiated during or shortly after menopause, but becomes ineffective when administered more than one year after menopause. As the onset of menopause dates back even longer in present study participants, it is possible that AI therapy did not influence hippocampal volumes because of a decreased sensitivity to hormonal influences. It is of note, that it is currently unknown to which extent the limited-time window hypothesis applies to hormonal *deprivation* caused by AI therapy, as the hypothesis is based on data about the *administration* of E2 or other estrogens in the course of HRT. Moreover, the influence of AI therapy on hippocampus-dependent memory suggests that certain E2-related mechanisms still occur in postmenopausal women. In fact, studies in aged rats suggest that certain cellular effects of E2 are still detectable as long as 15 months after OVX, roughly

equivalent to 26 human years[7] after menopause (Quinn, 2005; Smith, Vedder, Nelson, Bredemann, & McMahon, 2010). Similarly, E2 treatment in aged mice, well beyond reproductive senescence, still improved spatial memory but only when the dosage was high enough (Frick et al., 2002). Furthermore, the authors showed that E2 enhanced a synaptic marker, but left enzymatic activity unchanged. It is thus conceivable, that different cellular actions of E2 are differentially altered by periods of hormonal deprivation. As such, an alternative explanation could be that E2 levels were so low in present participants, that a further drop in E2 levels due to AI therapy had a ceiling effect on hippocampal volume but altered other hippocampal processes that in turn led to impairments in recollection.

One limitation of the present study is the failure to estimate process parameters from the visual memory task. Analysis of behavioral performance in this task was conducted on high-confidence answers, which are only a very rough indicator of recollection-based memory. Moreover, the inability of study subjects to use the full range of confidence levels, questions the validity of their confidence ratings in general. In fact, the association between confidence and accuracy is weaker in elderly compared with young subjects (Chua et al., 2007). It is therefore not possible to infer whether memory impairments are restricted to the verbal domain, or whether a reliable estimation of recollection parameters would yield significant impairments also in the visual domain. A second limitation concerns the interpretation of the current findings. Although decreases in hippocampal efficacy seem to be a sound explanation for the pattern of current results, structural and functional imaging failed to find direct evidence for this assumption. Firstly, this could be accounted for by the

7 Although highly hypothetical, results of the present study were translated from rat’s to human's years. Following Quinn (2005), 17.1 days in the life of an aged rat equal 1 year in a human's life.

fact that not all cellular processes, among which is LTP, are visible in fMRI. Secondly, hippocampal responsiveness to E2 decreases after menopause (Hamilton et al., 2011). Thirdly, small changes as a result of hormonal deprivation could be compensated by mechanisms within the hippocampus itself; Zhou et al., 2010). If hippocampal efficacy turns out to be unaffected by AI therapy, current findings are likely a result of altered functioning of extra-hippocampal areas such as the prefrontal cortex. Another limitation of the current study concerns the comparability of the two groups. In fact, patients showed better performance than the control group in several tasks across both measurement points. Furthermore, the time by group interaction for recollection in the inclusion-exclusion task is partly driven by changes in the control group. We therefore cannot rule out the possibility, that differences between the two groups as well as changes within the control group could have biased current results.

Altogether, current findings point to a restricted impairment in memory performance as a consequence of AI therapy and suggest compensatory mechanisms in a task where behavioral memory performance remained stable. As such, present results support previous findings of negative consequences of AI therapy on verbal memory performance (Collins et al., 2009). Differential effects on recollection compared to familiarity highlight the importance of using memory paradigms that test for clearly defined cognitive processes. Further research is necessary, to determine which mechanisms underlie present findings and whether cognitive consequences of AI therapy are of clinical significance.

6.2. Experiment 2: An aromatase polymorphism in healthy young men

6.2.1 Theoretical Background

Similar to the hippocampus in females, hippocampal neurons in male animals express aromatase as well as both estrogen receptor subtypes (Higo et al., 2009; Österlund et al., 2000, 1999; Stoffel-Wagner, Watzka, Schramm, Bidlingmaier, & Klingmüller, 1999). However, in sharp contrast to the large body of literature on the effect of E2 in female animals, surprisingly few studies have looked on how E2 affects the hippocampus in males. In fact, several effects of E2 seem to be highly sexually dimorphic, such as the E2-induced enhancement of spine density or LTP that occur exclusively in the female hippocampus (Barker & Galea, 2008; Fester et al., 2012; Miranda, Williams, & Einstein, 1999; Morse, Scheff, & DeKosky, 1986; Spritzer & Galea, 2007; Vierk et al., 2012). In contrast, E2-stimulated neuroprotection at hippocampal neurons has been observed in female as well as male animals (Azcoitia et al., 2001; Day et al., 2005; Veiga, Azcoitia, & Garcia-Segura, 2005). Likewise, E2 can positively influence spatial memory performance in male animals as well as visual and verbal memory in men (Beer et al., 2006; Cherrier et al., 2005; Kampen & Sherwin, 1996; Lagunas, Calmarza-Font, Grassi, & Garcia-Segura, 2011; Martin et al., 2003; Salminen, Portin, Koskinen, Helenius, & Nurmi, 2005; Zimmerman et al., 2011). Moreover, E2 decreases fear conditioning in male and female animals (Day et al., 2005; Zeidan et al., 2011).

Therefore, the present study aimed to explore potential estrogenic effects on macroscopic hippocampal morphology and hippocampus-dependent memory in men. For this purpose, the present study takes advantage of the single nucleotide polymorphism (SNP) *rs700518* (c.240A>G/p.=) in the gene coding for aromatase, *CYP19A1*, that has been repeatedly associated with interinvidual differences in serum E2 levels in men (Eriksson et al., 2008; Peter et al., 2008;

Olivo-Marston et al., 2010). More precisely, Peter et al. (2008) report a decrease of 9.59 % for the GA and of 11.93 % for the GG group in E2 serum level in comparison with the AA group. VBM was employed to investigate the association between rs700518 and hippocampal GM volume in two independent samples of healthy young men. Additionally, a spatial learning task, a verbal-source memory task and an emotional memory task were used to examine memory performance in a third independent sample.

6.2.2 Methods

Subjects

Three independent samples of healthy young men participated in the current study. They will be referred to as Cohort A, Cohort B and Cohort C. Whereas Cohort A and B contributed structural MR images, Cohort C contributed behavioral data. All participants signed informed consent, and the study proceeded as approved by the local ethics committee. For all samples, exclusion criteria were current or prior history of psychiatric or neurological illness and use of illicit substances (except for cannabis). All subjects were of Caucasian ancestry. Subjects of both samples were recruited by the Department of Systems Neuroscience in the Medical Center of Hamburg-Eppendorf.

Cohort A consisted of 77 men aged 18 to 44 years (M = 26.77, SD = 6.12). Of these, 22 subjects were smokers and eight were positively tested for cannabis use. All subjects were right handed. The structural scans of Cohort A have been acquired over the past 7 years as the standard anatomic scan during each subjects' participation in a variety of studies.

Cohort B consisted of 84 men aged 19 to 46 years (M = 26.69, SD = 5.51). None of the participants included in the sample had a positive drug test. In total, 15 participants were smokers

and eight reported to be left-handed. All structural scans of Cohort B have been acquired in the course of functional scanning for a previously published study (Yacubian et al., 2007).

The behavioral Cohort C consisted of 157 men aged 18 to 36 years (M = 24.16, SD = 3.27). Of these, 22 were smokers and 27 reported occasional cannabis use. All subjects were right handed. Behavioral data of Cohort C were acquired in the course of the neurodapt!-Core project. In this project, subjects performed a number of behavioral tasks on two consecutive days, filled various questionnaires and gave saliva samples for genoyping. Only data from a spatial learning task ('yellow cab task'), an emotional memory task and a verbal source memory task were used for the current study.

Genotyping

For Cohorts A and C, saliva was taken from all volunteers, and genomic deoxyribonucleic acid (DNA) was extracted by using the Oragene®•DNA sample collection kit (DNA Genotek, Ontario, Canada). Genotyping of the SNP *rs700518* was performed by amplifying the respective genomic region by polymerase chain reaction using primers CYP19A1-F 5`-CGTGATTCACAGATATACATCAC-3’ and CYP19A1-R 5’-AAGTGGAAAAAACTCCAGCCTCG-3’. The obtained 317-bp PCR products were directly sequenced.

For Cohort B, peripheral venous blood was drawn from all volunteers, and genomic DNA was extracted from peripheral blood lymphocytes by using the Qiagen FlexiGene DNA kit (Qiagen, Hilden, Germany). Genotyping was performed by the molecular laboratory BioGlobe GmbH (Hamburg, Germany).

To control for the effects of stratification, individuals of Cohorts A and B were also genotyped for

six unlinked SNPs: brain-derived neurotrophic factor (BDNF rs6265 or Val66Met), catechol-O-methyltransferase (rs4680), dopamine receptor (DRD4 120bp repeat) and transporter polymorphism (rs1042098, rs27072 & DAT2 40bp repeat). Other SNP's of Cohort C were not available. Pearson`s χ^2 was calculated to determine whether frequencies of demographic variables (i.e. smoking, left-handedness, education and cannabis use), and unlinked SNP differed between rs700518 genotype groups. Results of all analyses were considered significant at $p < .1$[8], Bonferroni-corrected for multiple comparisons.

MR-Image acquisition and preprocessing

MR scanning was performed on a 3T MRI system (Siemens Trio) with a standard 12-channel head coil. T1-weighted structural MRI of Cohort A was acquired for each subject by using a 3D-MPRAGE sequence (TR 2300 ms, TE 2.89 or 3.43 ms, flip angle 9°, 1 mm slices, field of view 256 x 192; 240 slices). Differences in TE were the result of different scan protocols and are used as a covariate in further analysis. T1-weighted structural MRI of Cohort B was acquired for each subject by using a 3D-FLASH sequence (TR 15 ms, TE 4.9 ms, flip angle 25°, 1 mm slices, field of view 256 x 256; 192 slices).

Data were analyzed using SPM8 (Statistical Parametric Mapping; Wellcome Deparment of Imaging Neuroscience, London, UK) in Matlab 7.8 (Mathworks). Preprocessing of T1-weighted structural images was performed using the VBM8 toolbox (Voxel Based Morphometry, http://dbm.neuro.uni-jena.de/vbm.html). The cross-sectional preprocessing stream provided by the VBM8 toolbox was used. Departing from default parameters, bias regularization was set to a value of 0.001 (i.e. light regularization). To correct for non-linear warping in the course of spatial

8 A more liberal threshold was chosen to increase power and to decrease the probability of type beta errors.

normalization, non-linear only modulation was performed on all images. The preprocessed GM segments were smoothed with a FWHM of the Gaussian kernel of 8 mm for all three directions.

Voxel-based morphometry analysis

Statistical analyses of Cohort A and Cohort B were conducted separately. Subjects of both cohorts were arranged according to rs700518 genotypes (i.e. AA, AG and GG). All analyses were performed on GM segments. Following the differences in E2 serum levels reported in association studies (Eriksson et al., 2008; Olivo-Marston et al., 2010; Peter et al., 2008), the CYP19_highE2 group (contrast weight: '2') was contrasted against the CYP19_lowE2 groups (contrast weights: '−1, −1'). To test for potential positive influences of testosterone levels that might also correlate with aromatase activity (Spritzer & Galea, 2007), the opposite contrast was applied (contrast weight '-2' for the CYP19_highE2 group and contrast weights '1, 1' for the CYP19_lowE2 groups).

To avoid possible edge effects between different tissue types, we excluded all voxels with GM values smaller than 0.15 (absolute threshold masking). Since age and the BDNF Val66Met polymorphism are known to influence hippocampal volume, both were included as single covariates in further analyses (Bueller et al., 2006; Walhovd et al., 2011). To remove potential variance caused by frequency differences in the DRD4 120bp repeat, this polymorphism was included as another covariate, summing up to a total of three covariates. The BDNF polymorphism and DRD4 120 repeat were coded as single covariates with the values '1', '2', and '3'.

Both cohorts were statistically compared in a cohort by genotype analysis with BDNF genotype, age and DRD4 120bp repeat as covariates, to identify GM differences associated with the CYP19A1 genotype. Results from all analyses were considered significant at $p < .05$, corrected for multiple comparisons at the entire scan volume and within the reduced search volume of interest,

i.e. an anatomical mask of the hippocampus (Amunts et al., 2005). Statistical results are reported for peak voxels, i.e. voxels showing the biggest GM distribution differences between genotype groups.

Behavioral Paradigms

The encoding phases of the emotional memory task and the verbal source memory task took place on the first testing day of the neurodapt!-Core project. Retrieval for these tasks was spread across two consecutive days, with each day testing one half of the stimulus material. The emotional memory task consisted of 240 negative, neutral and positive pictures, drawn from the International Affective Pictures System (IAPS; Lang, Bradley, & Cuthbert, 1999) as well as from a picture set provided by Talmi and colleagues (2007). For each subject, pictures were randomly assigned to either the target (n = 120) or the lure list (n = 120) in pseudo-randomized order. Every encoding trial began with a fixation cross lasting for 1 s, followed by the presentation of a target image in the middle of the screen for 1 s. Next, subjects judged on a 6-point scale whether the image would be suitable for publication in a magazine like ‘National Geographic’ within a time window of 2 s. In the following 2 s, subjects performed the arrow task (see 6.1.2) to ensure that emotional arousal returns to baseline levels after each picture. Total encoding trial length was 6 s. After encoding, subjects performed the 'divided attention I (auditive/visual)' sub-test from the TAP (Zimmermann & Fimm, 2002) for about 10 minutes to clear working-memory content[9]. During retrieval, subjects saw 60 target items intermixed with 60 lure items in the middle of the computer screen. For each picture, participants were required to indicate on a 6-point confidence scale, ranging from 'very sure new' to 'very sure old', whether the image has been presented during encoding and how sure they

9 Data of this task were not analyzed with respect to differences between genotype groups, as this task served only as a distractor in the present study.

were about their decision. Timing of retrieval trials was self-spaced. Model-free memory accuracy was assessed by means of d-prime. The parameter estimates recollection and familiarity were obtained by fitting the dual process signal detection model (Yonelinas, 2002) using maximum likelihood estimation (Dunn, 2010) in Matlab R2009a (Mathworks, Inc, Natick, MA, USA) to the single-subject confidence ratings, separately for each valence category. A practice run consisting of a short encoding and retrieval phase ensured that subjects understood the task.

The verbal source memory task was a modified version of a task published by Cansino and colleagues (2002). The stimulus material consisted of 200 concrete nouns with 4 to 7 characters (M = 5.43, SD = 0.97) and a word frequency ranging from 2 to 297 per million (M = 18.98, SD = 37.96). For each subject, words were randomly assigned to either the target (n = 100) or the lure list (n = 100) in pseudo-randomized order. Every encoding trial began with a fixation cross lasting for 1 s. Next, a target word was presented for 1200 ms in one of four screen positions (i.e. left-top, right-top, left-bottom and right-bottom) with equal probabilities. Subjects were asked to decide via button press whether the word describes a man-made (e.g. a table) or a natural object (e.g. a cat). During retrieval, half of the target items intermixed with 50 lure items was presented in pseudo-randomized order in the middle of the screen on each day. For each word, subjects had to judge via button press whether the item was presented before ('old') or not ('new'). For items that were judged as old, subjects were asked to remember the respective screen position. Timing of retrieval was self-spaced. Item memory accuracy was assessed by means of d-prime for the old-new decision. The percentage of correctly remembered word-locations, relative to the number of words that were correctly identified as old, served as a measure of associative-spatial memory. A practice run consisting of a short encoding and retrieval phase ensured that subjects understood the task.

Spatial learning was assessed by means of a shortened version of the yellow cab task published by

Newman and colleagues (2007). A detailed description of this task is provided in the methods section of the first experiment in Newman and colleagues (2007). In short, subjects had to navigate a taxi from a first-person point of view through a virtual town using a joystick. Each trial began with a search phase, in which subjects had to pick up virtual passengers who were randomly placed in the town. After a passenger had been picked up, subjects were informed that they should bring the passenger to a specific target store. A reward schedule using virtual money encouraged the subjects to prefer direct paths. During each day, subjects had to deliver 15 people to five different locations, so that every location was approached three times. Consequently, subjects used shorter delivery with each successive delivery in the original study. The virtual city was identical on both days, so that subjects could use spatial knowledge gained on day 1 for the task on day 2. In the current study, dependent measures were the time from pick-up to successful delivery ('delivery time') as well as the ratio between the length of the executed delivery path and the optimal delivery path ('path ratio'), averaged across all deliveries separately for each day. A practice run in a different virtual town ensured that subjects understood the task and got used to the joystick.

Subjects were considered as outliers and removed from further analyses when they had values deviating more than three standard deviations from the grand mean on any measure of a given task. The removal of outliers was conducted separately for each task. Results of all analyses were considered significant at a threshold of $p < .05$. To simplify behavioral analyses, statistical analyses were conducted on CYP19_highE2 and CYP19_lowE2 groups instead of single genotype groups (i.e. AA, AG and GG).

6.2.3 Results

Sample characteristics

In Cohort A, 16 subjects were homozygous for the A allele, 37 were homozygous for the G allele and 24 were heterozygous. Mean age, $F(2,75) = 0.658$, $p = .521$, level of education, $\chi^2(10, N = 77) = 6.45$, $p = .776$, proportion of smokers, $\chi^2(2, N = 77) = 2.69$, $p = .261$, and number of cannabis users, $\chi^2(2, N = 77) = 1.52$, $p = .468$, did not differ significantly between genotype groups.

In Cohort B, 18 subjects were homozygous for the A allele, 20 were homozygous for the G allele and 46 were heterozygous. Mean age, $F(2,82) = 1.37$, $p = .260$, level of education, $\chi^2(8, N = 84) = 7.35$, $p = .499$, proportion of smokers, $\chi^2(2, N = 84) = 2.39$, $p = .302$, and left-handed participants, $\chi^2(2, N = 84) = 0.62$, $p = .732$, did not differ between genotype groups. With respect to genotype frequencies, only the DRD4 120bp repeat polymorphism showed significant variations in allele frequency across *rs700518* genotypes in Cohort B, $\chi^2(4, N = 84) = 16.93$, $p = .002$, but not in Cohort A, $\chi^2(4, N = 77) = 1.55$, $p = .817$.

In Cohort C, 44 men were homozygous for the A allele, 52 were homozygous for the G allele and 61 were heterozygous. Genotype groups did not statistically differ in age, $F(17,139) = 0.54$, $p = .914$, level of education, $\chi^2(12, N = 157) = 15.45$, $p = .218$, proportion of smokers, $\chi^2(2, N = 157) = 0.48$, $p = .787$, or cannabis users, $\chi^2(2, N = 157) = 0.64$, $p = .726$.

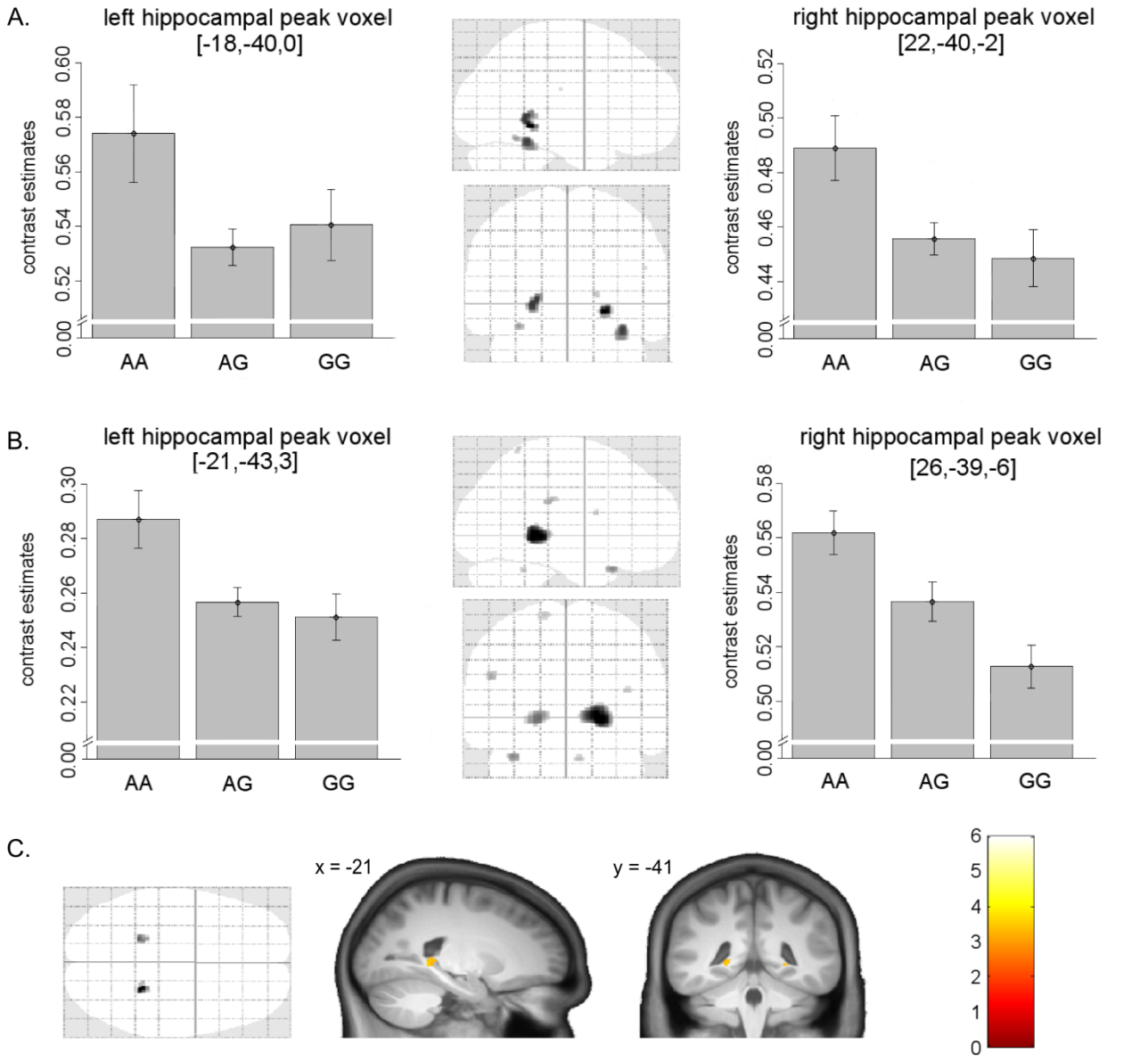

Figure 6. Results of voxel-based morphometry analyses (Experiment 2). A. and B. Regional GM distribution differences between CYP19_highE2 (AA) and CYP19_lowE2 groups (AG & GG) of Cohort A (A.) and Cohort B (B.). An uncorrected threshold of $p < .001$ was chosen for visualization purposes. Bar plots show contrast estimates for each genotype group. To circumvent a circular estimation of effect sizes, coordinates of peak voxels at the right and left hippocampus of cohort B were used for estimation of effect size for cohort A and vice versa (Kriegeskorte, Lindquist, Nichols, Poldrack, & Vul, 2010). Error bars represent standard error of the means. C. Overlapping GM differences between CYP19_highE2 and CYP19_lowE2 groups across both samples. Adapted from "Estrogen and the male hippocampus: Genetic variation in the aromatase gene predicting serum estrogen is associated with hippocampal gray matter volume in men," by J. Bayer, G. Rune, K. Kutsche, U. Schwarze, R. Kalisch, C. Büchel and T. Sommer, 2013, Hippocampus, 23, p. 117-121. Copyright 2012 by Wiley Periodicals, Inc.. Adapted with permission.

Voxel-based morphometry

CYP19_highE2 subjects showed significantly greater posterior hippocampal GM than CYP19_lowE2 in both cohorts (Figure 6, A. & B.). Analysis of Cohort A revealed a significant cluster within the left, x = -18, y = -40, z = 0, Z = 3.73, p_{SVC} = .017, and the right hippocampus, x = 22, y = -40, z = -2, Z = 3.76, p_{SVC} = .015. Similarly, analysis of Cohort B provided significant clusters within the left, x = -21, y = -43, z = 3, Z = 3.75, p_{SVC} = .016, and the right hippocampus, x = 26, y = -39, z = -6, Z = 4.40, p_{SVC} = .001. In both cohorts, no other GM difference between E2 disposition groups survived correction for multiple comparisons. The conjunction analysis of both cohorts (Figure 6, C.) confirmed that the CYP19A1 genotype effect on hippocampal GM anatomically overlapped substantially in both cohorts within the left, x = -21, y = -42, z = 1, Z = 3.41, p_{SVC} = .047, and the right hippocampus, x = 26, y = -39, z = -5, Z = 3.46, p_{SVC} = .041. The opposite contrast, mirroring potential differences in serum testosterone levels, did not reveal any GM differences that survived correction for multiple comparisons.

Emotional memory task

Data from 152 subjects were available for the emotional memory task. Outlier removal was conducted separately for the recognition accuracy d-prime and the process parameters familiarity and recollection, because outliers in the latter can be the result of modeling issues and are not necessarily true outliers in memory accuracy. To examine genotype differences, repeated-measures ANOVA's with the within-subject factors valence (positive, neutral, negative) and day (day 1, day 2) and the between-subject factor group (i.e. CYP19_highE2 vs. CYP19_lowE2) were calculated separately for all three measures.

For analysis of the recognition accuracy d-prime, six subjects were considered as outliers, leaving data from 40 CYP19_highE2 and 106 CYP19_lowE2 subjects for further analyses. Recognition

accuracy was higher on day 1 than day 2, $F(1,144) = 357.28$, $p < .001$, and showed a significant main effect of valence, $F(2,143) = 357.28$, $p < .001$ (Table 7). No further main effects or interactions were significant. Specifically, E2 disposition group did neither influence overall memory accuracy, $F(1,144) = 0.51$, $p = .476$, nor did it interact with day, $F(1,144) = 0.58$, $p = .448$, or valence, $F(2,143) = 0.76$, $p = .470$.

For analysis of the process parameters familiarity and recollection 23 subjects were considered as outliers, leaving data from 37 CYP19_highE2 and 92 CYP19_lowE2 subjects for further analyses. The high number of outliers can be partly accounted by unreliable estimates as a result of low item numbers in single item categories (e.g. when subjects did not use the full range of confidence levels). Analysis of recollection showed better performance on day 1 compared to day 2, $F(1,127) = 249.59$, $p < .001$, and a significant main effect of valence, $F(2,126) = 23.23$, $p < .001$ (Table 7). No further main effects or interactions reached statistical significance. Specifically, there was neither a main effect of group, $F(1,127) = 0.60$, $p = .440$, nor interactions with either day $F(1,127) = 0.54$, $p = .464$, or valence, $F(2,126) = 0.43$, $p = .651$. Analysis of familiarity yielded significant main effects of day, $F(1,127) = 77.17$, $p < .001$, and valence, $F(2,126) = 20.94$, $p < .001$ (Table 7). No further main effects or interactions reached statistical significance for familiarity. Specifically, the factor group did not show a significant main effect, $F(1,127) = 0.00$, $p = .975$, or interactions with either day, $F(1,127) = 0.13$, $p = .720$, or valence, $F(2,126) = 0.35$, $p = .705$. Means and standard deviations of d-prime and parameter estimates for the emotional memory paradigm are presented in Table 7.

Verbal Source Memory-task

Data from 155 subjects were available for the verbal memory task. Of these, three subjects were considered as outliers, leaving data from 43 CYP19_highE2 and 109 CYP19_lowE2 subjects for

further analyses. To examine genotype differences, a repeated-measures ANOVA with the within-subject factor day and the between-subject factor group (i.e. CYP19_highE2 vs. CYP19_lowE2) was calculated separately for the item memory accuracy d-prime and word location.

Analysis of d-prime revealed that subjects performed better on day 1 than on day 2, $F(1,150) = 1039.13$, $p < .001$ (Table 8). With respect to the factor group, neither the main effect, $F(1,150) = 0.40$, $p = .528$, nor the interaction term approached significance, $F(1,150) = 0.00$, $p = .956$. Similarly, analysis of memory performance for word location revealed better memory on day 1 compared to day 2, $F(1,150) = 216.21$, $p < .001$, but neither a significant main effect of group, $F(1,150) = 1.96$, $p = .164$, nor a E2 disposition group by day interaction, $F(1,150) = 1.37$, $p = .244$. Means and standard deviations of d-prime and percentage of correct word location responses for the verbal source memory task are presented in Table 8.

Yellow cab task

Data from 142 subjects were available for the yellow cab task. Of these, one subject was considered as an outlier, leaving data from 41 CYP19_highE2 and 100 CYP19_lowE2 subjects for further analyses. To examine genotype differences, a repeated-measures ANOVA with the within-subject factor day and the between-subject factor E2 disposition group (i.e. CYP19_highE2 vs. CYP19_lowE2) was calculated separately for delivery times and path ratios. Analyses showed faster delivery times, $F(1,139) = 267.97$, $p < .001$, and more efficient delivery pathways on day 2 compared with day 1 $F(1,139) = 246.23$, $p < .001$ (Table 9). However, genotype groups did not significantly differ with respect to delivery time, $F(1,139) = 0.03$, $p = .863$, or path ratio, $F(1,139) = 0.03$, $p = .856$, nor did this factor interact with testing day for delivery time, $F(1,139) = 0.93$, $p = .337$, or path ratio, $F(1,139) = 0.56$, $p = .456$. Means and standard deviations of delivery times and path rations for the yellow cab task are presented in Table 9.

Table 7. Behavioral performance in the emotional memory task (Experiment 2).

Group	Day	D-Prime						Recollection						Familiarity					
		Positive		Neutral		Negative		Positive		Neutral		Negative		Positive		Neutral		Negative	
		M	*SD*	*M*	*SD*	*M*	*SD*	*M*	*SD*	*M*	*SD*	*M*	*SD*	*M*	*SD*	*M*	*SD*	*M*	*SD*
AA (CYP19_highE2)	1	2.67	0.73	2.23	0.77	2.67	0.79	0.46	0.32	0.38	0.27	0.54	0.28	1.69	0.99	1.27	0.91	1.85	1.51
	2	1.55	0.76	1.18	0.51	1.75	0.77	0.23	0.20	0.15	0.14	0.24	0.19	0.93	0.60	0.79	0.40	1.17	0.60
AG (CYP19_lowE2)	1	2.55	0.88	2.38	0.91	2.90	0.79	0.44	0.30	0.40	0.25	0.53	0.31	1.73	1.62	1.22	0.73	1.73	1.38
	2	1.63	0.74	1.29	0.70	1.90	0.66	0.17	0.18	0.11	0.12	0.21	0.20	0.98	0.60	0.70	0.52	1.19	0.67
GG (CYP19_lowE2)	1	2.73	0.93	2.11	0.70	2.66	0.84	0.43	0.28	0.36	0.27	0.53	0.31	1.89	1.49	1.30	0.63	1.62	1.48
	2	1.57	0.65	1.30	0.64	1.93	0.80	0.18	0.17	0.14	0.14	0.21	0.16	1.02	0.50	0.83	0.54	1.24	0.68

Note. CYP19 = Gene coding for aromatase. AA, AG and GG = CYP 19 genotype groups. CYP19_highE2 = Subjects with a genetic disposition to high serum estradiol levels. CYP19_lowE2 = Subjects with a genetic disposition to low serum estradiol levels. D-Prime = Recognition accuracy. Recollection and Familiarity = Process parameter estimates.

Table 8. Behavioral performance in the verbal source memory task (Experiment 2).

Group	Day	D-Prime (Old/New Decision)		Word Location	
		M	*SD*	*M*	*SD*
AA (CYP19_highE2)	1	1.95	0.49	0.51	0.17
	2	0.83	0.33	0.30	0.10
AG (CYP19_lowE2)	1	2.00	0.48	0.57	0.15
	2	0.86	0.39	0.30	0.10
GG (CYP19_lowE2)	1	1.97	0.47	0.55	0.16
	2	0.89	0.36	0.30	0.11

Note. CYP19 = Gene coding for aromatase. AA, AG and GG = CYP 19 genotype groups. CYP19_highE2 = Subjects with a genetic disposition to high serum estradiol levels. CYP19_lowE2 = Subjects with a genetic disposition to low serum estradiol levels. D-Prime = Recognition accuracy. Word-Location = Source memory for the screen position of the target word.

Table 9. Behavioral performance in the spatial memory task (Experiment 2).

Group	Day	Delivery Time		Path Ratio	
		M	*SD*	*M*	*SD*
AA (CYP19_highE2)	1	1104.42	211.29	1.73	0.35
	2	870.63	212.64	1.33	0.37
AG (CYP19_lowE2)	1	1109.34	229.67	1.76	0.43
	2	870.63	212.64	1.31	0.31
GG (CYP19_lowE2)	1	1116.95	212.75	1.77	0.38
	2	845.61	209.15	1.32	0.37

Note. CYP19 = Gene coding for aromatase. AA, AG and GG = CYP 19 genotype groups. CYP19_highE2 = Subjects with a genetic disposition to high serum estradiol levels. CYP19_lowE2 = Subjects with a genetic disposition to low serum estradiol levels. Delivery Time = Time from pick-up to successful delivery. Path Ratio = Ratio between the length of the executed delivery path and the optimal delivery path.

6.2.4 Discussion

Male CYP19_highE2 subjects showed significantly larger bilateral posterior hippocampal GM volumes than CYP19_lowE2 subjects in both cohorts. GM distribution differences mirror reported E2 serum levels in subjects homozygous for the A-allele and carriers of the G-allele (Eriksson et al., 2008; Olivo-Marston et al., 2010; Peter et al., 2008). These results parallel the findings of previous VBM studies about E2 effects on the female hippocampus (Goto et al., 2011; Lord, Engert, Lupien, & Pruessner, 2010; Protopopescu et al., 2008). CYP19_highE2 and CYP19_lowE2 subjects did not differ with respect to behavioral performance in emotional memory, verbal source memory or spatial learning tasks.

Based on animal data, the genotype-dependent VBM difference in the current study is most likely a result of E2-stimulated neuroprotection. In fact, E2 stimulated neuroprotection is one of the few effects of E2 that have been observed in the male hippocampus (Azcoitia et al., 2001; Veiga et al., 2005). Other E2-induced modulations of hippocampal structure such as stimulation of fiber outgrowth, sprouting responses and spine density occur only in females and are thus likely not the cellular correlate of present findings (Barker & Galea, 2008; Fester et al., 2012; Morse et al., 1986; Spritzer & Galea, 2007; Woolley & McEwen, 1993). Enhanced hippocampal neuroprotection as a cause for higher hippocampal volumes in CYP19_highE2 subjects is further supported by the fact that the effects of E2 are modulated by treatment duration. Whereas neurogenesis and spine density are only enhanced after acute but not chronic E2 treatment (Miranda et al., 1999; Ormerod & Galea, 2001), neuroprotection is stimulated by acute as well as chronic E2 treatment in both male and female animals (Harms et al., 2001; Jover et al., 2002; McCullough & Hurn, 2003). Because chronic E2 treatment is more similar to genetic differences in serum E2 level than acute E2 administration, the latter findings also point to neuroprotection as the cellular correlate of the observed VBM differences. Whereas differences in hippocampal structure were highly reliable

across study cohorts, no differences were detected with respect to memory performance. Potential reasons for this discrepancy are (i) that genotypes affected hippocampal structure but not hippocampus-dependent memory function, (ii) that the memory tasks employed in the current task did not rely on the posterior hippocampus or (iii) that the sample size was too small to detect subtle behavioral differences.

With respect to the first hypothesis, it is important to note that the occurrence of differences in hippocampal structure in the absence of behavioral differences or vice versa is consistent with a number of studies (Cohen, Small, Lalonde, Friz, & Sunderland, 2001; Jager et al., 2007; Molendijk et al., 2012). Interestingly, the relationship between memory and hippocampal volume appears to be moderated by age, with no or even a negative association in young adults and a positive association in the elderly (Chantôme et al., 1999; Foster et al., 1999; Jager et al., 2007; Molendijk et al., 2012; Van Petten, 2004). This could be accounted for by the fact that not only the growth of neurons but also regressive events such as programmed cell death play an important role in the young brain (Foster et al., 1999). Beneath biological accounts, the first hypothesis concerns the difference between studies testing for within-subject versus between-subject. So far, only one study showed an association between E2-related changes in hippocampal structure and verbal memory (Protopopescu et al., 2008). Here, the authors employed a within-subject design to compare women in different phases of their menstrual cycle. One thus could hypothesize, that *intra-individual* changes in E2 levels are more critical for the modulation of memory performance than stable differences in E2 levels between subjects. In the present study, genetic differences most likely lead to *inter-individual* differences in E2 levels that occurred as early as during brain development or puberty. Therefore, individuals might have adjusted to their respective E2 level. In sum, it is in fact possible that CYP19 genotypes affected hippocampal structure but not hippocampus-dependent memory function. This might be explained either by a missing relationship between hippocampal volumes

and memory performance in healthy young man or by the minor importance of between-subject differences for E2-related modulations of memory performance.

With regard to the second hypothesis (i.e. that the memory tasks employed in the current task did not rely on the posterior hippocampus), results from imaging studies strongly suggest hippocampal involvement in all three memory tests used in the current study (Cansino et al., 2002; Dolcos, Denkova, & Dolcos, 2012; Fenker, Schott, Richardson-Klavehn, Heinze, & Düzel, 2005; Sperling et al., 2003; Suthana, Ekstrom, Moshirvaziri, Knowlton, & Bookheimer, 2011). More precisely, the anterior hippocampus is commonly activated during emotional and verbal-associative memory tasks and the posterior hippocampus is usually involved in spatial memory tasks. As such, the posterior hippocampus is *in general* involved at least in the spatial memory task. However, it is possible that differences in hippocampal efficacy could be masked by compensatory mechanisms. In fact, existing literature evidences that the recruitment of other brain regions can compensate for a reduced hippocampal efficacy (Bondi, Houston, Eyler, & Brown, 2005; Goshen et al., 2011). As such, imaging data are necessary to clarify whether potential deficits in hippocampal efficacy associated to CYP19 genotypes could be masked by compensatory activity in other brain areas.

To examine the third hypothesis of insufficient sample sizes, a power analysis was calculated for the biggest difference between genotype groups, i.e. memory for word locations on day 1. Given present means and standard deviations (i.e. CYP19_highE2: $M = 0.51$, $SD = 0.17$; CYP19_lowE2: $M = 0.56$, $SD = 0.15$) and an assumed power of 0.80, 204 subjects per group would have been necessary to achieve significant results for an unpaired two-tailed *t*-test using a significance threshold of $p < .05$. As this sample size is not unusual for genetic association studies (e.g. Peter et al., 2008), we cannot rule out the possibility that larger sample sizes would yield significant differences between genotypes.

In sum, the current study demonstrates that the polymorphism rs70058 on the gene coding for aromatase (CYP19) is associated with differences in hippocampal GM volume in two independent cohorts of healthy young men. Genotype groups did not differ with respect to memory performance, which could be related to a weak relationship between hippocampal volume and memory performance, the minor importance of between-subject differences in E2 levels, compensatory mechanisms or a lack of power due to insufficient sample sizes.

6.3. Experiment 3: Menstrual cycle in young women and emotional memory

6.3.1 Introduction

E2 and P4 modulate neuronal plasticity not only in the hippocampus, but also in further brain areas such as the amygdala and the prefrontal cortex (Foy et al., 1999; Hao et al., 2006; McEwen & Woolley, 1994; Murphy & Segal, 1996; Tang et al., 2004). These cellular effects of E2 and P4 stimulated research about hormonal influences on various cognitive and emotional functions. Consistent with their opponent effects on hippocampal neurotransmission, E2 exerts beneficial effects on memory that are reversed by P4 (Bimonte-Nelson et al., 2006; Gibbs et al., 2004; Harburger et al., 2007). However, hormonal influences differ across brain regions. For instance, E2 stimulates neural transmission in the hippocampus, but decreases neuronal excitability in the amygdala (Foy et al., 1999; Womble, Andrew, & Crook, 2002). Likewise, E2 and P4 act opponent on some cellular processes in the hippocampus but exert conjoint neuroprotective effects in the prefrontal cortex (Djebaili, Hoffman, & Stein, 2004; Foy et al., 2008; Hill, Chua, Jones, Simpson, & Boon, 2009; Kritzer & Kohama, 1998). The superior memory formation for emotionally arousing compared to neutral information (i.e. EEM effects) depends upon the interplay between all these brain structures (for a review, see Dolcos et al., 2012). Therefore, predictions concerning hormonal influences on this memory function are less straightforward.

Prefrontal areas, the hippocampus and the amygdala contribute differentially to the EEM, i.e. to distinct aspects and at different stages of emotional memory formation, and are all sensitive to hormonal influences (see 3.4.2). It is thus conceivable, that hormonal fluctuations modulate EEM at various stages. In fact, animal studies suggest hormonal effects on basic affective processes, because both hormones exert anxiolytic and antidepressant actions in the laboratory (Frye, Petralia,

& Rhodes, 2000; Frye & Walf, 2004; Lund, Rovis, Chung, & Handa, 2005; Reddy, O'Malley, & Rogawski, 2005; Shirayama et al., 2011; Walf & Frye, 2006). Likewise, functional imaging in humans reveals that E2 and P4 change the reactivity to emotional stimuli in the amygdala, the hippocampus and the medial prefrontal cortex (Derntl et al., 2008; Goldstein et al., 2005; Guapo et al., 2009; Ossewaarde et al., 2010; Sripada et al., 2013; van Wingen et al., 2007). Moreover, clinical research points to hormonal effects on affective disorders such as depression (Cohen, Soares, Vitonis, Otto, & Harlow, 2006; Freeman & Sammel, 2004). Studies using fear conditioning paradigms in animals and humans further attest hormonal influences on the consolidation of fear memories (Gupta et al., 2001; Markus & Zecevic, 1997; Milad et al., 2006, 2010; Milad, Igoe, Lebron-Milad, & Novales, 2009; Zeidan et al., 2011). Hormonal effects on the consolidation of *declarative* EEM effects have been investigated so far only behaviorally, by contrasting women in the first (i.e. menstruation to ovulation) and second half of the menstrual cycle, with a lower number of negative pictures recalled by women in the second half (Ertman et al., 2011). Another study, in which women were post hoc sub-divided into a high and low P4 group, found group differences in emotional memory only when a stressor was present but not under rest (Felmingham et al., 2012).

The current study aims to further explore how natural hormonal variations affect the superior consolidation of emotional arousing information in declarative recognition memory. Therefore, functional neuroimaging was performed in healthy young women while encoding positive, negative and neutral pictures once during menstruation in the early follicular phase, where both hormones are low, and once in the middle of the luteal phase where the concentration of P4 is at its maximum and E2 reaches a second peak. In order to assess the effects of hormones on arousal-stimulated consolidation rather than the initial processing of emotional stimuli, memory was tested two days after encoding. To examine not only changes in memory accuracy but also quality, recollection and

familiarity estimates were estimated based on Yonelinas' dual-process model (Yonelinas, 2002). Additionally, arousal ratings were acquired for target images, so that stimuli could be grouped according to arousal ratings into a high (HA) or low arousing (LA) stimulus category. According to the goal of the study, analyses were primarily conducted on HA emotional and LA neutral stimuli. Menstrual cycle phases were verified by assessments of E2 and P4 in saliva.

6.3.2 Material and methods

Subjects

Twenty-three healthy, naturally cycling female volunteers, aged 19 to 33 years (M = 26, SD = 3.25), were each tested twice during their menstrual cycle (counterbalanced order over the sample of participants); once during the *early follicular* and once during the *luteal phase.* A telephone screening prior to the first test session ensured that no subject met criteria for premenstrual disphoric disorder as defined by the Diagnostic and Statistical Manual of Mental Disorders IV (American Psychiatric Association, 2000). All subjects reported to be free of neurological or psychiatric diseases, use of illicit drugs and central nervous system medication and did not smoke more than two cigarettes per week. Date of the last menstruation and length of the menstrual cycle (M = 28.18, SD = 1.42) were assessed during the first interview to determine adequate time points for testing. During the time period between the first contact and last testing, all subjects informed the experimenter about their menstruation dates. One subject was excluded due to menstrual cycle irregularities, leaving 22 subjects for further analysis.

Memory encoding inside the scanner was scheduled at day 0 to day 4 after onset of menstruation in the early follicular phase (M = 1.52, SD = 1.34) and at 5 to 11 days before estimated onset of next menstruation (M = 8.14, SD = 2.08) in the luteal phase. After encoding and a 10 minutes break,

women performed a reward paradigm which is reported elsewhere (Bayer, Bandurski, & Sommer, 2013). Memory retrieval was conducted 2 days after the encoding session outside the scanner.

Participants received financial compensation for the time spent at the institute. All participants gave written informed consent according to the Declaration of Helsinki. Ethics approval was obtained from the ethics committee of the medical association of Hamburg.

Emotional Memory-task

In total, 576 colored photographs depicting positive, negative or neutral contents were used for the current study. Images were drawn from the IAPS repository (Lang et al., 1999) and internet search. The three valence categories contained an equal number of images showing animals and people and image contents were visually matched across valence categories to equalize semantic coherence and complexity. Images with similar gist were grouped pairwise, so that each target image had a corresponding lure image. Two lists were created for every subject, each assigning stimulus pairs to cycle phases and determining which item of the stimulus-pair would be a target or lure.

During each cycle phase, subjects encoded 144 stimuli spread over four runs inside the scanner. Stimuli were pseudo-randomized with the restriction that not more than three pictures of the same valence category were presented in a row. Participants were informed about the memory test 2 days later. Each trial began with a fixation cross lasting for 3 seconds, followed by the presentation of the image for 2 seconds. Next, subjects judged on a 6-point scale whether the image would be suitable for publication in a magazine like ‘National Geographic’ within a time window of 2 seconds. In the following 4 seconds, subjects performed the arrow task described above (see 6.1.2). The arrow task served as an active baseline (Stark & Squire, 2001) and ensured that emotional arousal returns to baseline levels after each picture.

During retrieval, participants were shown the 144 previously learned stimuli intermixed with 144 new stimuli in pseudo-randomized order. While viewing each image separately on the screen, participants were required to indicate on a 6-point scale ranging from 'very sure new' to 'very sure old' whether the image was presented during encoding and how sure they were about this decision. Timing of retrieval-trials was self-spaced.

After testing in both cycle phases was accomplished, participants rated all 288 target stimuli on subjective emotional arousal. Ratings were accomplished by using a 9-point pictorial 'Self-Assessment-Manikin' scale (Bradley & Lang, 1994). Because the assignment of items to target or lure lists was pseudo-randomized, every subject rated a different set of stimuli. As a result, every single stimulus was rated at least 8 times. Therefore, average z-standardized arousal ratings derived from the study sample could be used as approximate values for subjective arousal of lure items.

Neuroimaging

Event-related functional MRI was performed on a 3 T system (Siemens Trio) with an echo planar imaging T2*-sensitive sequence in 30 contiguous axial slices (2 mm thickness with 1 mm gap; TR, 1.8 s; TE, 25 ms; flip angle, 70°; field of view, 216 x 216; matrix 108 x108). The chosen MR sequence covered a region from the superior frontal gyrus to the parietal superior lobule and ventral to the cisterna pontis (see Figure 9 A, red frame). For spatial normalization, a high-resolution T1-weighted structural MR image was acquired by using a 3D-MPRAGE sequence (TR 2300 ms, TE 2.89 ms, flip angle 9°, 1 mm slices, field of view 256 x 192; 240 slices).

MRI data were preprocessed and analyzed using Statistical Parametric Mapping (SPM8; Wellcome Department of Imaging Neuroscience, London, UK), running under Matlab R2009a (Mathworks, Inc, Natick, MA, USA). To prevent biases due to spin saturation, the first five

functional images were discarded. All functional images were slice time corrected, realigned and unwarped. Individual structural T1 images were coregistered to functional images and segmented using the 'New Segment' algorithm provided by SPM8. The DARTEL toolbox was used to normalize structural and functional images to MNI space. Finally, images were smoothed with FWHM Gaussian kernel of 8 mm for all directions. Functional MR data from three subjects were excluded from further analysis due to scanning artifacts, leaving data from 19 subjects for further functional image analysis.

Event-related BOLD responses were analyzed employing the GLM as implemented in SPM, using a mass univariate approach. On the individual subject level, 12 separate regressors for the factors valence (positive, neutral, negative), arousal (HA, LA) and memory (hit, miss), plus a regressor containing all rating events during encoding were created for each cycle phase by convolving the onset regressors with the canonical HRF.

The second level analyses focused on HA emotional and LA neutral categories, since hormones were expected to interact with arousal-stimulated memory consolidation and its neural substrates, but not so much on semantic, elaborate processing that is involved in the EEM for low arousing stimuli (Dolcos et al., 2012; Kensinger & Corkin, 2004). The individual beta images corresponding to hits and misses in the HA positive and negative as well as the LA neutral conditions were included, resulting in a design matrix comprising 12 regressors. The main effects of emotional processing were tested by linear contrasts, assigning a weight of -1 to all HA positive/negative categories and 1 to all LA neutral categories. To test whether menstrual cycle phase affects emotional arousal processing irrespective of encoding success, the HA emotional vs. LA neutral × cycle phase interactions were formulated as contrast vectors for both valences and both interaction directions, i.e. luteal > early follicular and vice versa. The EEM_{neg} and EEM_{pos} effect was estimated

by applying the contrast 'negative emotion (negative > neutral) x memory (hit > miss)' and 'positive emotion (positive > neutral) x memory (hit > miss)', respectively. The *main hypothesis*, whether menstrual cycle phase modulates neural correlates of EEM effects, was computed by contrast vectors that formulated the interaction of cycle phases with the EEM_{neg} and EEM_{pos}, i.e. 'negative emotion (negative > neutral) x memory (hit > miss) x cycle phase' and 'positive emotion (positive > neutral) x memory (hit > miss) x cycle phase', respectively. Results of all analyses were considered significant at $p < .05$, FWE corrected for multiple comparisons at the entire scan volume and within predefined anatomical regions of interests, i.e. the hippocampus, the amygdala and prefrontal regions. All anatomical masks were created using the Harvard-Oxford cortical and subcortical structural atlases as implemented in FSL (www.fmrib.ox.ac.uk/fsl), except of the ACC mask which was derived from the Anatomical Automatic Labeling toolbox for SPM8 (Tzourio-Mazoyer et al., 2002).

Mood ratings

A mood questionnaire was applied in the course of the two sessions prior to scanning (Pessiglione, Seymour, Flandin, Dolan, & Frith, 2006). The questionnaire consisted of 16 pairs of opposite feelings which were all translated into German. The dimensions were *'alert/drowsy', 'calm/excited', 'strong/feeble', 'clear-headed/muzzy', 'well-coordinated/clumsy', 'energetic/lethargic', 'contented/discontented', 'tranquil/troubled', 'quick-witted/mentally slow', 'relaxed/tense', 'attentive/dreamy', 'proficient/incompetent', 'happy/sad', 'amicable/antagonistic', 'interested/bored'* and *'gregarious/withdrawn'*. Every word pair was presented separately on the screen and subjects were asked to rate their feelings by moving a cursor continuously between the two extremes.

Assessment and analysis of hormone concentrations

During each testing day, three saliva samples were collected with 30 minutes apart. The three samples were pooled and analyzed by IBL (Innovation Beyond Limits, Hamburg, Germany) with highly sensitive luminescence assays in terms of E2, P4 and cortisol (sensitivity E2: 0.3 pg/ml, P4: 2.6 pg/ml, Cortisol: 0.06 ng/ml). Repeated measurement ANOVA's with the factors testing day and cycle phase were calculated to examine whether hormone levels would differ between cycle phases. Statistical results were considered significant at a threshold of $p < .05$.

6.3.3 Results

Hormone concentrations and mood ratings

As expected, analysis of E2 concentrations yielded a significant main effect of cycle phase, $F(1,21) = 21.50$, $p < .001$, with higher E2 concentrations in the luteal than in the early follicular phase (Figure 7). Similarly, analysis of P4 concentrations showed a significant main effect of cycle phase, $F(1,21) = 47.63$, $p < .001$, with higher P4 concentrations in luteal compared to early follicular phase.

Cortisol levels were assessed to control for confounding effects of menstrual cycle-related fluctuations in cortisol levels (Andreano, Arjomandi, & Cahill, 2008; Walder, Statucka, Daly, Axen, & Haber, 2012). Analysis showed a significant main effect of testing day, $F(1,21) = 7.34$, $p = .013$, with higher cortisol concentrations on day 2 ($M_{EF} = 0.35$ pg/ml, $SD = 0.34$; $M_{LUT} = 0.30$ pg/ml, $SD = 0.16$) than on day 1 ($M_{EF} = 0.24$ pg/ml, $SD = 0.16$, $M_{LUT} = 0.23$ pg/ml, $SD = 0.18$). No other main effects or interactions reached statistical significance (all $p > .1$). As another control measure, the assessment of mood on 16 dimensions by means of a questionnaire served as a control for menstrual cycle-related changes in mood. Paired-sample *t*-tests did not show significant

associations with menstrual cycle phase on any dimension (all $p > .1$).

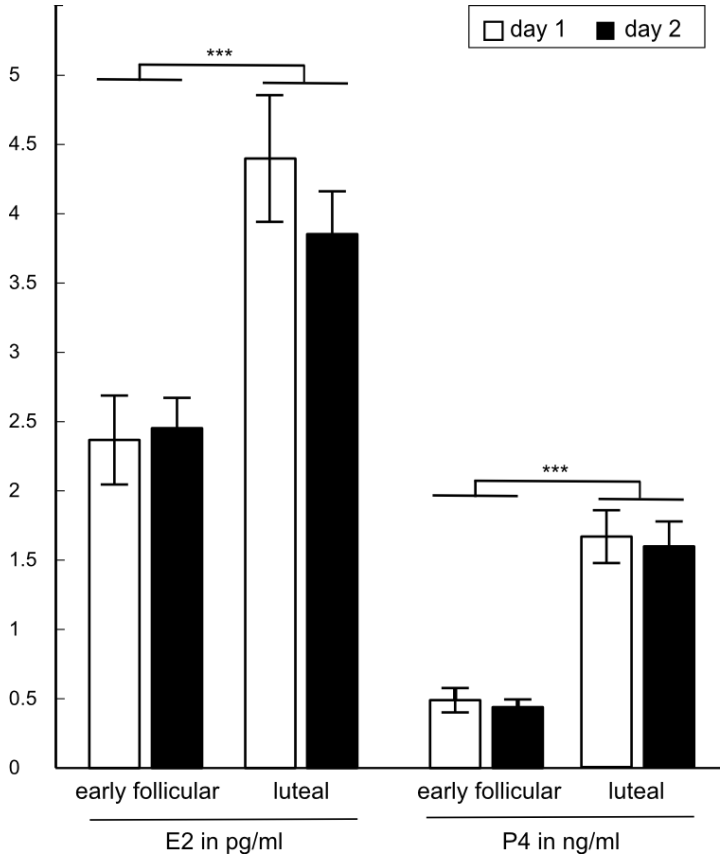

Figure 7. Saliva steroid hormone levels for both testing days (Experiment 3). Depicted are mean estradiol (E2; in pg/ml) and progesterone (P4; in ng/ml) concentrations during early follicular and luteal phase. Concentrations of E2 and P4 were higher during luteal than during early follicular phase. Error bars represent the standard error of the mean. Adapted from "Menstrual-cycle dependent fluctuations in ovarian hormones affectemotional memory," by J. Bayer, H. Schultz, M. Gamer, T. Sommer, 2014, Neurobiology of Learning and Memory, 110, p. 55–36. Copyright 2014 by Elsevier. Adapted with permission.

Arousal ratings

Arousal ratings were on average 6.42 points (SD = 0.80) for negative, 3.05 points (SD = 1.13) for neutral and 5.02 points (SD = 1.27) for positive target images on the 9-point arousal scale. A repeated measures ANOVA revealed a significant main effect of valence, $F(2,20) = 323.77$, $p <$

.001, but neither a significant main effect of cycle phase, $F(1,21) = 1.00$, $p = .329$, nor a cycle by valence interaction, $F(2,20) = 1.68$, $p = .212$. Because subjects rated only their individual set of target items, mean ratings derived from all subjects were used as an approximation of subjective arousal in response to lures. In detail, arousal ratings were first *z*-standardized within each subject and then averaged across subjects. Median split based categorization of images into low (LA; arousal $\leq$ $arousal_{MED}$) and high arousal (HA; arousal > $arousal_{MED}$) within each subject, resulted in an assignment of on average 28.75 % (*SD* = 8.28) negative, 96.44 % (*SD* = 3.35) neutral and 50.60 % (*SD* = 8.26) positive images to the LA category. As a very small number neutral items fell into the HA category, HA neutral images were removed from all analyses.

Based on the goal of the study, analyses were primarily conducted on a reduced stimulus set consisting of HA emotional and LA neutral stimuli. All behavioral and neuroimaging results are reported for this stimulus set unless stated otherwise. However, arousal ratings in this reduced set were still higher for negative HA ($M_z = 0.95$, $SD_z = 0.11$) than for positive HA images ($M_z = 0.63$, $SD_z = 0.12$). Therefore, additional analyses were conducted on a third further reduced stimulus set with matched arousal levels, to confirm that valence specific effects were not driven by differences in mean arousal between negative and positive pictures. Statistically equal arousal ratings for negative and positive items, $t(21) = 0.90$, $p = .378$, were achieved by removing negative images with arousal ratings higher than the 97.5 percentile for targets and to the 82.5 percentile for lures. The percentage of negative items that were excluded was on average 25.86 % (*SD* = 2.30).

Memory performance

Recognition accuracy as measured by d-prime revealed a significant main effect of valence, $F(2,20) = 43.47$, $p < .001$ (Figure 8, A). The main effect of cycle, $F(1,21) = 0.79$, $p = .384$, and the cycle by valence interaction did not reach significance, $F(2,20) = 0.23$, $p = .797$. Closer inspection

using planned paired two-tailed *t*-tests showed that d-prime was higher for positive compared to neural, $t(21) = 6.19$, $p < .001$, as well as negative compared to neutral stimuli, $t(21) = 5.53$, $p < .001$. Recognition accuracy for positive and negative stimuli did not differ, $t(21) = -0.58$, $p = .568$.

Using maximum likelihood estimation (Dunn, 2010) in Matlab R2009a (Mathworks, Inc, Natick, MA, USA), parameter estimates of recollection and familiarity were obtained by fitting the dual process signal detection model (Yonelinas, 2002) to the single-subject confidence ratings, separately for each valence category and cycle phase. Analysis of recollection values yielded a significant main effect of valence, $F(2,20) = 15.03$, $p < .001$, as well as a significant valence by cycle interaction, $F(2,20) = 5.76$, $p = .011$, but not a main effect of cycle, $F(1,21) = 0.91$, p = .351 (Figure 8, C). Planned paired two-tailed *t*-tests revealed that recollection for negative stimuli was marginally higher in the low-hormone early follicular compared to high-hormone luteal phase, $t(21) = 2.04$, $p = .055$. No significant differences between cycle phases were detected in recollection for neutral, $t(21) = 1.60$, $p = .125$, or positive stimuli, $t(21) = -1.39$, $p = .178$. Visual inspection of recollection suggests changes for negative and positive stimuli changes in opposite directions across cycle phases (Figure 8, C). Because estimation of the process parameters familiarity and recollection can be problematic when the number of items in each category is low, process parameters were additionally estimated using the full stimulus set (i.e. the stimulus set that included all stimuli irrespective of arousal category). Confirming previous results, analyses of recollection yielded even higher significance levels for the interaction term ($p < .001$) and the *t*-test ($p < .05$). Parameter estimation, using the stimulus set constituted of HA emotional and LA neutral stimuli with further exclusion of very high arousing negative pictures (see above), did not yield reliable estimates due to the small number of items. Nevertheless, results showed qualitatively the same pattern as the two other sets.

Analysis of familiarity showed a significant main effect of valence, $F(2,20) = 13.25$, $p < .001$, but neither a main effect of cycle phase, $F(1,21) = 0.07$, $p = .791$, nor a valence by cycle interaction, $F(2,20) = 0.59$, $p = .564$ (Figure 8, C). Analysis of the full set confirmed that familiarity did not show a significant interaction between valence and cycle phase.

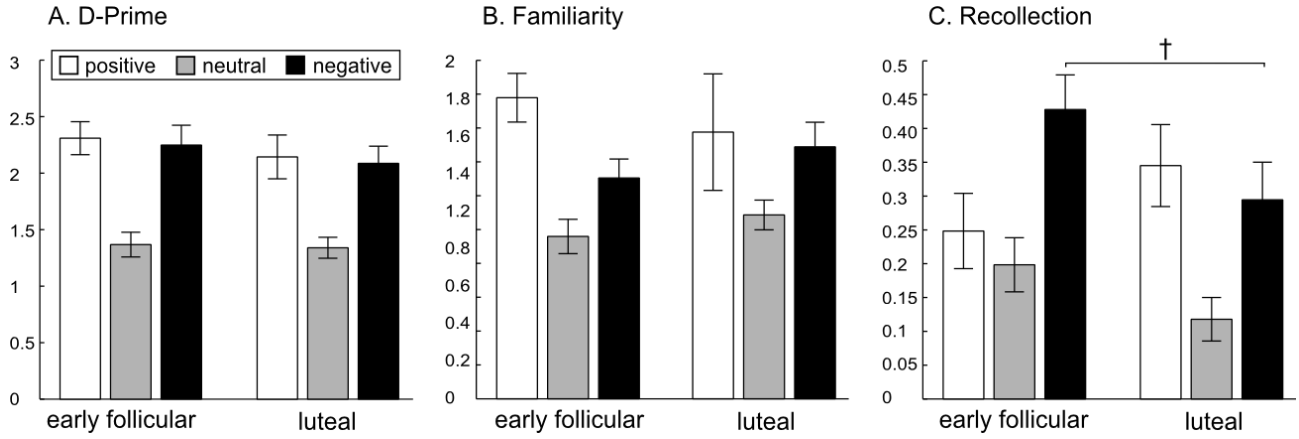

Figure 8. Behavioral performance in the emotional memory task (Experiment 3). Recognition accuracy (d-prime) and process parameters were estimated from the stimulus set consisting of high arousing emotional and low arousing neutral items. A. D-prime was higher for emotional compared to neutral stimuli, but does not vary with cycle phases. B. Familiarity varied between valence categories but not cycle phases. C. Recollection for negative stimuli showed a significant time by group interaction. Planned paired *t*-tests showed that recollection was lower in luteal compared to early follicular phase with marginal significance (†). This comparison reached significance when the full stimulus set was used. Error bars represent the standard error of the mean. Adapted from "Menstrual-cycle dependent fluctuations in ovarian hormones affectemotional memory," by J. Bayer, H. Schultz, M. Gamer, T. Sommer, 2014, Neurobiology of Learning and Memory, 110, p. 55–36. Copyright 2014 by Elsevier. Adapted with permission.

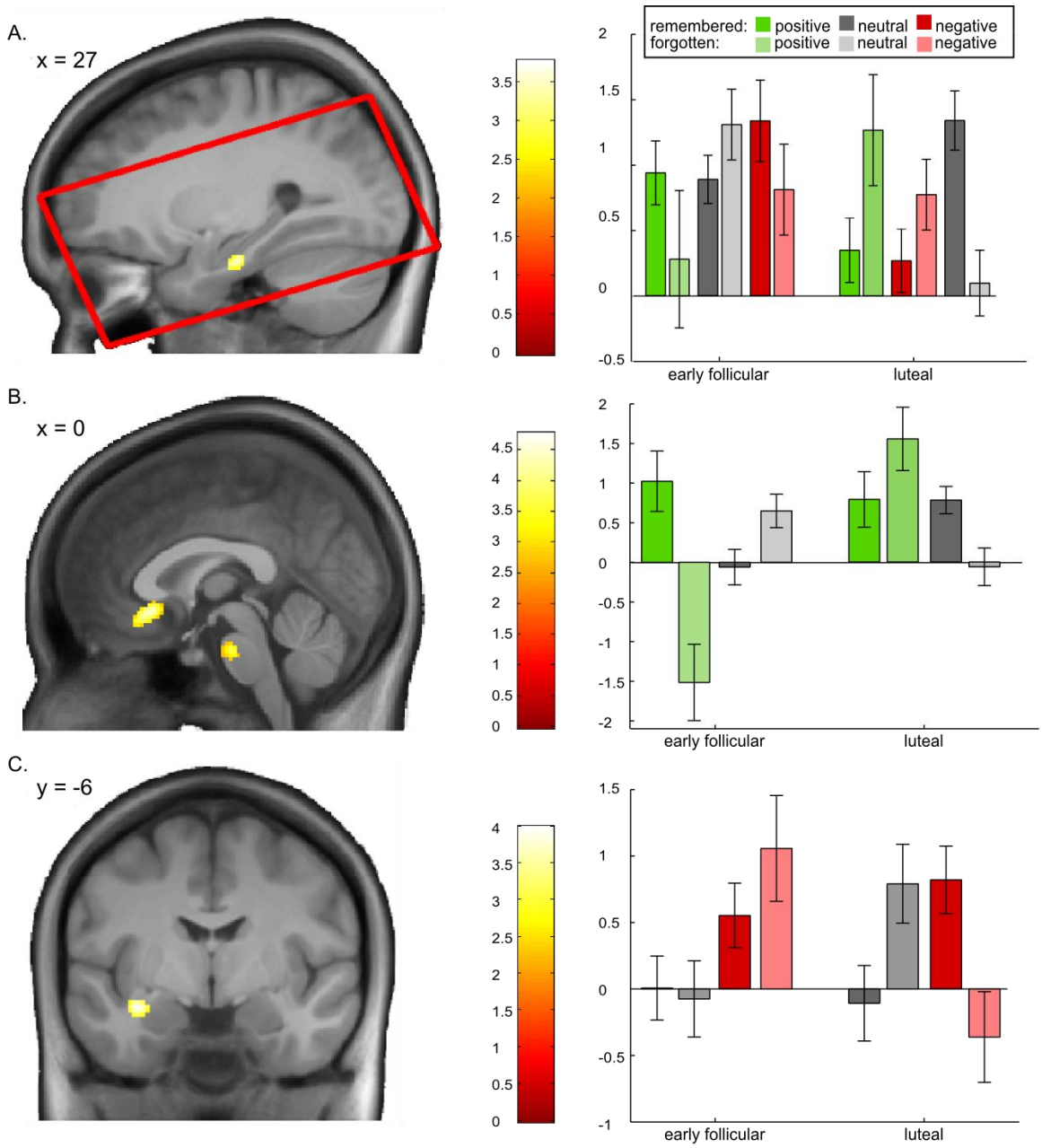

Figure 9. Results of functional neuroimaging (Experiment 3). Depicted are statistical maps (left column) and corresponding beta values (right column) of emotionally enhanced memory (EEM) effect by menstrual cycle interactions. An uncorrected threshold of $p < .005$ was chosen for visualization purposes. A. The conjunction analysis of EEMpos and EEMneg effects (EEM effect for positive/negative stimuli) revealed higher activity in the right hippocampus during early follicular compared to luteal phase. The red frame highlights the coverage of the functional MR sequence. Beta values show, that this interaction was driven by activity differences for emotional as well a neutral categories. B. Neural activity associated to the EEMpos effect was higher in a cluster within the ACC, extending into both hemispheres, during early follicular compared with luteal phase C. Neural activity related to the EEMneg effect was higher in the left amygdala in luteal compared to early follicular phase. Error bars represent the standard error of the mean.

Neuroimaging

Irrespective of cycle phase, the hippocampus, the amygdala, the medial prefrontal cortex (mPFC), the orbitofrontal cortex (OFC) and the ACC showed higher BOLD responses to positive compared to neutral stimuli, as well as to negative compared to neutral stimuli (Table 10, Appendix). A weak association of the EEM_{pos} effect with activity in the right hippocampus did not reach statistical significance, $x = 38$, $y = -12$, $z = -26$; $Z = 2.90$, $p_{SVC} = .241$. The weak effect can be accounted for by relatively strong menstrual-cycle dependent variations in BOLD responses associated to the EEM_{pos} effect (see below). The EEM_{neg} effect was associated with significant BOLD responses in the left amygdala, $x = -26$, $y = -4$, $z = -26$; $Z = 3.31$, $p_{SVC} = .039$, extending into the left hippocampus, $x = -36$, $y = -10$, $z = -26$; $Z = 3.52$, $p_{SVC} = .044$.

Activity related to emotional processing irrespective of encoding success did not change across cycle phases within regions of interest or at the whole brain level. The EEM_{pos} effect, i.e. (pos_{hit} > pos_{miss}) > ($neut_{hit}$ > $neut_{miss}$), elicited significant higher activity during the early follicular phase compared to the luteal phase in the right anterior hippocampus, $x = 32$, $y = -16$, $z = -26$; $Z = 3.98$, $p_{SVC} = .010$, and a cluster within the ACC extending into the left, $x = -8$, $y = 28$, $z = -6$, $Z = 4.08$, $p_{SVC} = .006$, and the right hemisphere, $x = 0$, $y = 22$, $z = -8$, $Z = 4.10$, $p_{SVC} = .004$ (Figure 9, B). On the other hand side, the EEM_{pos} was associated with higher BOLD responses during luteal relative to early follicular phase within the left, $x = -26$, $y = -38$, $z = 2$; $Z = 3.70$, $p_{SVC} = .025$, and the right posterior hippocampus, $x = 32$, $y = -26$, $z = -12$; $Z = 3.32$, $p_{SVC} = .037$. The EEM_{neg} effect, i.e. (neg_{hit} > neg_{miss}) > ($neut_{hit}$ > $neut_{miss}$), was associated with higher BOLD responses in early follicular compared to luteal phase within the left, $x = -22$, $y = -16$, $z = -28$; $Z = 3.72$, $p_{SVC} = .023$, and the right anterior hippocampus, $x = 28$, $y = -12$, $z = -28$; $Z = 3.95$, $p_{SVC} = .011$. A cluster within the left amygdala, $x = -32$, $y = -8$, $z = -16$; $Z = 3.45$, $p_{SVC} = .026$, provided a greater EEM_{neg} during luteal compared to early follicular phase (Figure 9, C).

A conjunction analysis (Nichols, Brett, Andersson, Wager, & Poline, 2005) confirmed that the clusters in the right hippocampus for the EEM_{pos} and the EEM_{neg} by cycle interaction were overlapping, i.e. EEM's were stronger for both valences in the early follicular compared to luteal phase, $x = 26$, $y = -18$, $z = -24$; $Z = 3.78$, $p_{SVC} = .020$ (Figure 9, A). Results of all analyses were replicated with slightly different *Z*-values in the further reduced stimulus set, where negative pictures with very high arousal ratings were excluded so that arousal for positive and negative pictures was matched. As such, differences in the cycle dependent-modulation of EEM_{pos} and EEM_{neg} cannot be attributed to differences in mean arousal between positive and negative pictures.

6.3.4 Discussion

The current results demonstrate valence-specific effects of menstrual cycle phases on behavioral memory performance and neural correlates of emotional memory. Behaviorally, menstrual cycle phase affected recognition quality, i.e. the amount of contextual information recollected with an event, but not overall recognition accuracy. In detail, recognition for negative items was more driven by recollection during low-hormone early follicular than high-hormone luteal phase, whereas recognition of positive items was potentially based more on recollection in the luteal compared with early follicular phase. The contribution of familiarity to recognition memory was unaffected by menstrual cycle phases, which is consistent with previously reported effects. For example, a dopamine antagonist and also the catechol-o-methyltransferase val158met genotype exclusively affected recollection but not familiarity of emotional arousing stimuli (Gibbs, Naudts, Spencer, & David, 2010; Naudts, Azevedo, David, Heeringen, & Gibbs, 2012). With respect to neural activity, the enhanced memory formation for emotionally arousing pictures of both valences was associated with higher activity in the low-hormone early follicular compared to the high-hormone luteal phase.

In other brain regions, hormones modulated EEM-related brain activity in a valence specific manner. In detail, successful encoding of positive relative to neutral items elicited higher activity in the ACC during early follicular phase. The opposite pattern of activity, i.e. a higher activity increase in luteal compared to early follicular phase, was observed in the posterior hippocampus during successful encoding of positive and in the left amygdala during successful encoding of negative stimuli. In sum, cycle phase not only affected the degree of recollection in a valence-specific manner, but also the neural substrates involved in the EEM for negative and positive pictures.

Behaviorally, recollection for negative stimuli was lower during high-hormone luteal compared to low-hormone follicular phase. Lower recollection in the high-hormone phase are in line with anxiolytic actions of E2 and P4, as well as with studies showing decreased contextual fear conditioning when hormone levels are high (Frye et al., 2000; Gupta et al., 2001; Lund et al., 2005). The valence-specificity of present findings connect to the study of Ertman and colleagues (2011), in which memory between subjects in the two halves of the menstrual cycle differed only for negative but not neutral slides. Moreover, current findings of changes in recollection but not familiarity go well with the fact that Ertman et al. (2011) found menstrual cycle-dependent variations only for the free recall but not the recognition memory test. While free recall and recollection rely on the hippocampus and the prefrontal cortex, recognition can also be supported by familiarity which is mediated by regions like the entorhinal cortex (Ranganath et al., 2004; for a review, see Yonelinas, 2002). Thus, both studies showed that high hormone levels specifically affect arousal-related consolidation which is mediated by the hippocampus and/or the prefrontal cortex. It is of note, that Ertman and colleagues (2011) report better memory in subjects with high P4 levels (second half group) compared to subjects with low P4 levels (first half group), which stands in contrast to present results. However, critical differences in study designs limit direct comparisons between the results published by Ertman and colleagues (2011) and the present study. Ertman and colleagues

(2011) divided participants post hoc broadly according to the first and second half of the menstrual cycle and averaged thereby across a broad range of hormonal increases and decreases. Furthermore, free recall relies on a broader network of prefrontal structures than recollection, on which hormonal fluctuations can exert different actions than on the hippocampus (Djebaili et al., 2004; Staresina & Davachi, 2006; Zhou et al., 2010).

Neural activity associated to the negative EEM was higher within the left amygdala in the high-hormone luteal phase and within the anterior hippocampus in the low-hormone follicular phase. Thus, heightened activity in the amygdala during luteal phase coincided with decreased recollection for negative pictures. This pattern of enhanced amygdala activity but reduced behavioral recollection is consistent with the notion that arousal and amygdala activity supports the encoding of single item features but not contextual details (Kensinger & Schacter, 2006; Murray & Kensinger, 2013). Moreover, the present findings parallel the observation that a particular Catechol-o-Methyl-Transferase genotype is associated with worse recollection for negative stimuli as well as heightened amygdala activity (Naudts et al., 2012; Smolka, 2005). Additionally, the positive association between activity in the *left* amygdala and hormone levels matches previous reports about women showing higher activity in the left and men in the right amygdala during successful encoding of negative stimuli (Cahill, 2004; Canli, Desmond, Zhao, & Gabrieli, 2002). The enhanced hippocampal activity during early follicular phase coincided with increased recollection. Again, this coincidence is in line with literature as the hippocampus is known to be specifically involved in the encoding of contextual details and recollection, respectively (Ranganath et al., 2004).

The positive EEM effect was associated with higher activity in the anterior hippocampus and the ACC during early follicular phase. During luteal phase, activity related to the positive EEM shifted

to the posterior hippocampus, accompanied by a descriptive increase in recollection for positive pictures (i.e. the opposite pattern than recollection for negative pictures). In other words, whereas recollection for negative items covaries with anterior hippocampal activity, recollection for positive items is positively associated with posterior hippocampal activity. Animal studies provide evidence that this pattern of hormonal influences is well plausible. More precisely, the observed differential effect of hormonal fluctuations on the anterior and posterior hippocampus mirror differential effects of E2 on the dorsal and ventral proportion of the hippocampus in animals (Buterbaugh & Hudson, 1991). Furthermore, consistent with present changes in ACC activity across the menstrual cycle, evidence for hormonal actions also exists within the ACC (Fink, Sumner, Rosie, Grace, & Quinn, 1996; Parsons, Rainbow, MacLusky, & McEwen, 1982; Xiao et al., 2013). On the conceptual level, the present results pattern suggests that recollection is supported by different underlying processes for negative and positive pictures.

One factor that differentially influences the consolidation of the two valences is emotional arousal. While increased arousal-stimulated processing supports the consolidation of negative stimuli, it can decrease consolidation of positive stimuli that rather benefit from a semantic encoding mode (Kensinger & Schacter, 2008; Mickley & Kensinger, 2008). A potential explanation for current findings could thus be that the effects of arousal were lower during high-hormone luteal phase, which lead to decreased recollection for negative but increased recollection for positive items. As a matter of fact, increased sensitivity to arousal during early follicular phase has already been reported elsewhere (Goldstein et al., 2005). More support for an increased sensitivity to emotional arousal when hormones are low is delivered by the increased responsivity of the ACC during low-hormone early follicular phase in the present study. The ACC is associated with heightened emotional awareness as well as with referring external emotional input to a person's own emotional state (Canli et al., 2002; Kensinger & Schacter, 2008; Lane et al., 1998). Additionally, heightened

ACC activity during follicular phase is related to approach behavior to male faces (Roberts, Newell, Simões-Franklin, & Garavan, 2008). Further evidence for a switch from an emotionally-focused processing during early follicular to a more semantic processing in luteal phase, is provided by the shift from activity in anterior hippocampal to posterior hippocampal regions. While the anterior part of the hippocampus has been associated with emotional functions, the posterior part is known to be heavily involved in 'cold' cognitive functions such as spatial memory and the successful encoding of verbal material (Fanselow & Dong, 2010; Fernández et al., 1998). In sum, the observed differential effect of hormonal fluctuations on the EEM for positive and negative pictures adds evidence to the notion that both effects are supported by partly distinct processes.

The present study demonstrates that EEM-related hippocampal activity as well as hippocampus-dependent recollection for negative pictures varies intra-indivually across the menstrual cycle. Furthermore, current results show that hormonal effects can differ between hippocampal subparts and valence categories.

6.4. Experiment 4: Menstrual cycle in young women and hippocampal structure

6.4.1 Introduction

In animal research, hormone-induced structural changes at hippocampal neurons are well evidenced. For instance, E2 increases spine as well as synapse density and alters the shape of hippocampal spines (Li et al., 2004; Woolley et al., 1990; Woolley & McEwen, 1992). P4 administered alone can increase synapse density but has detrimental effects when administered after or together with E2 (Choi et al., 2003; Gould et al., 1990; Murphy & Segal, 1996; Silva et al., 2000). For a more detailed discussion of this topic see chapter 3.4. Based on these results, the human menstrual cycle (Figure 10) should reveal higher hippocampal GM volume during the middle/late follicular phase (E2 rising, P4 low), and lower GM volume in the luteal phase (E2 at a medium peak, P4 at its maximum). A comparison between hippocampal GM volumes during early follicular phase (both hormones low) and luteal phase should yield higher GM volumes during early follicular phase, because P4 can down-regulate spine density nearly to a level observed after OVX (Woolley & McEwen, 1993).

In humans, local changes in brain structure have so far been investigated in three studies, each employing different cycle phases (Figure 10). In support of animal studies, results of Protopopescu and colleagues (2008) point to higher hippocampal GM values when E2 is unopposed by P4, compared to when E2 and P4 are both high. In detail, they found higher para-/hippocampal volumes in the late follicular phase when E2 is rising, compared to the late luteal phase when E2 and P4 decline sharply. Using similar menstrual cycle phases, Osseewaarde and colleagues (2013) found higher GM volumes only in the amygdala during late follicular phase. Because Osseewaarde and

colleagues (2013) employed a relatively narrow time window within the late luteal phase and did not verify cycle phases by assessments of E2 and P4 values, inconsistencies could be accounted for by differences in actual hormonal states of the two study samples. Results from Pletzer and colleagues (2010) support the hypothesis that P4 down-regulates hippocampal GM volumes in the luteal phase to a level below hippocampal GM volume when hormones are low. More precisely, the authors report higher (para)hippocampal GM values during low-hormone early follicular phase than during high-hormone luteal phase.

The first goal of the present study was to replicate previous findings of higher hippocampal volumes during the early follicular compared to the luteal phase, with menstrual cycle phases verified by hormone concentrations in saliva. The second goal of the study was to investigate the relationship between gray matter changes and hormone levels via correlation analyses.

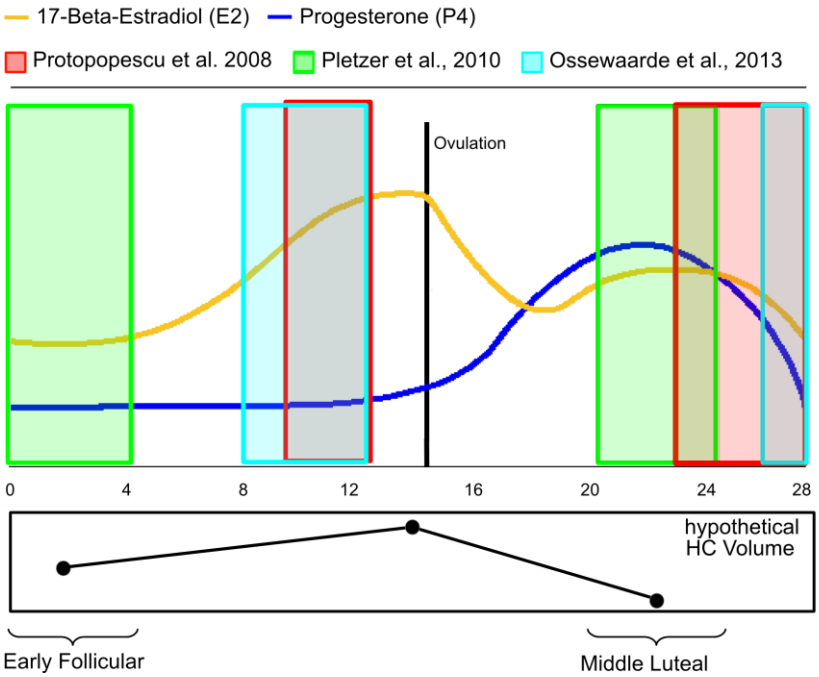

Figure 10. Schematic representation of the menstrual cycle (Experiment 4). The yellow and blue lines represent concentrations in ovarian hormones. Colored boxes show time windows used in existing studies. The white box at the bottom illustrates hypothetical changes (ordinally scaled) in hippocampal volume.

6.4.2 Material and methods

Subjects and procedure

Acquisition of structural images for the current study was accomplished in the course of functional scanning of Experiment 3. Please refer to chapter 6.3.2 for details about the study sample and experimental procedure.

Structural Neuroimaging

High-resolution T1-weighted structural MR image were acquired by using a standard 3D-MPRAGE sequence (TR 2300 ms, TE 2.89 ms, flip angle 9°, 1 mm slices, field of view 256 x 192; 240 slices).

MRI data were preprocessed and analyzed using the VBM8 for SPM8 (Wellcome Department of Imaging Neuroscience, London, UK), running under Matlab R2009a (Mathworks, Inc, Natick, MA, USA). The default longitudinal preprocessing approach integrated into the VBM8 toolbox was used. Finally, gray matter segments were smoothed with a FWHM Gaussian kernel of 8 mm for all directions. A sample homogeneity check as provided by the VBM8 toolbox revealed one outlier, leaving data from 21 subjects for further structural image analysis.

To examine differences in local gray-matter volumes across cycle phase, structural images from the early follicular phase were contrasted against structural images from the luteal phase and vice versa using paired *t*-tests. Results of all analyses were considered significant at $p < .05$, corrected for multiple comparisons at the entire scan volume and within regions of interest. Regions of interest were anatomical masks of regions that showed volume changes across the menstrual cycle in previous studies, i.e. the hippocampus, the amygdala and the parahippocampus (Amunts et al., 2005; Ossewaarde et al., 2013; Pletzer et al., 2010; Protopopescu et al., 2008). Statistical parameters

are reported for peak voxels, i.e. voxels showing the biggest GM distribution differences between cycle phases.

6.4.3 Results

Higher GM volumes during low-hormone early follicular compared with high-hormone luteal phase were found in a cluster within the right hippocampus, x = 24, y = -16, z = -27, Z = 3.86, p_{SVC} = .025, that extended into the amygdala, x = 24, y = -7, z = -26, Z = 3.52, p_{SVC} = .034, and the parahippocampus, x = 24, y = -7, z = -26, Z = 3.97, p_{SVC} = .015 (Figure 11). Additionally, another cluster within the amygdala (extended amygdala) showed higher GM volumes during early follicular compared with luteal phase within the left, x = -26, y = -12, z = -5, Z = 4.28, p_{SVC} = .002, and the right hemisphere, x = -26, y = -12, z = -5, Z = 4.28, p_{SVC} = .002 (Figure 11). Moreover, a cluster in the right parahippocampus showed higher GM volumes during early follicular compared with luteal phase, x = 24, y = -18, z = -27, Z = 3.97, p_{SVC} = .015. A cluster in the left parahippocampus reached only marginal significance for this contrast, x = -21, y = -7, z = -35, Z = 3.56, p_{SVC} = .051. No region showed higher GM volumes during luteal compared with early follicular phase.

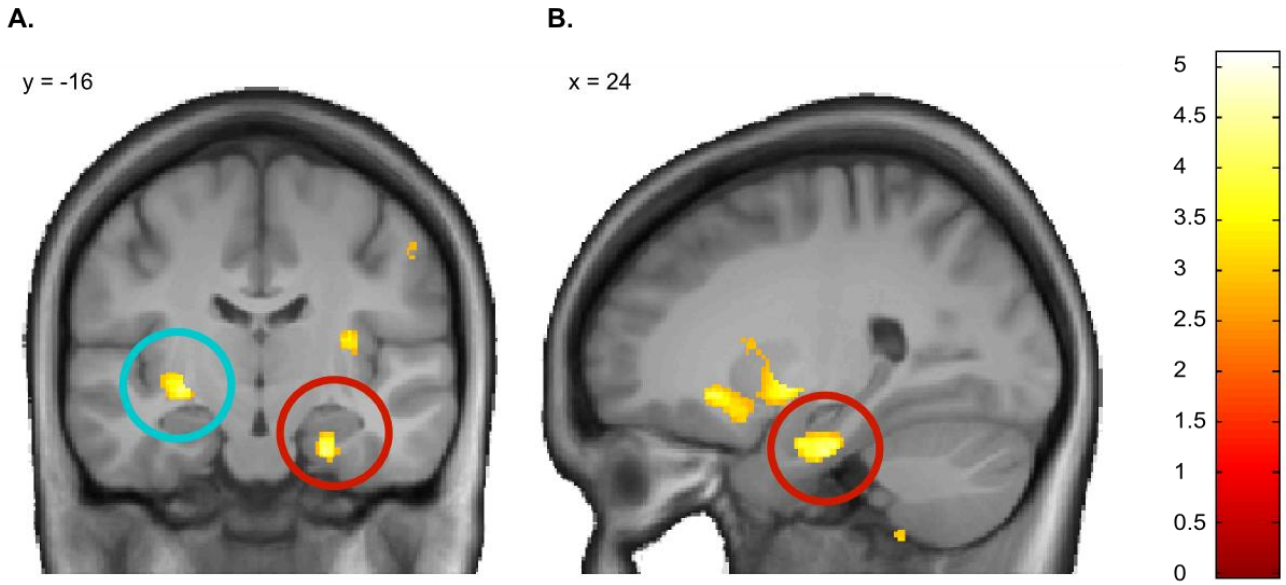

Figure 11. Results of voxel-based morphometry (Experiment 4). Statistical maps of the regional gray matter (GM) distribution differences between early follicular and luteal phase. An uncorrected threshold of $p < .005$ was chosen for visualization purposes. A. A cluster within the left extended amygdala (blue circle) and the right hippocampus (red circle), extending into the amygdala and the parahippocampus, showed higher GM volumes during early follicular than luteal phase. B. Sagittal view on the significant cluster within the right hippocampus (red circle).

To assess whether hippocampal GM volume changes were associated with changes in hormone concentrations, a correlation analysis between the difference in GM volume at the peak voxel of the cluster within the right hippocampus and the difference in E2 and P4 between early follicular and luteal phase was calculated. Lillietests confirmed that difference scores for E2 and P4 followed a normal distribution. Pearson's product-moment correlation coefficient showed that hippocampal GM volumes did not correlate significantly with either E2, $r(19) = -.19$, $p = .409$, or P4, $r(19) = -.16$, $p = .488$.

6.4.4 Discussion

The current study replicates previous findings of increased hippocampal volume during the low-

hormone early follicular phase, relative to the luteal phase when E2 and P4 are both high. GM volumes in the amygdala showed the same pattern of higher volumes in early follicular as in luteal phase. In contrast to previous studies, menstrual cycle phases were verified by the assessment of E2 and P4 levels in saliva (see 6.3.3). Moreover, current analyses revealed significant differences in a greater network of brain regions than previous studies. Hippocampal GM changes did not correlate with changes in E2 and P4 across the menstrual cycle.

Higher hippocampal GM volumes during low-hormone early follicular compared with high-hormone luteal phase replicate previous VBM studies and go well with the finding that P4 can counteract the stimulating effects of E2 on hippocampal spine growth (Murphy & Segal, 2000; Woolley & McEwen, 1993). GM volume changes in the amygdala are in line with animal studies reporting decreases in dendritic spines when E2 and P4 peak during proestrus (Rasia-Filho, Fabian, Rigoti, & Achaval, 2004). While the role of E2 is widely acknowledged in its effects on hippocampal morphology, the current results together with the findings obtained by Pletzer and colleagues highlight the critical role of P4 in macroscopic GM changes. If only E2-related effects would be relevant for the regulation of hippocampal structure, volumes would be higher in the high-hormone luteal than the low-hormone early follicular phase. Consistent with animal research, current findings suggest that P4 not only counteracts E2-related structural increases, but further down-regulates hippocampal volume to a level that undercuts the GM volume during low-hormone early follicular phase.

Correlation analyses show that neither changes in E2 nor P4 were significantly associated with hippocampal GM volume. Moreover, contradictory to findings from animal studies, correlation coefficients for both hormones went descriptively into the same direction. Potential reasons for the missing link between hormone levels and hippocampal GM volume are that (i) there is no linear

relationship *across subjects* between hippocampal GM and hormone changes, or that (ii) hormone concentrations in saliva are not a good approximation to hippocampal hormone levels.

In regard to the first hypothesis, it is important to emphasize that the current correlation analysis tested whether the size of hormonal differences is linearly related to the size of differences in hippocampal GM across subjects. A positive relationship would mean that women who exhibit high hormonal fluctuations also exhibit high fluctuations in hippocampal GM volume. Although this relationship is theoretically possible, one might argue that the organism likely adjusts to a certain level of hormonal fluctuations so that differences between subjects are of minor importance. Moreover, although *some kind of a positive relationship* between E2/P4 and hippocampal spine density is well evidenced in animal models, no study has yet reported a significant *linear* relationship between hormone concentrations and hippocampal spine density (Gould et al., 1990; Woolley and McEwen, 1993; Murphy and Segal, 2000). Another point worth mentioning, is that hormonal modulations of structural processes such as synapto- and spinogenesis require a certain amount of time, so that previous hormonal levels could be more predictive than actual ones (for a review, see Yuste & Bonhoeffer, 2004). In summary, the exact relationship between changes in hormone levels and cellular events at hippocampal neurons is relatively unknown, so that further research is necessary to determine to which extent correlations between hormone levels and macroscopic hippocampus volume can be expected.

The concern raised in the second hypothesis, i.e. that hormone levels in saliva might be different from hormone levels in the hippocampus, pertains not only the relationship between hippocampal and peripheral hormone levels but also the reliability and validity of hormone assessments in saliva compared with serum. In premenopausal women, hormone levels assessed in saliva are in good agreement with serum hormone levels (Bellem, Meiyappan, Romans, & Einstein, 2011; Evans,

Stewart, & Merrick, 1980). Moreover, saliva assessments have the advantage over serum assessments that they measure the fraction of biological available hormone concentrations, i.e. the amount of hormones that can enter tissues (for a review, see Taves, Ma, Heimovics, Saldanha, & Soma, 2011). With respect to the relationship between peripheral and hippocampal E2 levels, a high linear correlation between menstrual cycle-dependent hippocampal E2 and serum E2 has been observed in female rats (Hojo et al., 2009; Prange-Kiel et al., 2008). To sum up, saliva concentrations should mirror hippocampal E2 concentrations quite well, but further research is necessary to clarify this relationship with respect to P4.

Whereas previous studies reported GM volume differences located *either* in the hippocampus, the parahippocampus *or* in the amygdala, all of these regions showed significant clusters in the current study. Pletzer and colleagues (2010) speculated, that different foci of structural differences could be related to testing in slightly different menstrual cycle phases in the respective studies. Current results suggest that all of these regions are sensitive to hormonal influences but the size of effects depends on details in study design and image processing. It is conceivable, that findings of Ossewaarde and colleagues (2013) as well as Protopopescu and colleagues (2008) were more restricted to specific regions, because they tested subjects in cycle phases that differed less in hormone concentrations than the cycle phases used in the current study. Additionally, all three previous studies used the VBM toolbox of SPM5, whereas the current study employed the longitudinal VBM toolbox of SPM8. Therefore, different algorithms for image segmentation and normalization were used in the present study. Thus, more extended structural differences in the current study are most likely the result of differences in the definition of menstrual cycle phases and/or the use of newer image preprocessing algorithms.

Results of the current study replicate previous findings concerning higher hippocampal volumes

during high-hormone luteal than low-hormone early follicular phase. Moreover, they highlight the importance of progesterone in the regulation of hippocampal structure.

7 General Discussion

7.1. Summary of the experimental part

The present thesis investigated the influence of E2 and P4 on the human hippocampus. For this purpose, four experiments were conducted that assessed hormonal modulations of hippocampus-dependent memory as well as hippocampal activation and structure by using several complementary approaches. Results obtained in these studies illustrate the variety of hormonal effects on the human memory system and their dependence upon experimental factors.

An observational pharmacological approach was used in *Experiment 1*. In this experiment, postmenopausal women suffering from hormone-sensitive breast-cancer were tested before onset of AI therapy, leading to decreases in E2 levels, as well as 3 to 6 months after continous AI therapy. A group of healthy age-matched controls was tested with a matched delay between the two measurement points. Behavioral measures were performances in two verbal source-memory tasks, a visual memory task, two attention tasks and a word-color interference task. Structural MRI as well as functional MRI during encoding of a word-color association task was recorded on both measurement points. Behaviorally, deprivation of E2 lead to decreased recollection in a specific sub-domain of verbal source memory. Performances in other memory tasks as well as executive functions remained unaffected by AI therapy. On the neuronal level, AI therapy increased activity in the ACC and DLPFC related to successful encoding in a word-color association task, but did not alter hippocampal activity or structure. Impairment in recollection could be accounted for by decreases in the efficacy of the hippocampus or prefrontal areas, due to the decline in E2 levels after AI therapy, whereas increased activity in the ACC and DLPFC are likely the result of compensatory mechanisms.

A genetic approach was used in *Experiment 2*. Here, three cohorts of healthy young men were

genotyped for a single-nucleotide polymorphism (rs700518) on the gene coding for aromatase, which has been repeatedly associated with E2 serum levels in men (e.g. Peter et al., 2008). Structural T1 images were acquired from two cohorts. Behavioral performance in a verbal, emotional and spatial memory task was assessed in a third cohort. Independent analysis of structural T1 images from the two MRI cohorts showed that the genotype group with a genetic disposition to higher E2 serum levels (AA) had also higher hippocampal volumes than the other two genotype groups (AG and GG). Analyses of behavioral data showed no significant associations between rs70018 genotype and memory performance. Higher hippocampal volumes in the genotype group with a disposition to higher E2 levels are likely caused by increased hippocampus neuroprotection in this group, as the stimulation of spine growth by E2 does typically not occur in the male hippocampus (Azcoitia et al., 2001; Fester et al., 2012).

The natural menstrual cycle was used as an independent variable in *Experiment 3.* In this experiment, healthy naturally-cycling women were tested once in the low-hormone early follicular phase and once in the high-hormone luteal phase. On both occasions, subjects encoded photographs depicting negative, neutral or positive contents inside the scanner. A recognition memory test was performed two days later outside the scanner. Menstrual cycle phases were verified by the assessment of E2 and P4 in saliva. Behaviorally, women showed higher recollection-based memory for negative pictures in the low-hormone phase than in the high-hormone phase. No significant differences across cycle phases were found for the other two valence categories or familiarity-based memory. On the neuronal level, the EEM effect for positive pictures was associated with higher activity in the anterior hippocampus and the ACC during low-hormone early follicular compared with high-hormone luteal phase. During luteal phase, the EEM effect for positive pictures was associated to higher activity in the posterior hippocampus compared with early follicular phase. Similar to the positive valence category, the EEM effect for negative pictures was associated with

higher activity in the anterior hippocampus during early follicular compared with luteal phase. A conjunction analyses confirmed that an overlapping cluster within the left anterior hippocampus showed increased activity during early follicular phase for positive as well as negative pictures. A cluster within the left amygdala showed higher activity during luteal compared with early follicular phase associated to the EEM effect for negative pictures. Present results indicate that high levels of E2 and P4 levels might reduce the effect of negatively valenced arousal on hippocampus-dependent consolidation.

Similar to Experiment 3, the natural menstrual cycle was used as an independent variable in *Experiment 4.* Here, structural neuroimaging was performed in healthy young women once during early follicular phase and once during the luteal phase. Consistent with previous literature, GM volumes of the hippocampus, the amygdala and the parahippocampus were higher during early follicular phase when E2 and P4 are low than during luteal phase when E2 and P4 are high. Hippocampal volume changes were not significantly correlated to changes in the concentration of E2 or P4. These results suggest, that P4 can regulate hippocampal volumes during high-hormone luteal phase down to a level beyond low-hormone early follicular phase.

7.2. Reflection of current results on the ground of existing literature

The theoretical background of the present thesis showed that existing literature does not provide sufficient data to judge whether P4 or E2 modulates hippocampus-dependent memory in humans. Classical psychological theories about the role of the hippocampus in recognition memory, suggest hippocampal involvement particularly in spatial and verbal episodic memory. Further refinements have been postulated by advocates of dual process models, stating that memory performance can be differentiated into hippocampus-dependent recollection and hippocampus-independent familiarity.

These refinements accommodate findings of varying degrees of hippocampal involvement within the same memory domain, among which is verbal memory (for a review, see Schacter & Wagner, 1999). The idea that E2 particularly targets processes within the hippocampus, was stimulated by the observation that hippocampal neurons synthesize E2 de novo from cholesterol and by profound E2-related modulations of cellular mechanisms at hippocampal neurons (Foy et al., 1999; Kretz et al., 2004; Rune et al., 2006). Among others, E2 regulates hippocampal spine- and synapse density as well as LTP, or in other words neural correlates of hippocampus-dependent memory. Later on, similar effects have been observed for P4, although some go into the opposite direction (Choi et al., 2003; Foy et al., 2008). Fitting to data on the cellular effects of E2, animal research clearly indicates beneficial effects of E2 on spatial memory (Gibbs et al., 2004; Harburger et al., 2007). Human research delivers evidence for positive effects of E2 on verbal and spatial memory, but is much more inconsistent compared with animal literature (Bartholomeusz et al., 2008; Hogervorst & Bandelow, 2010; Resnick, 1998b; Solis-Ortiz & Corsi-Cabrera, 2008). A potential reason for this inconsistency is that no distinction is made between hippocampus-dependent and -independent memory functions. Furthermore, human research often operationalizes hormonal status as an independent variable with questionable degrees of validity and reliability. For instance, menstrual cycle phases were employed without verification by hormonal assessments or administration methods were used that produced highly variable hormonal increases across study participants (e.g. Ossewaarde et al., 2013; Wolf et al., 1999). In regard to the influences of P4 on hippocampus-dependent memory, animal as well as human research are controversial and do not provide sufficient data to draw proper conclusions.

Experiments in the current thesis therefore used hormonal manipulations, for which the effects on hormonal levels are either very well known (i.e. AI therapy in Experiment 1, aromatase polymorphism in Experiment 2), or employed assessments of E2 and P4 to verify natural hormonal

variations (i.e. menstrual cycle phases Experiment 3 and Experiment 4). Independent variables include an acute pharmacologically-induced decrease in E2 production (Experiment 1), long-term differences in E2 serum levels between genotype groups (Experiment 2) as well as acute endogenous fluctuations of E2 and P4 (Experiment 3 & Experiment 4). Hormonal effects were investigated not only behaviorally, but also with respect to modulations of hippocampal activity and structure. To disentangle hippocampus-dependent from -independent memory on the behavioral level, the process parameters recollection and familiarity were estimated from overall memory performance.

Data of the current thesis support the view, that *hormones specifically target hippocampus-dependent memory*. In detail, deprivation of E2 due to AI therapy (Experiment 1) as well as hormonal fluctuations in the course of the menstrual cycle (Experiment 3) affected recollection but not familiarity. In other words, hormonal variations particularly modulated those memory functions that highly depend on hippocampal function. It has to be emphasized, that no differences were detected in overall memory performance, so that no significant hormonal modulations of memory performance would have been detected without estimation of process parameters. Moreover, hormonal manipulations did not affect executive functions (Experiment 1), mood or the subjective emotional arousal of pictures (Experiment 3), suggesting that hormonal effects were specific to the memory domain in the current thesis. With respect to existing literature, present results suggest that at least a part of the inconsistencies concerning hormonal effects on human memory can be accounted for by not differentiating between hippocampus-dependent and -independent memory functions.

E2 does not modulate all forms of hippocampus-dependent memory equally. In Experiment 1, AI therapy decreased recollection in the verbal domain, but did not alter the free recall of words in a

different task. Similarly, recollection in Experiment 3 was affected by menstrual cycle phase only for negative but not for neutral or positive stimuli. The pattern of results obtained in the two studies have in common that performance in both memory paradigms (i.e. recollection-based recognition vs. free recall in Experiment 1), as well as for the three valence categories (negative vs. neutral or positive in Experiment 3), is known to rely on hippocampal functioning, but differs with respect to exact underlying mechanisms (Kensinger & Schacter, 2006, 2008; Mickley & Kensinger, 2008; Staresina & Davachi, 2006). As such, hippocampus-dependent memory processes are more likely sensitive to hormonal influences than hippocampus-independent memory processes, but hippocampal involvement does not guarantee the sensitivity to hormonal fluctuations. Moreover, hormonal effects are most likely not restricted to hippocampal areas, as hormone-dependent variations in neural activity or structure were also significant in the ACC, the dorsolateral prefrontal cortex, the amygdala and the parahippocampal cortex.

The pattern of present results further highlight that hormonal effects on hippocampus-dependent memory are *critically influenced by how hormonal status is operationalized as an independent variable*. While decreased E2 levels due to AI therapy (Experiment 1), as well as natural fluctuations in E2 and P4 across the menstrual cycle (Experiment 3), were associated with significant changes in memory performance, a genetic difference in E2 metabolism did not show a significant association to any form of hippocampus-dependent memory performance (Study 2). Importantly, the null-findings of Experiment 2 can not be solely attributed to the sex of study participants, as hormonal modulations of memory performance have been observed in women as well as men (e.g. Bartholomeusz et al., 2008; Beer et al., 2006; Phillips & Sherwin, 1992a). A potential explanation for these results could be that certain types of hippocampal functions can adjust to a specific hormonal status or a particular range of hormonal fluctuations. This biological concept is known as allostasis, i.e. the ability to maintain stability under changing circumstances

(McEwen & Wingfield, 2003). The polymorphism in E2 metabolism is associated with the height of E2 levels most likely for a long time, so that hippocampal memory functions had enough time to adjust. Likewise, the magnitude of hormonal fluctuations is fairly stable across different cycles within the same woman, so that the female body undergoes similar hormonal changes every cycle (Shultz, Wideman, Montgomery, & Levine, 2011). As such, specific types of hippocampal processes might show a different sensitivity to deviations from hormonal allostasis. Next to biological considerations, the choice of the independent variable and the related study design determines the chance of detecting true effects, i.e. the study power. Firstly, common single genetic variations are likely associated with smaller effect sizes than hormonal treatments (Experiment 1) or endogenous hormonal fluctuations (Experiment 3). Secondly, longitudinal designs (Experiments 1, 3 &4) are usually associated with a greater statistical power than cross-sectional ones (Experiment 2) (Maxwell & Delaney, 2004). This issue is also critical when interpreting null-findings from cross-sectional studies that investigated the cognitive consequences of HRT or AI's. Thus, another account for behavioral null findings in Experiment 2 could be a lack of power due to insufficient sample sizes. Again, this problem might affect distinct hippocampal functions differentially, because unlike memory performance, hippocampal volume was significantly associated to genotypes.

Current results illustrate that the *assessment of behavior, hippocampal activity and hippocampal structure can lead to diverging results*. To complicate matters even more, this issue interacts with the choice of the independent variable. In Experiment 1, AI therapy was associated with significant decreases in hippocampus-dependent verbal memory, but neither significant changes in hippocampal activity nor structure. On the other hand, the polymorphism in E2 synthesis was associated with hippocampal volume but not hippocampus-dependent memory (Experiment 2). In Experiment 3, menstrual cycle phase modulated recollection for stimuli of negative valence but not

other valence categories. Hippocampal activity in contrast showed menstrual-cycle dependent variations for the emotional memory effect for negative as well as positive stimuli. As such, the examination of hormonal influences on the human brain should be performed on the behavioral as well as the neuronal level to draw proper conclusions (Berga, 2008). This issue is also pertaining basic science, as it is still not fully clarified how single neuronal processes relate to BOLD signals and how cellular processes relate to behavioral memory performance (Ekstrom, 2010; Erickson, 2013; Logothetis et al., 2001; Logothetis, 2008).

Sample characteristics influence whether and how hormonal modulations affect the hippocampus. While menstrual cycle phases (Experiment 4) as well as a the polymorphism in E2 synthesis (Experiment 3) were significantly associated to hippocampal volumes in young subjects, a reduction in E2 levels after AI therapy in postmenopausal women was not (Experiment 1). Likewise, hippocampal BOLD signals varied across cycle phases in young women (Experiment 3) but were not related to AI therapy in postmenopausal women (Experiment 1). Next to age or menopausal status, other factors are known to influence the actions of E2 on neural processes. Several cellular actions of E2 are sex-specific, so that some of the most popular actions of E2 do not occur in the male sex (Barker & Galea, 2008; Fester et al., 2012; Vierk et al., 2012). In rats, reproductive experience has been shown to modulate the influence of E2 on the choice between a hippocampus-dependent or -independent strategy to solve a spatial memory task (Hussain, Hoehne, Woodside, & Brake, 2013). Complicating matters even more, evidence exists that the effectiveness of hormonal treatments is determined by a complex interaction between sample and treatment characteristics. For instance, E2 administration facilitated spatial memory in aged mice only at a dosage that has impairing consequences in young mice (Markham, Pych, & Juraska, 2002). As all these mechanisms are far from being understood, care should be taken when generalizing results from a specific study sample to a population.

7.3. Limitations and open questions

Results from the present thesis contribute data to the question how the neurosteroids E2 and P4 affect the human hippocampus. However, several important questions remain open. To name only a few, it is still unclear whether the specificity in consequences of AI therapy (Experiment 1) is related to the treatment itself, or the age of the study participants. Moreover, data from Experiment 1 do not provide the opportunity to examine the clinical relevance of impairments in hippocampus-dependent verbal memory. We further do not know, whether hippocampal activity evoked by other memory types (e.g. spatial memory) would be modulated by AI therapy. Similarly, none of the experiments covered all aspects of hippocampus-dependent memory. It could be possible that the aromatase polymorphism is only associated to memory tasks with a very high degree of hippocampal dependence, such as tasks that require the associative binding across large temporal- and spatial discontinuities (Staresina & Davachi, 2009). Moreover, genetic differences in E2 synthesis were not investigated in women, so that it remains unclear whether the lack of significant findings could be specific to the male sex. It further remains undetermined, whether results obtained in Experiment 3 and 4 are specific to natural endogenous fluctuations in hormones, or whether the same results could be obtained by exogenous hormonal treatments. It is also unclear, whether a significant linear relationship between hormone levels and hippocampal volumes could be obtained when the same woman would be repeatedly tested in all states of her menstrual cycle. Answers to these and many other questions would have required a massive battery of studies, which would require high economical and practical effort.

7.4. Future directions

Present results, together with existing literature, highlight the importance of clearly defining the

processes which are assessed and carefully choose which hormonal manipulation or natural variation is used as an independent variable. However, success in these points requires further research on the neural basis of specific behavioral memory tasks, as well as more insight into the consequences of peripheral hormonal manipulations or natural variations on hippocampal hormone levels. As unveiling exact neural processes or measuring hippocampal hormone levels are usually not feasible in humans, adequate animal studies are necessary that consider species-related differences (Lagace et al., 2007). As far as possible, studies aiming at higher cognitive functions should be conducted in animals and humans at once to uncover cellular mechanisms that underlie hormonal modulations in cognitive functions or macroscopic neuronal correlates. Moreover, to increase the comparability of animal and human research, pharmacological manipulations verified by hormonal assessment should be employed in human research under tight medical control.

8 References

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9 Appendix

Table 10. Main effects of negative and positive emotion (Experiment 3, Appendix).

Region	Side	*x*	*y*	*z*	*Z*-value
Main Effect Of Negative Emotion (Negative > Neutral)					
Hippocampus	L	-26	-10	-14	3.53*
	R	38	-8	-32	3.39
Amygdala	L	-22	-6	-14	4.39**
	R	24	-4	-14	3.86**
Medial Prefrontal Cortex	L	-2	46	-18	4.90***
	R	0	46	-18	4.44***
Orbitofrontal Cortex	L	-28	34	-12	7.27***
	R	38	32	-13	5.73***
Anterior Cingulate	L	-8	50	14	4.16**
	R	0	4	32	3.45
Main Effect Of Positive Emotion (Positive > Neutral)					
Hippocampus	L	-20	-34	0	3.72*
Amygdala	L	-20	-8	-14	3.54*
Medial Prefrontal Cortex	L	-2	32	-18	3.79**
	R	0	32	-18	3.56*
Orbitofrontal Cortex	L	-38	24	-10	4.55**
	R	28	18	-18	4.30**
Anterior Cingulate	L	-8	38	18	4.43**
	R	0	38	-2	3.88*

Note. † = $p < .001$, uncorrected.* = $p < .05$, family-wise error corrected for the reduced search volume. ** = $p < .01$, family-wise error corrected for the reduced search volume. **$p < .001$, family-wise error corrected for the reduced search volume. ****= $p < .05$, family-wise error corrected for the whole volume. L, left. R, right.

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Erklärung nach § 9 Abs. 1, Nr. c der Promotionsordnung zur Doktorin/ zum Doktor der Philosophie oder der Naturwissenschaften des Fachbereichs Psychologie der Universität Hamburg vom 03. Februar 2004

Hiermit erkläre ich, dass die von mir vorgelegte Dissertation nicht Gegenstand eines anderen Prüfungsverfahrens gewesen ist.

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Eidesstattliche Erklärung nach § 9 Abs. 1, Nr. d der Promotionsordnung zur Doktorin/ zum Doktor der Philosophie oder der Naturwissenschaften des Fachbereichs Psychologie der Universität Hamburg vom 03. Februar 2004

Hiermit erkläre ich an Eides statt, dass ich die vorliegende Arbeit selbständig und ohne fremde Hilfe verfasst habe. Andere als die angegebenen Quellen und Hilfsmittel habe ich nicht benutzt und die wörtlich oder inhaltlich übernommenen Stellen als solche kenntlich gemacht.

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